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CLONING, CHARACTERIZATION AND SUBCELLULAR LOCALIZATION OF THE N (NUCLEOCAPSID) AND P (PHOSPHOPROTEIN) PROTEIN OF THE SYDV (POTATO YELLOW DWARF VIRUS SANGUINOLENTA STRAIN)

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ABSTRACT OF DISSERTATION

Debasish Ghosh

The Graduate School
University of Kentucky

2007

CLONING, CHARACTERIZATION AND SUBCELLULAR LOCALIZATION OF
THE N (NUCLEOCAPSID) AND P (PHOSPHOPROTEIN) PROTEIN OF THE SYDV
(*POTATO YELLOW DWARF VIRUS – SANGUIOLENTA* STRAIN)

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences at the
University of Kentucky

By
Debasish Ghosh

Lexington, Kentucky

Director: Dr. Judith A. Lesnaw, Professor of Biology

Lexington, Kentucky

2007

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CLONING, CHARACTERIZATION AND SUBCELLULAR LOCALIZATION OF THE N (NUCLEOCAPSID) AND P (PHOSPHOPROTEIN) PROTEIN OF THE SYDV (*POTATO YELLOW DWARF VIRUS – SANGUIOLENTA* STRAIN)

Potato yellow dwarf virus (PYDV) is the type member of the genus *Nucleorhabdovirus*. The virus replicates in the nuclei of infected cells and mature virions accumulate in the perinuclear space after viral cores bud through the inner nuclear membrane. The virus was first described as an extremely destructive pathogen of potato (*Solanum tuberosum*) and other members of family *Solanaceae*. There are two different strains of PYDV based on their insect-vector specificity, namely SYDV (*sanguinolenta* strain) and CYDV (*constricta* strain). PYDV is considered a model system to study virus-vector relationship, particularly for agriculturally harmful rhabdoviruses. However, very little is known about the molecular aspects and cell biology of PYDV. Preliminary studies showed that infection of transgenic *Nicotiana benthamiana* plants that constitutively express GFP targeted to endomembranes with SYDV and SYN (*Sonchus yellow net virus*, another member of genus *Nucleorhabdovirus*) results in increased accumulation of GFP and membrane within the infected nuclei, though the pattern of GFP accumulation is completely different for the two viruses. GFP accumulation was found mainly in the external and internal loci of the nucleus in SYDV-infected cells, where as, in the case of SYN infection, the GFP accumulation was scattered throughout the nucleus of the infected cell. Molecular characterization of SYDV was undertaken to better understand the cellular difference between these two members of *Nucleorhabdoviruses*. This dissertation describes the determination of the complete nucleotide and ORF (open reading frame) sequences of N (nucleocapsid) and P (Phosphoprotein) gene of SYDV from cDNA clones of both viral genomic and messenger RNAs. Analyses of sequence showed that SYDV-N mRNA contains an 11 nucleotide (nt) untranslated region followed by a 1416 nt ORF encoding a 472 amino acid (aa) protein and P-mRNA contains an 18 nt 5' untranslated region followed by 840 nt ORF encoding a 280 aa protein. Characterization of SYDV-N and P protein using bioinformatic algorithms predict basic hydrophilic and coiled coil regions that may possess the putative nuclear localization signal and protein-protein interaction domain, respectively. Comparison of the SYDV-N ORF with orthologous regions from other plant and animal rhabdoviruses showed

statistically significant identity. Phylogenetic analysis based on consensus N-ORFs placed SYDV into the same group with other *Nucleorhabdoviruses*. Localization studies of SYDV-N and P protein as autofluorescent protein fusions revealed that both proteins are exclusively nuclear localized. Taken together, this dissertation reports a detailed analysis of the biology of SYDV-N and P protein at the molecular and cellular level for the first time towards the long term goal to characterize the entire SYDV genome and to better understand SYDV-host interaction.

Keywords: rhabdovirus, SYDV, SYNV, nucleus, transgenic “16c” plant,

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Dedicated to the loving memory of my grandfather, Late Mr. Chitta Ranjan Ghose

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Chapter 1

1. Comprehensive literature review of rhabdoviruses

1.1 Introduction:

Rhabdoviridae is a family of viruses whose members infect vertebrates and invertebrates as well as plants. The family includes four genera of animal infecting virus viz. *Vesiculoviruses*, *Lyssaviruses*, *Ephemeroviruses* and *Novirhabdoviruses*, some of which are important human and animal pathogens such as *Rabies virus* (RV) (human pathogen), *Bovine ephemeral fever virus* (BEFV), *Vesicular stomatitis virus* (VSV) (livestock pathogens) and *Infectious haematopoietic necrosis virus* (IHNV) (fish pathogen). The plant adapted rhabdoviruses are divided into the *Nucleorhabdovirus* and *Cytorhabdovirus* genera (Table 1.1). They have a characteristic bullet or bacilliform shape and a host-derived lipid envelope. In general, the chemical composition of rhabdoviruses varies from 65% to 75% protein, 1% to 2% RNA, 15% to 25% lipid, and 3% G protein carbohydrate (Jackson et al., 2005). The rhabdovirus genome encodes at least five major proteins; the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the RNA-dependent RNA polymerase (L). One of the unique features of the negative strand RNA genome of a rhabdovirus is that it can serve as the template for both transcription (mRNA synthesis) and replication (genome RNA synthesis). The linear single-strand RNA genome is tightly associated with monomers of N protein. Together with two other proteins, L and P, the genome RNA plus N complex constitutes the transcription as well as genome replication ribonucleoprotein particle (RNP) (Figure 1.1). The matrix (M) protein and the glycoprotein (G) are the major

structural proteins. Within the virion, the RNP is coiled to form a tight helix and is associated with the matrix protein (M).

1.2 Genomic organization of plant rhabdoviruses:

The plant-infecting rhabdoviruses contain a genome consisting of non segmented negative-sense, single-stranded RNA, with a length in the range of 11,000-13,000 nucleotides. Although about 100 plant rhabdoviruses have been described so far (Jackson et al., 2005), complete nucleotide sequences of only 8 plant-adapted rhabdoviruses are available, namely *Rice yellow stunt virus* (RYSV) (Huang et al., 2003), *Sonchus yellow net virus* (SYNV) (Heaton et al, 1989), *Maize fine streak virus* (MFSV) (Tsai et al., 2005), *Maize mosaic virus* (MMV) (Reed et al., 2005), *Taro vein Chlorosis virus* (TaVCV) (Revill et al. 2005) - genus *Nucleorhabdovirus*, *Lettuce necrotic yellow virus* (LNYV) (Wetzel et al., 1994), *Northern cereal mosaic virus* (NCMV) (Tanno et al., 2000) - genus *Cytorhabdovirus* and *Orchid fleck virus* – genus *Dichorhabdovirus* (proposed) Kondo et al., 2006). Partial nucleotide sequence is also available for *Strawberry crinkle virus* (SCV) (Posthuma et al., 2002). Amongst all the sequenced plant rhabdoviruses, SYNV is the most extensively characterized at both the molecular as well as the cellular level (Jackson et al., 1999, Tsai et al., 2005). *Potato yellow dwarf virus* (PYDV) is the type member of the genus *Nucleorhabdovirus* of the family *Rhabdoviridae*, yet no sequence or cell biology data are available to date for further classification.

The genomes of almost all sequenced plant rhabdoviruses possess more than five open reading frames (N, P, M, G and L). The additional gene in SYNV is “sc4”, which is considered a putative viral cell-to-cell movement protein that facilitates

virus movement through plasmodesmata and is indicative of adaptive modification in regard to rhabdovirus host-plant cell interaction (Heaton et al, 1989) (Figure 1.2). This 37 kDa protein is membrane-associated and is rich in serine and threonine residues (16%). LNYV contains a novel gene called “4b” whose function is still unknown (Wetzel et al., 1994). There are two additional genes in RYSV genome: gene 3 and gene 6. Gene 3 is found between the P and M genes where additional ORFs have been identified in all plant rhabdoviruses genomes examined so far. However, RYSV gene 6 is located in between G and L genes, where the presence of the novel gene(s) has been detected in some animal rhabdoviruses only (Huang et al., 2003). The genome of NCMV contains 4 novel ORFs in between the P gene and M gene and these ORFs are named gene 3, gene 4, gene 5, and gene 6, respectively. Though the organization and their size is very similar, there is no homology of these regions as compared to other rhabdoviruses (Tanno et al., 2000).

Another striking feature of rhabdovirus genome is the presence of nearly identical gene junction sequences. The gene junctions of the rhabdovirus genome have short, virtually identical nucleotide sequences that provide signals for transcription initiation, termination and polyadenylation of viral mRNA (Table 1.4). The gene junction sequences are composed of three distinct regions, a poly-U tract near the 3' end of each gene (element I), a dinucleotide “spacer” (element II) and a conserved region at the 5' end of subsequent gene (element III). Homologies are found in the intergenic regions of different rhabdoviruses, especially at the 3' ends of the mRNAs (Heaton et al., 1989, Wetzel et al., 1994). The gene junction sequences are not only highly conserved amongst plant rhabdoviruses (Jackson et al., 2005), but show marked similarity when compared with the gene junction sequence of animal rhabdoviruses. The first three nucleotides in

the element III of gene junction sequences are UUG in case of SYNV, VSV and Rabies virus (Heaton et al., 1989), which shows the evidence of conserved gene junction sequences amongst plant and animal rhabdoviruses.

1.3 Properties of rhabdoviral Nucleocapsid (N) and Phosphoprotein (P):

The Nucleocapsid protein (N)

The N protein encapsidates the entire genomic RNA and packages it into a core that serves as template for both viral mRNA transcription and genomic/antigenomic RNA replication. The N protein is an important part of the viral ribonucleoprotein (RNP) core. The assembly of N protein with viral RNA genome was explicitly described in VSV. The 2.9 Å VSV N-RNA complexes consists of 10 molecules of N protein with 90 bases of RNA and over 1200 copies of N protein tightly associated with genomic RNA (Green et al., 2006). SYNV N protein has been shown to contain a bipartite nuclear localization signal (NLS) – RKRR and KPKK at the proximity of carboxy (C) terminal (Goodin et al., 2001). Similar NLS's have been found near the C-terminus of the MFSV, RYSV and OFV-N protein (Table 1.2). No such signal was detected in the case of the TaVCV and MMV-N protein. In the absence of the P protein, N is localized throughout the nucleus of the infected cell in case of SYNV (Goodin et al., 2001) and MFSV (Tsai et al., 2005), where as interaction with the P protein results in subnuclear (restricted to a region inside the nucleus) and nucleolar localization, respectively.

The Phosphoprotein (P):

The P protein is a part of the RNP core and comprises the viral RNA dependent polymerase complex along with L protein (Jayakar et al., 2004). The

polymerase complex is responsible for both viral transcription and replication. The P protein can be found in soluble form in the infected cells. The P protein in fact, acts as chaperone for both N and L polymerase proteins (Qanungo et al., 2004, Green et al., 2000). The SYN V and the VSV- P protein forms complex with L and N proteins which might function as the viral transcriptase and replicase complex, respectively (Jackson et al., 2005, Qanungo et al., 2004). The SYN V and VSV-P protein are found to be phosphorylated in threonine and serine residues, respectively (Jackson et al., 2005; Chen et al., 1997). Phosphorylation of the VSV-P protein in specific residues is important in regulating viral transcription and replication. Studies with the VSV-P protein showed that phosphorylation of precise serine residues at amino terminal acidic domain I and domain II are required for viral transcription and replication respectively (Hwang et al., 1999). Subcellular localization studies of the autofluorescent protein fusion of SYN V-P revealed localization of P in the nucleus as discrete ring-shaped structure along with detection of some cytoplasmic fluorescence around the peripheries of the cell (Goodin et al., 2007 and 2001). In contrast, MFSV-P localized throughout the cell (Tsai et al., 2005). SYN V P protein contains a bipartite nuclear localization signal (NLS) – RKRK and RKHR at the proximity of carboxy (C) terminal. Similar NLS's have been found near the C-terminus of the MMV, RYSV and OFV-P protein (Table 1.2). No such signal was detected in the case of the TaV CV and MSFV-P protein.

A comparison of nucleotide and ORF sequences of rhabdoviral N and P proteins is discussed more in detail in chapter 3.

1.4 Biology of N and P protein in brief:

This dissertation reports the complete nucleotide and deduced amino acid sequence of N and P mRNA of *sanguinolenta* strain (SYDV) of PYDV along with their characterization, subcellular localization and comparative sequence analysis of SYDV-N and P protein with other animal and plant rhabdoviruses in an attempt to characterize SYDV at both the molecular as well as the cellular level. N and P proteins are considered as very important structural proteins and crucial for replication of genomic RNA and transcription of mRNA of rhabdoviruses. The N protein encapsidates the genomic RNA along its entire length and the concentration of the N protein regulates the switch of transcription and replication. The structure of N protein and its interaction pattern with P protein and RNA has been extensively characterized for VSV. In VSV, 10 molecules of N protein binds with 90 bases of RNA forming a 2.9 angstrom complex where RNA is tightly sequestered in a cavity between two lobes of N protein and the ratio of VSV N: P is 2: 1 for N/P/RNA complex (Green et al, 2006). Along with its role as an important part of the viral transcriptase and replicase complex, the P protein also acts as a chaperone protein for N and L proteins and forms N-P, L-P and P-P interactions. Furthermore, phosphorylation of VSV-P protein regulates the transcription and replication of viral mRNA and genomic RNA, respectively (Pattnaik et al, 1997). Extensive research on VSV-P protein showed that VSV-P is phosphorylated by cellular Caesin kinase II (Gupta et al., 1995) and phosphorylation of serine residues at the amino terminal acidic domain I and domain II of the P protein are required for viral transcription and replication respectively (Figure 1.3) (Hwang et al., 1999). In addition, phosphorylation of specific serine residues in domain I and II showed that VSV-P protein is vital for virus

morphogenesis (Das et al, 2004). Mutations in the phosphate acceptor sites in domain I and II of VSV-P protein negatively impact virus morphogenesis (Figure 1.3). Virus recovery was completely abolished when the phosphate acceptor sites in domain I and II of P protein were replaced with alanine (Das et al., 2004). In addition to replication and morphogenesis, phosphorylation of specific residues of P protein is also important for protein-protein interactions. The P protein of *Chandipura virus* (CHP), another member of genus *Vesiculovirus*, is found to have an N terminal domain of 46 amino acids, which is phosphorylated by Caesin kinase II and the phosphorylation is required for P-P interactions (Raha et al., 2000). In VSV, the interaction between N and P does not require phosphorylation of P protein. Five amino acids at the C terminus (Val-Glu-Phe-Asp-Lys) of N protein and N terminal domain I along with C terminal domain III of P protein are important for N-P interaction in VSV (Takacs et al., 1993). The most extensively studied plant rhabdovirus, SYNV, also showed a similar kind of N-P interaction and P-P interaction like their animal counterparts, although the interacting domains in the case of SYNV are different when compared to VSV. An N terminal 72 amino acid domain of N protein and N terminal 80 amino acid domain of P protein are involved in N-P interaction for SYNV, where as a centrally located domain is essential for P-P interactions (Goodin et al., 2001). This central domain (aa position 40-124) also serves as the karyophilic domain for SYNV-P (Goodin et al., 2001). Recently, a study with *Newcastle disease virus*, a member of the genus *Avulavirus*, family *Paramyxoviridae* showed a C terminal 45 amino acid domain (aa 247-291) of the P protein is required for both N-P and P-P interactions (Jahanshiri et al., 2005). Taken together, available data for N-P and P-P

interactions for both plant and animal viruses in the order *Mononegavirales* suggest that there may also be similar domains necessary for protein-protein interactions in SYDV.

1.5 Comparison of the replication cycle of *Nucleo-* and *Cytorhabdoviruses*:

As stated earlier, members of plant rhabdoviruses are divided into two genera – *Nucleo-* and *Cytorhabdoviruses*, based on their sites of replication and maturation (Figure 1.4). Most plant rhabdoviruses enter into their host cells by insect vector feeding because of the rigid cell wall in plant cells. Upon entry, uncoating of the lipid envelope takes place in the endoplasmic reticular (ER) membrane and the ribonucleoprotein (RNP) core released into the cytoplasm. At this stage, members of *nucleo-* and *cytorhabdovirus* follow two different paths to complete their replication cycle. In the case of *cytorhabdoviruses*, the RNP core associates with the ER and become transcriptionally active to synthesize viral messenger RNAs (mRNAs). Translation of viral mRNAs occur in the cytoplasm and the resulting viral proteins (N, P and L) accumulated in the viroplasm and take part in antigenome and genome RNA synthesis. Viral G protein is directed to the cytoplasmic membrane, or outer nuclear membrane. The maturation of virion occurs through M protein mediated condensation of RNP core at the site of G protein accumulation in the ER membrane. It is hypothesized that in case of the *nucleorhabdoviruses*, RNP core moves into the nucleus through nuclear pore complexes (NPCs), and transcription of viral mRNAs occurs in the nucleus. Following transcription, viral mRNAs export into the cytoplasm, where they are translated into viral proteins with the help of host cell protein synthesis machinery. Viral proteins are then transport to the nucleus and initiate replication of genomic and antigenomic RNA. Morphogenesis of

nucleorhabdoviruses occurs either by invagination of inner nuclear membrane (e.g. SYNV) or expansion of outer nuclear membrane (e.g. PYDV) (Goodin et al., 2005).

1.6 Vector specificity of plant rhabdoviruses:

Most plant rhabdoviruses are transmitted by their insect vector (Table 1.3). The virus-vector relationship is very specific and in most of the cases, the virus is propagated by three distinct insect families, *Aphidae* (aphids), *Cicadellidae* (leafhoppers) or *Delphacidae* (planthoppers). For example, SYDV is transmitted by *Aceratagallia sanguinolenta*, and MMV is transmitted by *Peregrinus maidis* (Jackson et al., 2005). Recently, another putative member of genus *Nucleorhabdoviruses*, Sorghum Stunt Mosaic virus (SSMV) was reported to be transmitted by a leafhopper vector, *Graminella sonora* (Family *Cicadellidae*) (Creamer et al., 1997). Although some viruses are found to be spread by mechanical injuries in the leaves of host plants, insect mediated propagation is the major controlling factor of virus distribution. The plant rhabdoviruses are equally capable of replicating in their specific insect vectors. It is believed that epithelial lining of insect midgut cells are the major sites for virus replication with the viruses passed on from the infected midgut cells to the salivary gland via transport through hemolymph (Jackson et al., 2005, Black L.M., 1979; Nault L.R., 1997; Sylvester et al., 1992). To date, very little is known about the multiplication events of plant rhabdoviruses in their insect vector and the biology of virus-vector interaction. Further research in the field of virus-vector biology will provide a better understanding of the relationship between plant rhabdoviruses and their specific insect vector, which will prove beneficial for examining ways to control the transmission of agriculturally harmful viruses.

1.7 Potato yellow dwarf virus (PYDV) – type species of genus *Nucleorhabdoviruses*:

PYDV is the type species of genus *nucleorhabdoviruses*, which was first reported in the common potato plant, *Solanum tuberosum* from the U.S.A. (Barrus and Chupp; 1922). The PYDV virion is enveloped, rhabdo- or bullet-shaped, and usually straight, with a length of 380 nm and width of 75 nm. Virions contain 2 % nucleic acid, 78 % protein and 20 % lipid (*source: VIDE database*). The genome consists of a single-stranded linear negative sense RNA with a total genome size of 12.6 kb. Virion replication does not depend on a helper virus and virions are found in the perinuclear space mainly after replication and morphogenesis. Viral inclusions are found in the leaves, roots, stem, trichome, and phloem tissue of infected plant (Black 1938, 1970). Experimentally, PYDV-infected plants exhibit symptoms which include vein clearing, stunting, leaf malformation, mosaic (intermingling patches of normal and light green to yellow color, delineated by veins, angular areas against a yellow background) and chlorotic local lesions (Black, 1970) (Figure 1.5 A). *Nicotiana rustica* is widely used as the virus maintenance and propagation host as well as the viral assay host. Recently we have shown in our lab that the PYDV successfully infects *N.benthamiana* (1.5 B).

1.8 Vector specificity, host range and strains of PYDV:

PYDV is transmitted in a persistent manner by an insect (leafhopper) vector (Family: Cicadellidae) and by manual inoculation to *Nicotiana rustica*. There are two different strains of PYDV, namely SYDV (transmitted by *Aceratagallia sanguinolenta*) and CYDV (transmitted by *Agallia constricta*). *Agallia quadripunctata* sometimes poorly transmits both strains of PYDV (Black L.M. 1970). Viruses are generally retained when the vector molts and multiplies in the vector. The nymph and the

adult male and female can transmit the virus. SYDV (Black L.M., 1943) (Nagaraj and Black L.M., 1962) has not been found to be transmitted through egg or sperm of the vector, but in some strains of CYDV, it is occasionally transmitted through the egg (Black L.M., 1953). The experimental mode of virus transmission is by mechanical inoculation. All known plant hosts of SYDV are dicotyledons (Black L.M., 1970). Although plants of the *Solanaceae* family are the most susceptible to virus infection, other possible hosts include members of the family *Compositae*, *Cruciferae*, *Labiatae*, *Leguminosae*, *Polygonaceae* and *Scrophulariaceae* (Younkin, 1942; Frampton et al., 1942). The diagnostically susceptible hosts of SYDV are *Nicotiana glutinosa*, *Nicotiana rustica* and *Trifolium incarnatum*. The ox-eye daisy, (*Chrysanthemum leucanthemum*) also acts as one of the principal host plant for SYDV. The infection intensity of SYDV in the potato plants is temperature dependent. It was reported that high temperature favors rapid virus infection as compared to lower temperature (Walker and Larson, 1939; Black L.M. 1970).

1.9 Geographical distribution of PYDV:

PYDV is generally found in regions of Northeastern United States and adjacent parts of Canada. The SYDV and CYDV strains were first identified in New York (NY) and New Jersey (NJ), respectively (The New York and New Jersey isolates of PYDV. Black L.M. 1970; Falk and Weathers, 1983). Later another SYDV strain was identified in California (CA) (California isolate of PYDV, Falk and Weathers, 1981). Serological studies indicate that SYDV-NY and SYDV-CA are more closely related to each other than CYDV-NJ (Falk and Weathers, 1981). The virus had not been seen in the

mid-west of the USA for forty years, until an outbreak in Minnesota in between 1986-88 on ornamental plants (Lockhart, 1989).

1.10 Serological relationship between PYDV strains:

There are two different strains of PYDV based on their insect vector specificity, SYDV (*sanguinolanta* strain) and CYDV (*constricta* strain) (Black L.M. 1970, Falk and Weathers, 1983). As discussed, two isolates of SYDV (SYDV-NY and SYDV-CA) and one isolate of CYDV (CYDV-NJ) have been described. Early serological studies between these three isolates showed SYDV-NY and SYDV-CA are more closely related to each other than CYDV-NJ (Falk and Weathers, 1983). The serological differentiation study between these two strains revealed that CYDV has greater cross-reactivity with SYDV encoded proteins when compared to reciprocal cross-reaction. Western immunoblot analysis of total protein isolated from SYDV and CYDV infected *N. benthamiana* leaves probed with CYDV primary antibody (obtained from Dr. Hei-Ti-Hsu, USDA) showed that CYDV primary antibody cross-reacts with at least two SYDV proteins, the G (Glycoprotein) and N (Nucleocapsid) proteins. Similar blot failed to cross-react with any CYDV encoded protein when probed with SYDV primary antibody (obtained from Dr. Hei-Ti-Hsu, USDA) (Figure 1.6). Another serological immunoblot of PYDV was conducted to verify the serology data obtained so far using SYDV and CYDV polyclonal antibodies from different source (Dr. Andrew Jackson's lab UC, Berkley). These antibodies were raised in rabbits against disrupted virions and antibodies from two different bleeds of rabbits were obtained. Western immunoblot analysis of total protein isolated from SYDV and CYDV infected *N. benthamiana* leaves probed with SYDV and CYDV primary antibody from both bleeds repeated the

serological cross-reactivity between those strains (Figure 1.7 A,B,C,D) when compared with the data obtained from similar analysis but using different source of antibodies (Figure 1.6). The CYDV primary antibody cross-reacts with SYDV-G and N protein, but no reciprocal cross-reaction is detected with the SYDV primary antibody. Cross-reaction with total protein isolated from mock and SYNIV infected *N. benthamiana* leaves were also negative. The serological relationship data of SYDV and CYDV will be helpful for the effective and reliable diagnosis of the symptoms of SYDV and CYDV infection in the host plant along with widely used electron microscopy.

Following the validation of the antibodies, a series of western immunoblot were conducted with total protein isolated from SYDV and CYDV infected *N. benthamiana* leaves probed with different dilutions of antibodies from both bleeds to determine the best working antibody dilution. Three different dilutions, 1:500, 1:1000 and 1: 2000 were tested. Three major proteins, G, N and P were detected in the blot probed with SYDV and CYDV polyclonal antibodies in 1:500 as well as 1:1000 dilutions from both bleed (Figure 1.7 E, F, G, and H). Therefore, antibodies raised from both bleeds will be useful in 1:1000 working dilutions for any future immunoblotting experiments. Taken together, these two serologically distinguishable strains of PYDV may serve as models to study the comparative cell biology between themselves as well as with other plant rhabdoviruses and biology of virus-host interaction. Because in addition to their vector specificity, SYDV and CYDV may differ in their titers in infected plants, ease of purification, timing of symptom development and other biological properties that may contribute their differential reaction with host cells.

In this dissertation, cloning and complete sequence analysis of Nucleocapsid (N) and Phosphoprotein (P) mRNA from cDNA clones derived from both SYDV genomic and messenger RNAs are reported. The deduced amino acid sequence of SYDV “N” protein and the N proteins of other well characterized plant as well as animal rhabdoviruses were compared in order to establish a phylogenetic relationship between these viruses. Further, *in planta* subcellular localization studies of SYDV “N” and “P” protein showed the localization of those proteins in the infected nucleus of *Nicotiana benthamiana* plant cells. Successful completion of cloning and sequencing of PYDV strains will be helpful to develop virus specific antibodies and a plant expression vector for use in tobacco (*N. tabacum*).

Table 1.1: Examples of Animal and Plant infecting rhabdoviruses.

Animal infecting rhabdoviruses

Genus	Example
Vesiculovirus	<i>Vesicular stomatitis virus (VSV)</i>
Lyssavirus	<i>Rabies virus (RV), Australian bat lyssavirus (ABLV), Mokola virus (MV)</i>
Ephimerovirus	<i>Bovine ephemeral fever virus (BEFV)</i>
Novirhabdovirus	<i>Infectious haematopoietic necrosis virus (IHNV), Snakehead rhabdovirus (SRV)</i>

Plant infecting rhabdoviruses

Genus	Example
Nucleorhabdovirus	<i>Potato yellow dwarf virus (PYDV), Sonchus yellow net virus (SYNV), Maize fine streak virus (MFSV), Taro vein chlorosis virus (TaVCV)</i>
Cytorhabdovirus	<i>Lettuce necrotic yellow virus (LNYV), Northern cereal mosaic virus (NCMV)</i>

The family *Rhabdoviridae* is divided into six genera, four of which are animal-infecting and two are plant-infecting rhabdoviruses. Plant rhabdoviruses are classified based on their site of replication and maturation, either in nucleus (*Nucleorhabdoviruses*) or in cytoplasm (*Cytorhabdoviruses*). PYDV is the type species of genus *Nucleorhabdoviruses*.

Table 1.2: Nuclear localization signal (NLS) of N and P protein of plant *Nucleorhabdoviruses*.

Virus	N-NLS	P-NLS
<i>Potato yellow dwarf virus-sanguinolenta</i> strain (SYDV)	None	None
<i>Sonchus yellow net virus</i> (SYNV)	RKRR, KPKK	RKRK, RKHR
<i>Maize fine streak virus</i> (MFSV)	KRSSDGTGNVSKKSRK	None
<i>Maize mosaic virus</i> (MMV)	None	KRPR
<i>Rice yellow stunt virus</i> (RYSV)	KKLGPPRANAHSRRKE	RKDSHHYRTVVSRIEKK
<i>Taro vein chlorosis virus</i> (TaVCV)	None	None
<i>Orchid fleck virus</i> (OFV)	RKRH	PKRK, KRKH

ORFs of N and P protein of the members of genus *Nucleorhabdovirus* were analyzed using pSORT algorithm (Nakai et al., 1995) for their respective putative NLS's. SYNV had a putative bipartite NLS for both N and P protein, and OFV had a putative bipartite NLS for its putative P (ORF2) protein. Other viruses have monopartite putative NLS's for N or P protein unless otherwise stated. Analysis of SYDV and TaVCV revealed no putative NLS for N and P protein.

Table 1.3: List of plant rhabdoviruses (*Nucleo-* and *Cytorhabdoviruses*) and their specific insect vector.

Virus	Insect vector type
Genus <i>Nucleorhabdoviruses</i>	
<i>Potato yellow dwarf virus-sanguinolenta</i> strain (SYDV)	Cicadellid leafhopper <i>(Aceratagallia sanguinolenta)</i>
<i>Sonchus yellow net virus</i> (SYNV)	Aphid <i>(Aphis coreopsidis)</i>
<i>Maize fine streak virus</i> (MFSV)	Cicadellid leafhopper <i>(Graminella nigrifrons)</i>
<i>Maize mosaic virus</i> (MMV)	Delphacid planthopper <i>(Peregrinus maidis)</i>
<i>Rice yellow stunt virus</i> (RYSV)	Cicadellid leafhopper
<i>Taro vein chlorosis virus</i> (TaVCV)	unknown
Genus <i>Cytorhabdoviruses</i>	
<i>Lettuce necrotic yellows virus</i> (LNYV)	Aphid <i>(Hyperomyzus lactucae)</i>
<i>Northern cereal mosaic virus</i> (NCMV)	Delphacid planthopper <i>(Laodelphax striatellus)</i>

Only those viruses used in this study for the purpose of comparative analysis were taken into account. [Data source: VIDE (Virus Identification Data Exchange); *Biology of plant rhabdoviruses. Annu Rev Phytopathol* (2005)43:623-60]

Table 1.4: Comparison of gene junction sequences between plant and animal rhabdoviruses.

Virus	Element I	Element II	Element III
<i>Sonchus yellow net virus</i> (SYNV)	AUUCUUUUU	GG	UUG
<i>Lettuce necrotic yellows virus</i> (LNYV)	AUUCUUUUU	G(N) ^x	CUU
<i>Vesicular stomatitis virus</i> (VSV)	ACUUUUUUU	TU	UUG
<i>Rabies virus</i> (RV)	ACUUUUUUU	T(N) ^x	UUG

Example of members from *Nucleo-* (SYNV) and *Cytorhabdovirus* (LNYV) were compared with members of animal rhabdoviruses (VSV, genus *Vesiculoviruses* and RV, genus *Lyssaviruses*). The rhabdoviral gene junction is divided into three distinct elements – I, II and III (please see page 21 for detail explanation). Note that element III is conserved (UUG) for SYNV, VSV and Rabies virus. “T” represents either “G” or “C” nucleotide. “N” represents the presence of either “A”, “U”, “G” or “C” nucleotide. “X” denotes occurrence of variable number of nucleotides in that position.

(Data source: Jackson et al., *Annu Rev Phytopathol* (2005)43:623-60; Heaton et al., *Proc. Natl. Acad. Sci. USA* (1989) 86:8665-8668)

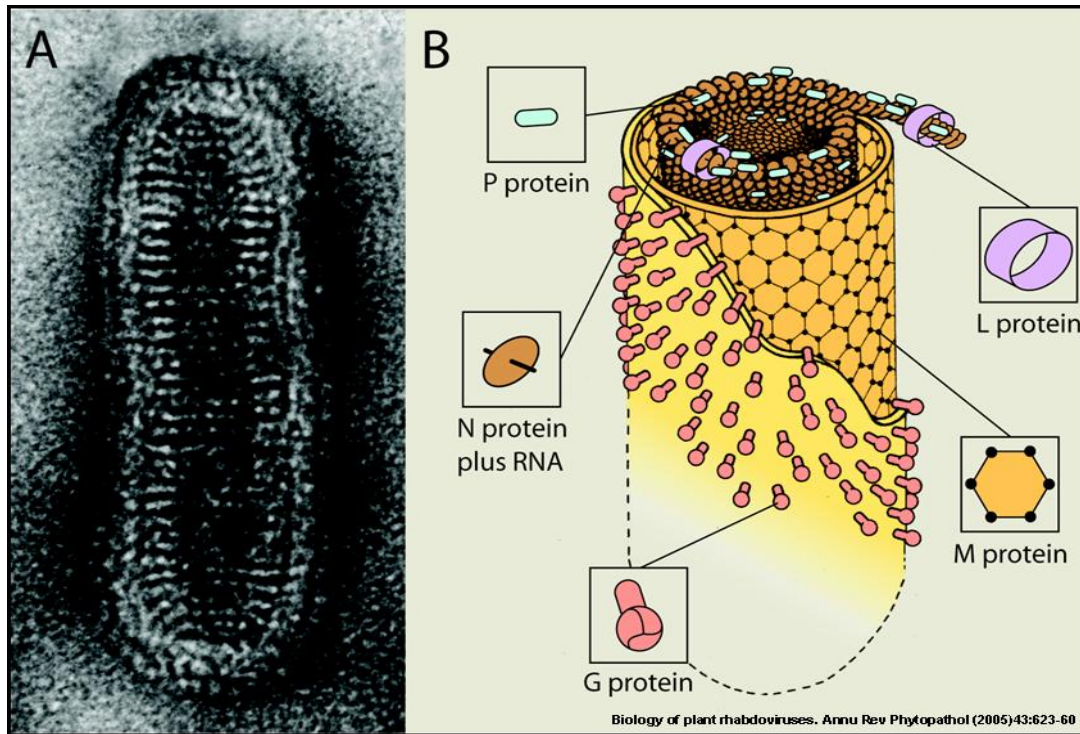


Figure 1.1: Generalized morphology of rhabdoviruses. **A.** Transmission electron micrograph of *Lettuce necrotic yellows virus* showing the bacilliform shaped virus particle. **B.** Diagram illustrating the structure of a virion. The RNP core consists of negative-sense and single stranded genomic RNA encapsidated with N, P and L protein. The M protein is responsible for RNP core condensation and attached with G protein. The G protein is a surface glycoprotein associated with host derived lipid envelop.

(Picture courtesy: *Biology of plant rhabdoviruses. Annu Rev Phytopathol (2005)43:623-60*)

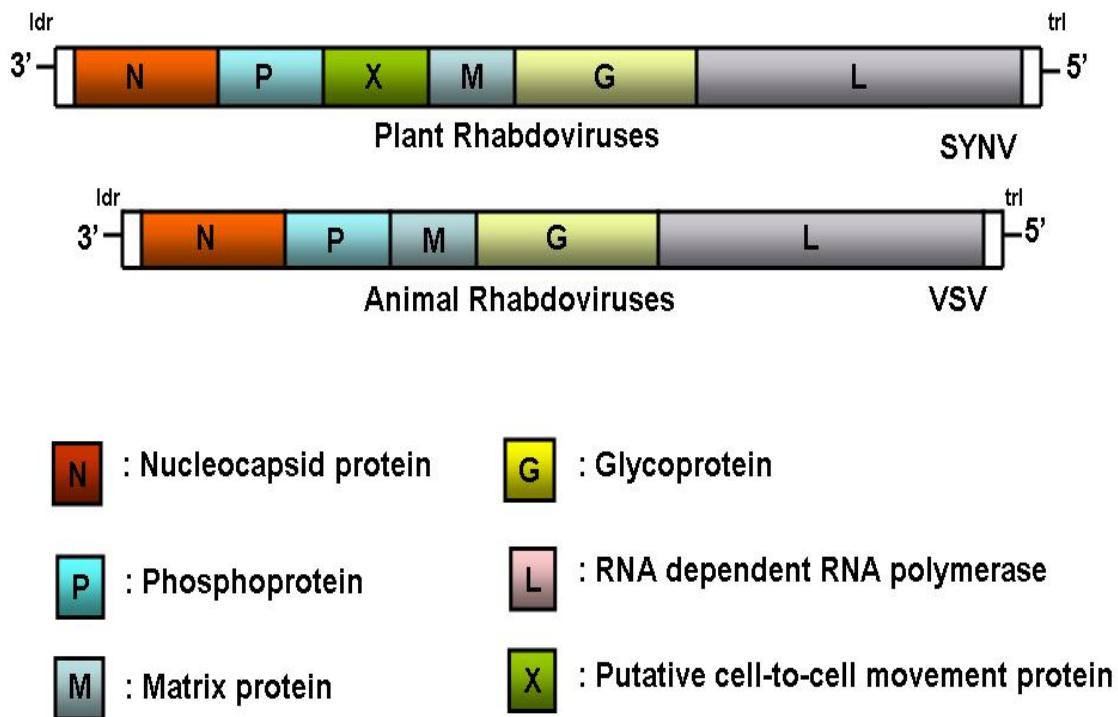


Figure 1.2: Comparison of negative-sense genomic organization between plant (SYNV) and animal (VSV) rhabdovirus. The genome of rhabdovirus primarily comprises of five genes *viz.* N, P, M, G and L gene with a 3'-leader and 5'- trailer sequences. Plant rhabdoviruses contain additional genes (X), which are thought to encode the putative cell-to-cell movement protein. In case of SYNV, the putative cell-to-cell movement protein gene is SC4.

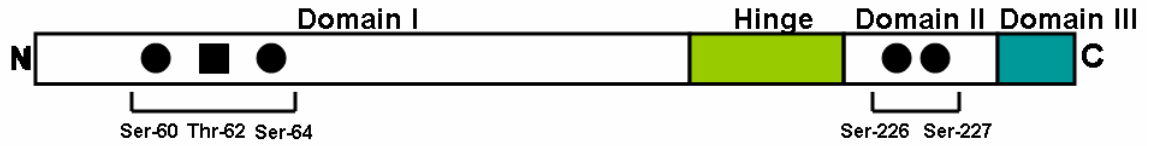


Figure 1.3: Structure of VSV-P protein. VSV-P protein is divided into three functional domains, Domain I, II, III and a hinge region. The phosphate acceptor sites are Serine residues at amino acid position 60, 64 in domain I and at amino acid position 226 and 227 in domain II (showed in solid black circle).

(Figure adapted from Das et al, 2004 Journal of Virology Vol. 78 No. 12)

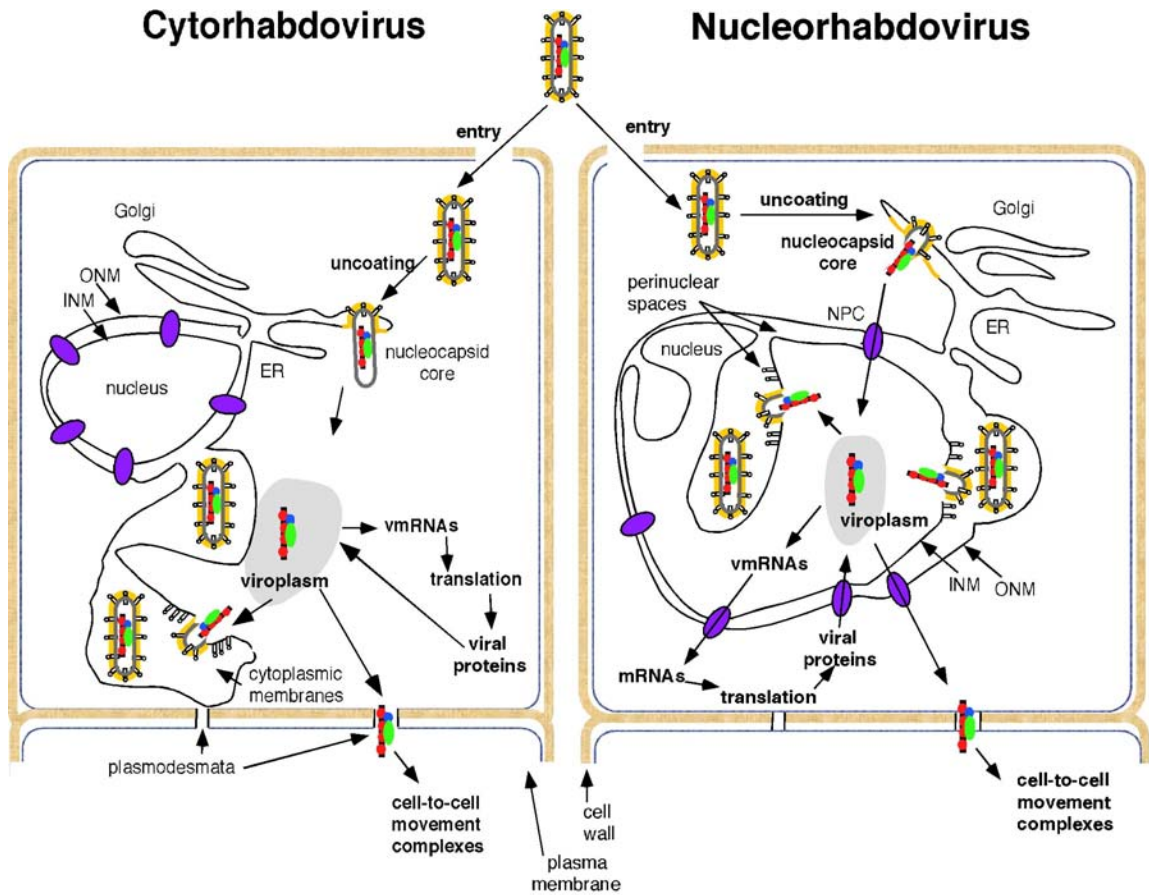


Figure 1.4: Comparison of multiplication cycle between *Nucleo-* and *Cytorhabdoviruses* in the host cell. Upon infection, uncoating takes place in the ER membrane and the RNP core is released into the cytoplasm. In case of *Cytorhabdoviruses*, transcription of viral mRNAs and replication of viral genomic and anti genomic RNAs occur in the cytoplasm, whereas in case of *Nucleorhabdoviruses*, the RNP core is transported into the nucleus, transcription of viral mRNAs and replication of viral genomic and antigenomic RNAs takes place in the nucleus only. [INE: Inner Nuclear membrane, ONE: Outer Nuclear Membrane]

(Picture courtesy: *Biology of plant rhabdoviruses. Annu Rev Phytopathol (2005)43:623-60*)

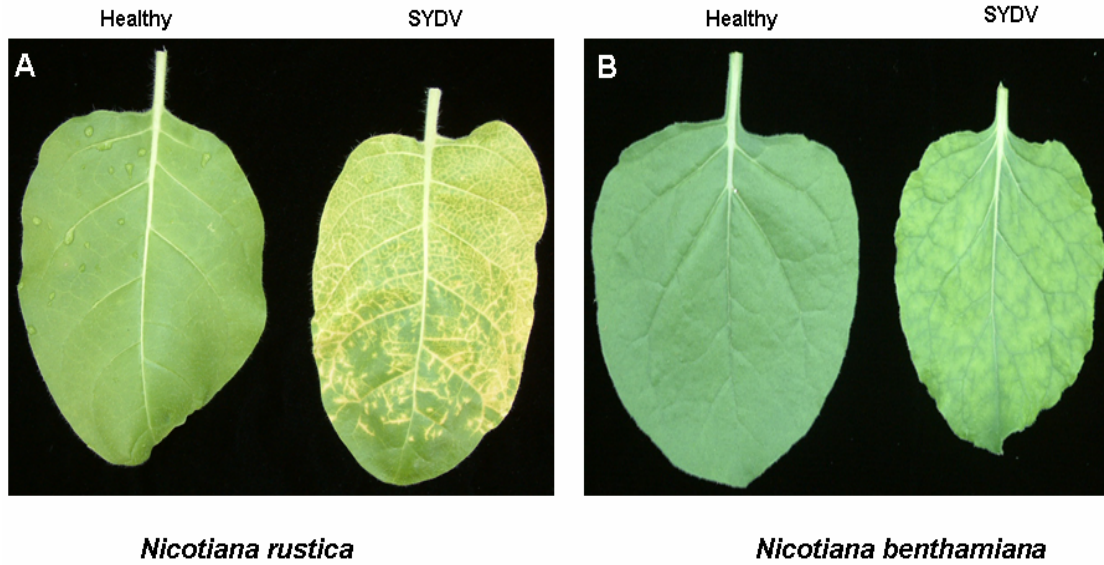


Figure 1.5: Symptoms of SYDV in infected leaves of **A.** *Nicotiana rustica* and **B.** *Nicotiana benthamiana*. Leaves from mock-inoculated plants of the same age are shown for comparison. The symptoms mainly include intermingling patches of light green to yellow color, delineated by veins, and chlorotic local lesions

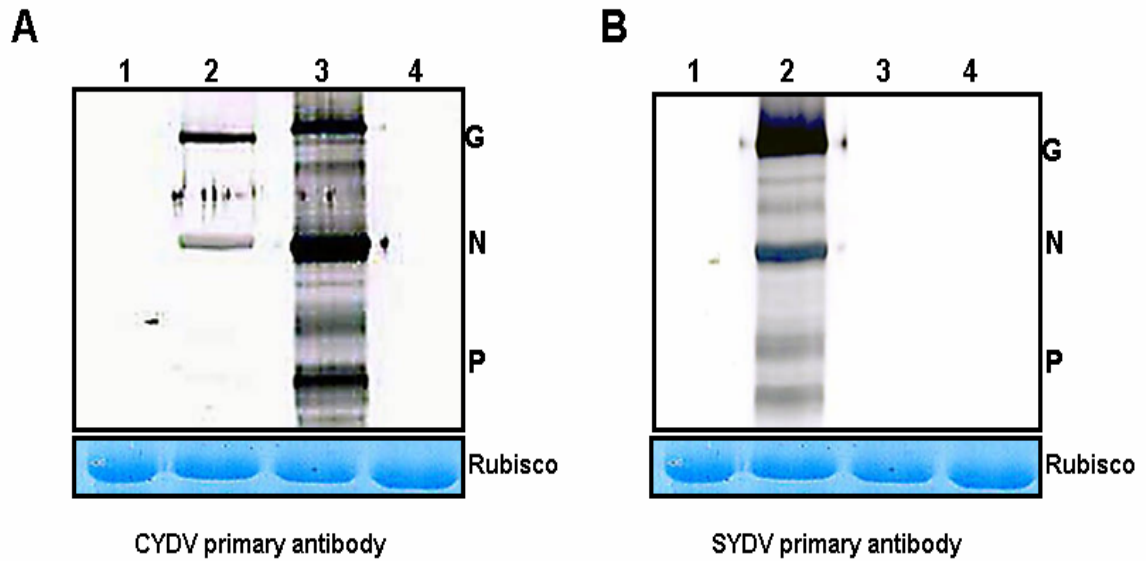


Figure 1.6: Western immunoblots demonstrating the serological relationship between the SYDV and CYDV. Following separation on 12% SDS-PAGE gels, equivalent amounts of protein isolated from healthy *N. benthamiana* (lane 1) SYDV (lane 2), CYDV (lane 3) and SYNIV-infected (lane 4) *N. benthamiana* leaves were transferred to nitrocellulose membranes and probed with **A.** anti-CYDV or **B.** anti-SYDV polyclonal antibodies. Note that the CYDV antibodies cross-react with SYDV G and N protein. A coomassie stained gel image showing rubisco (50 kD) were used as loading controls.

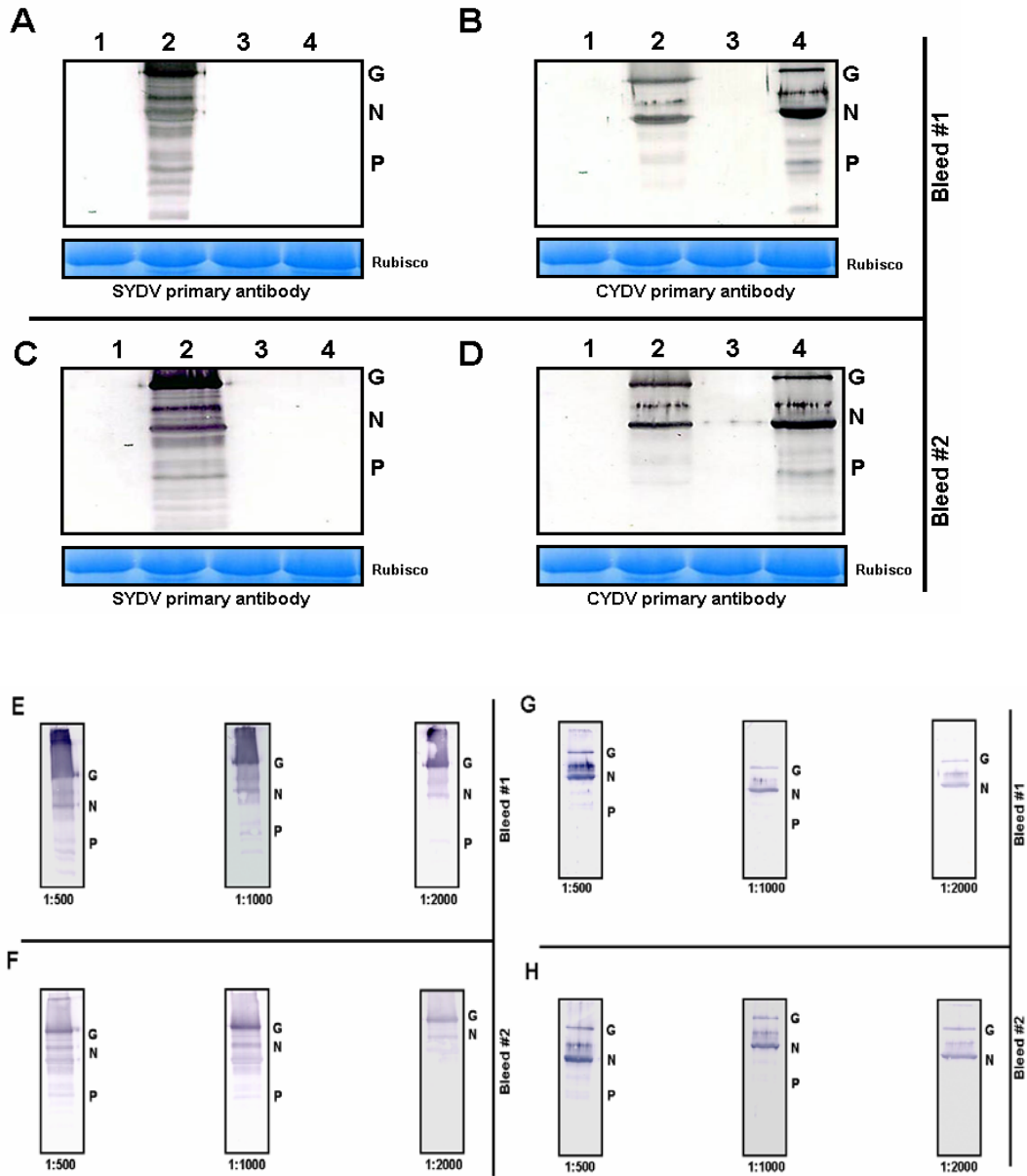


Figure 1.7: Serological western immunoblot analysis of SYDV and CYDV using different source of anti-SYDV and CYDV antibodies. Top panel (A, B) and lower panel (C, D) shows the immunoblot image using PYDV primary antibodies raised in rabbits to disrupted virions from bleed #1 and bleed #2 respectively. Following

separation on 12% SDS-PAGE gels, equivalent amount of protein samples isolated from healthy *Nicotiana benthamiana* (lane 1) SYDV (lane 2), SYNIV (lane 3) and CYDV-infected (lane 4) *Nicotiana benthamiana* leaves were transferred to nitrocellulose membranes and probed with **A.** and **C.** anti-SYDV or **B.** and **D.** anti-CYDV polyclonal antibodies. Note that the CYDV antibodies cross-react with SYDV proteins. A coomassie stained gel image showing rubisco (50 kD) were used as loading controls. **E, F.** Western immunoblot analysis of equivalent amount of protein from SYDV infected *Nicotiana benthamiana* leaves probed with different dilutions (1:500, 1:1000 and 1:2000) of anti-SYDV primary antibody. **G, H.** Western immunoblot analysis of equivalent amount of protein from CYDV infected *Nicotiana benthamiana* leaves probed with different dilutions (1:500, 1:1000 and 1:2000) of anti- CYDV primary antibody.

Chapter 2

2. Preliminary experiments: The infection pattern of SYDV in host cells

2.1 Introduction:

The effect of plant rhabdovirus infection on host cell biology is poorly understood. To determine the possible effects of such infection on the endomembrane system of infected host cells, a series of experiments was conducted with two serologically distinct plant rhabdoviruses, SYDV and SYNV. The fact that both SYDV and SYNV infect *N. benthamiana* is important because it will help in comparing the cytopathology and genomics of SYDV with the well-characterized plant nucleorhabdovirus, SYNV (Heaton et al., 1989). A major characteristic of nucleorhabdovirus infections is the formation of large proteinaceous inclusions or “viroplasms” in nuclei of infected cells (Christie and Edwardson, 1977). The viroplasms induced by SYNV have been found to contain the N, P, and L proteins in addition to the viral genomic and anti-genomic RNA (Martins et al., 1998).

To study the infection pattern of SYDV in the host plant cell, experiments were conducted to monitor the pattern of GFP accumulation in nuclei of infected cells in *Nicotiana benthamiana* 16c plants that constitutively express GFP targeted to endomembrane (GFP-HDEL). The increased accumulation of GFP and membrane in the infected nuclei of different tissue types is an indication of virus infection (Figure 2.1), which was monitored by epifluorescence as well as Laser Scanning Confocal Microscopy (Goodin et al, 2005). The microscopy and counter-staining with the DNA-selective dye DAPI confirmed the accumulation of GFP within nuclei in rhabdovirus-infected tissues (Goodin et al., 2005). These studies also demonstrated that the pattern of GFP

accumulation is completely different for the two different species of nucleorhabdoviruses.

2.2 Plant material, growth conditions and virus inoculation procedures:

The SYDV strain was obtained from Dr. Hei-ti-Hsu at USDA (United States Department of Agriculture) with permission from Animal and Plant Health Inspection Service (APHIS; Permit # 62615).

Transgenic (16c) *N. benthamiana* plants, (Ruiz et al., 1998) that constitutively express the mgfp5-ER variant of the GFP were grown in the greenhouse under ambient conditions (please see Table 2.1 for relative temperature and humidity). Plants were inoculated with virus when they had 4-6 fully-expanded leaves. Virus-infected leaves were used as sources of inocula for all viruses, except TRV (*Tobacco rattle virus*) was “agroinoculated” as described by Liu et al (2002). SYNV or INSV -infected plant tissues were grounded with inoculation buffer (10 mM sodium phosphate, pH 6.9, 0.5% w/v NaSO₃, 1% celite) in a mortar and pestle. SYDV-infected tissues were grounded in approximately 2 volumes of 0.1 M sodium phosphate, pH 6.9. Leaf homogenate was applied to carborundum dusted leaves of *N. benthamiana* plants from which the apical meristem has been removed prior to inoculation. TEV (*Tobacco etch virus*) infected material was ground in 10 mM sodium phosphate buffer, pH 6.9. Mock-inoculations were conducted using virus-free leaves processed in the same buffer condition.

Results

2.3 SYDV infection induce increased accumulation of GFP in the infected nuclei of

Nicotiana benthamiana cells

Increased GFP accumulation was detected in SYDV as well as in SYNV infected cells, though the pattern of GFP accumulation was completely different in these two *nucleorhabdoviruses*. The GFP accumulation was found mainly in the external and internal loci of the infected nucleus in SYDV infected cells, where as, in the case of SYNV infection, GFP accumulated throughout the nucleus, mostly within the DAPI-stainable portion of the nucleus (Goodin et al., 2005) (Figure 2.2). The results of the experiments conducted in this study represent the demonstration of the ability to monitor rhabdovirus infections *in planta* by live-cell imaging which provides the means to study rhabdovirus infection in living cells and will thus facilitate studies of virus cell-to-cell movement. To confirm rhabdovirus-induced relocalization of GFP is associated with the membrane, live cell imaging was conducted by using fluorescent dyes (DAPI and BODIPY-TRme) to mark the location of nuclei and endomembranes respectively. The endoplasmic reticulum and nuclear membrane were stained and the GFP expressed in 16c *N.benthamiana* plants was targeted to endomembrane via the ER-retention “HDEL” sequence. The results confirmed that GFP expressed in 16c plants was membrane-associated and that this association was maintained in the virus-infected plants (Goodin et al, 2005). To show that GFP accumulation in nucleus in rhabdovirus infected plants is consistent with the site of virus accumulation, a coimmunolocalization experiment was carried out using anti-N (of SYNV) and anti-GFP polyclonal antibodies. Because N protein of SYNV is exclusively nucleus localized and tightly associated with single-

strand genome RNA of the virus (Wagner and Jackson, 1996; Wagner et al., 1997; Martins et al. 1998; Goodin et al., 2001, 2002), it can be used as an ideal marker for sites of virus accumulation. This experiment clearly showed that nuclei regions that reacted with N antibodies were coincident with the sites of increased GFP accumulation in DAPI-stained nuclei (Goodin et al., 2005). In contrast, there was no fluorescence signal detected by the N antibodies in mock-inoculated tissues.

2.4: GFP accumulates in both external and internal loci of nuclei in SYDV-infected tissue

Three dimensional optical projections were made from the SYDV-induced GFP accumulation. Optical sections were made through x, y and z planes of the projections. Careful observations through these optical slices of GFP accumulation clearly established that GFP was accumulated within as well as in the external foci of the infected nuclear periphery, which is a unique characteristic compared to SYNV-induced GFP accumulation scattered throughout the nucleus (Goodin et al., 2005).

2.5: Virus specific pattern of GFP and membrane accumulation in SYDV and SYNV-infected nuclei

It is hypothesized that the differences in the patterns of increased GFP accumulation and membrane relocalization depend on the manner in which SYDV and SYNV undergo morphogenesis. Invaginations of the inner nuclear membrane occur upon SYNV infection, through which condensed core particles bud into the perinuclear space (VanBeek et al., 1985), resulting nuclear accumulation of GFP. In contrast, SYDV buds into the perinuclear space resulting in expansion of the outer nuclear membrane with an apparent accumulation of GFP on the “outside” of the nucleus (Goodin et al., 2005).

Such accumulation of GFP on the external foci of the nucleus is consistent with the site of accumulation of mature SYDV particles determined by electron microscopy (MacLeod, et al., 1966). In contrast to nucleorhabdovirus-mediated GFP accumulation, infection of plants by viruses that replicate in cytoplasm, *Tobacco etch virus*, a potyvirus (TEV) and *Impatiens necrotic spot virus*, a tospovirus (INSV), induces proliferation of ER membranes by activity of their replicase proteins or G proteins.

2.6: Enlargement of nuclei occurs in only rhabdovirus-infected tissues

Another striking feature was that the rhabdovirus-infected nuclei in which GFP had accumulated were larger compared to the nuclei of mock-inoculated plants. The cross-sectional areas of nuclei in micrographs obtained by epifluorescence microscopy were measured to determine the size difference of nuclei in mock and virus infected cells. Statistical comparative analysis using SAS 9.0 reveals that the mean area of nuclei in mock-inoculated cells was much smaller when compared to the size of the nucleus of SYN and SYDV infected cells (Figure 2.3). Despite the presence of accumulation of GFP surrounding the nucleus of TEV and INSV infected cells, their area did not increase significantly as compared to those in mock-inoculated cells.

2.7: Hypothesis:

The infection pattern study showed that both SYDV and SYN induce accumulation of membrane-associated GFP (mgfp5-ER) in the nuclei of infected transgenic *Nicotiana benthamiana* cells. Since nucleorhabdovirus induced viroplasms contain polymerase associated N and P proteins as it was evident for SYN (Martins et al., 1998, Deng et al., 2007) and SYN induced increased accumulation of GFP in the nuclei of infected *Nicotiana benthamiana* cells is consistent with the site of viroplasm

formation (Martins et al., 1998, Goodin et al., 2005, Deng et al., 2007), N and P protein of SYDV should localize to the nucleus when expressed as autofluorescent protein fusions as these proteins are an inherent part of nuclear viroplasm.

Specific aims:

Specific aim 1: To clone and characterize the N and P gene of *Potato yellow dwarf virus -Sanguinolenta* (SYDV) strain at molecular level.

Specific aim 2: Study the subcellular localization of SYDV-N and SYDV-P protein in order to gain a better understanding of the biology of virus-plant interactions.

2.8: Significance

This live-cell imaging study is the first to document rhabdovirus-induced changes in the morphology of plant endomembranes during viral replication (Goodin et al, 2005) and will facilitate the study of rhabdovirus infection in living cells including virus cell-to-cell movement, infection processes and effects on host gene expression. The pattern of GFP accumulation in the infected nuclei was completely different for SYDV and SYNIV infection; although both viruses are belong to genus *Nucleorhabdovirus*. This different pattern of GFP and membrane accumulation between SYNIV and SYDV encourages further exploration in the area of molecular and cell biology of SYDV. Given the fact that SYNIV is well characterized at molecular level, the molecular data of SYDV should permit to explain the difference of their infection pattern at the cellular level. In addition to that, the live cell imaging technique is an efficient and non-destructive way to study the endomembrane dynamics of plant nucleorhabdovirus and virus-host interactions. Moreover, these series of experiments also determined that 16c *Nicotiana*

benthamiana plants that constitutively express GFP targeted to their endomembrane system could be used to monitor rhabdovirus-mediated change in the host cell.

Table 2.1: SYDV cage condition in greenhouse

Greenhouse 1	Zone4	Cage#2
Date	Time	Specific condition
22 nd February'05	9:00 a.m	Temperature: 26°C Relative Humidity: 38% Temperature Range: 20°C – 28°C
	3:00 p.m	Temperature: 29° C Relative Humidity: 27% Temperature Range: 26°C – 31°C
23 rd February'05	9:00 a.m	Temperature: 28° C Relative Humidity: 18% Temperature Range: 20°C – 29°C
	3:00 p.m	Temperature: 29° C Relative Humidity: 28% Temperature Range: 27°C – 31°C
24 th February'05	9:00 a.m	Temperature: 25°C Humidity: 34% Temperature Range: 20°C – 31°C
	3:00 p.m	Temperature: 27°C Humidity: 21% Temperature Range: 22°C – 30°C

Greenhouse Set points: Temperature: Day - 25°C
Night - 22°C
14 hour day length

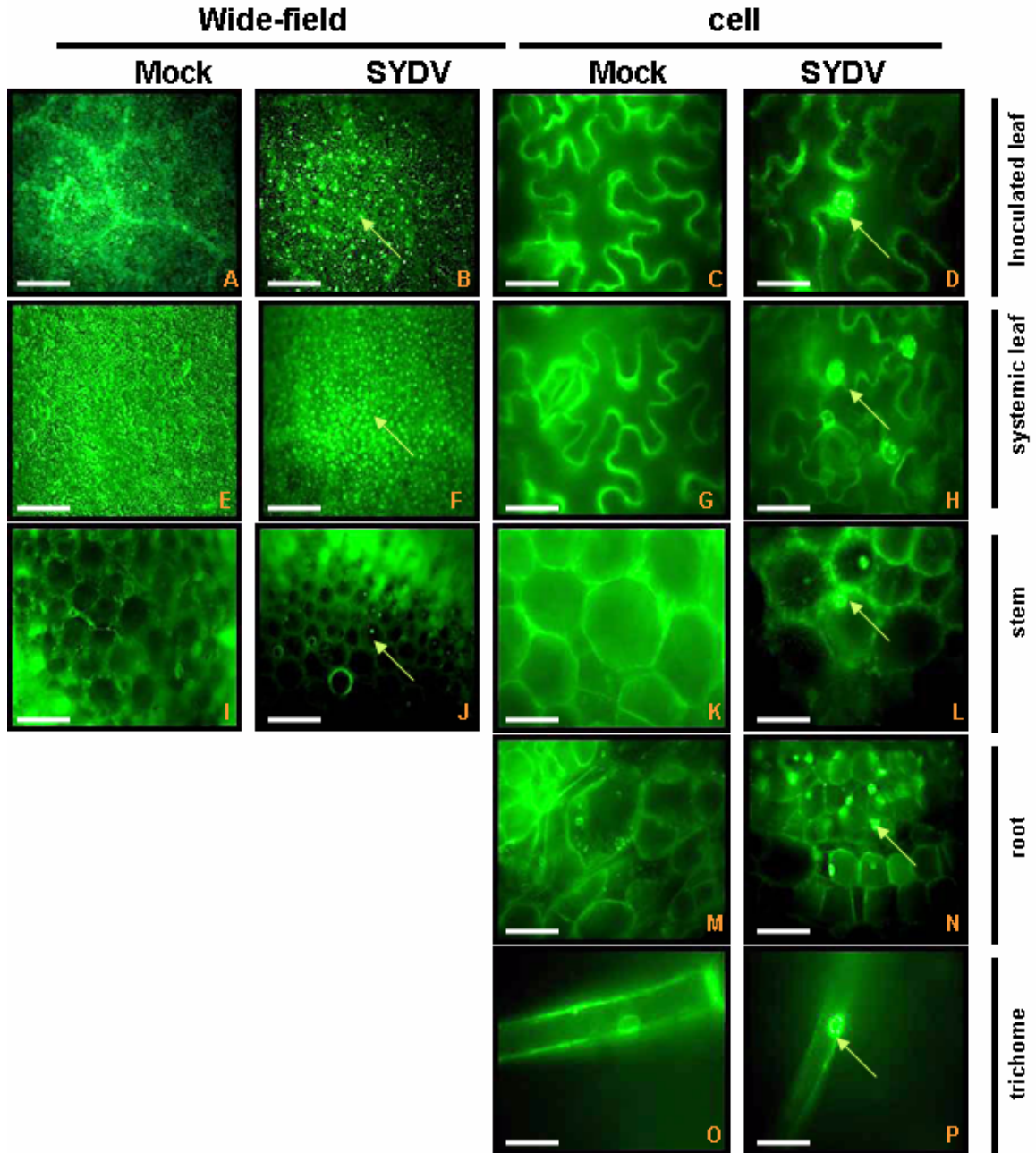


Figure 2.1: Detection of GFP fluorescence in SYDV infected “16c” *Nicotiana benthamiana* tissue. Epifluorescence micrograph of relocalized membrane-associated GFP fluorescence observed in the nuclei of SYDV infected (B, D). epidermal cells in inoculated leaf, (F, H). epidermal cells in systemic leaf (J, L). stem, (N). root and (P) trichome tissue of *Nicotiana benthamiana* (yellow arrow). In contrast, no detectable

fluorescence of relocalized GFP was observed in the nuclei of mock-inoculated *Nicotiana benthamiana* cells (**A, C, E, G, I, K, M and O**).

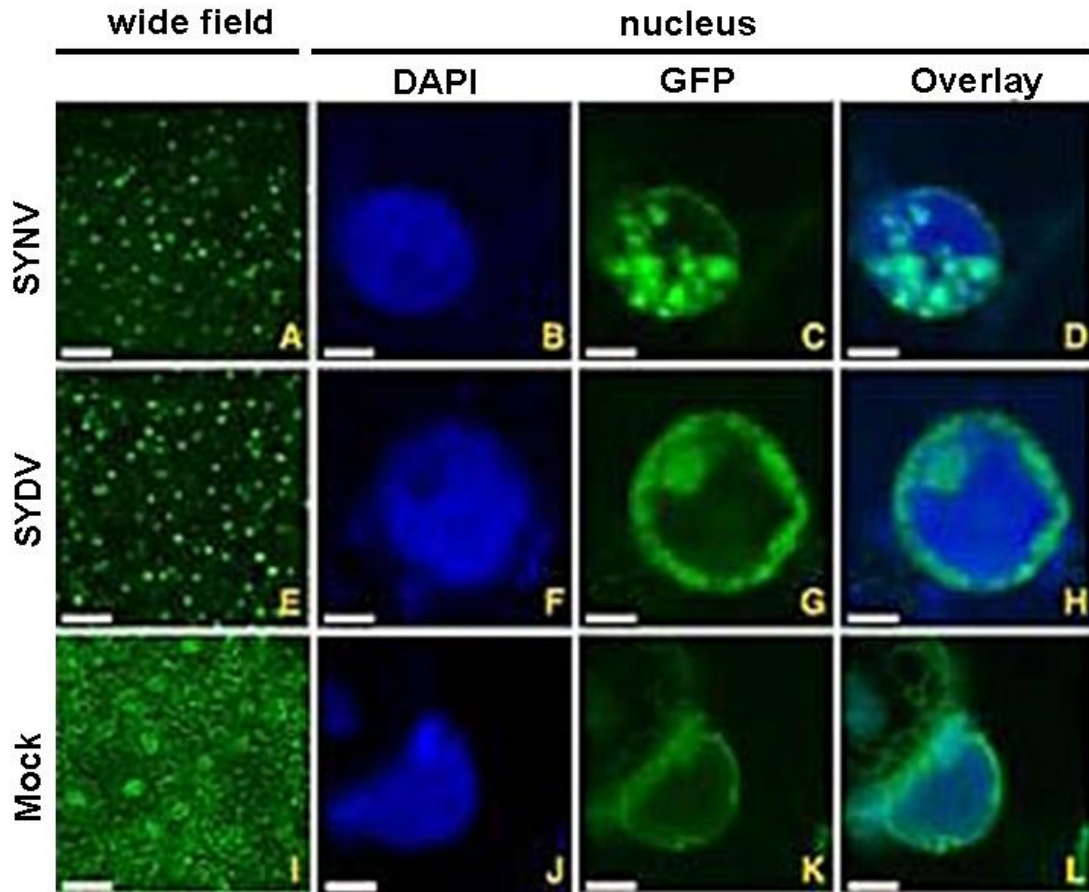


Figure 2.2: Increased accumulation of membrane-associated GFP in the nuclei of SYDV-infected cells.

(A-L). Confocal micrograph of SYNV, SYDV and mock-inoculated *Nicotiana benthamiana* leaves.

(A, E, I). Wide field confocal micrograph showing the GFP fluorescence as punctate loci in SYNV and SYDV *Nicotiana benthamiana* leaves (E, I). In contrast, increased accumulation of GFP was not observed in mock-inoculated *Nicotiana benthamiana* leaves (A).

(B-D, F-H and J-L). Detection of DAPI, GFP fluorescence and overlay of these images in the nucleus of SYNV, SYDV and mock-inoculated *Nicotiana benthamiana* cells, respectively.

(B-D). Relocalization of membrane-associated GFP primarily observed as punctuate loci within the DAPI stained area of the nucleus of SYNV infected cells relative to mock inoculated cells.

(F-H). GFP relocalization relative to mock inoculated cells was observed at the periphery of infected nucleus around the DAPI stained region and an internal region that excludes DAPI in case of SYDV infection.

(J-L). Relocalization of membrane-associated GFP was not observed in mock-inoculated *Nicotiana benthamiana* nucleus.

(Goodin et al, 2005)

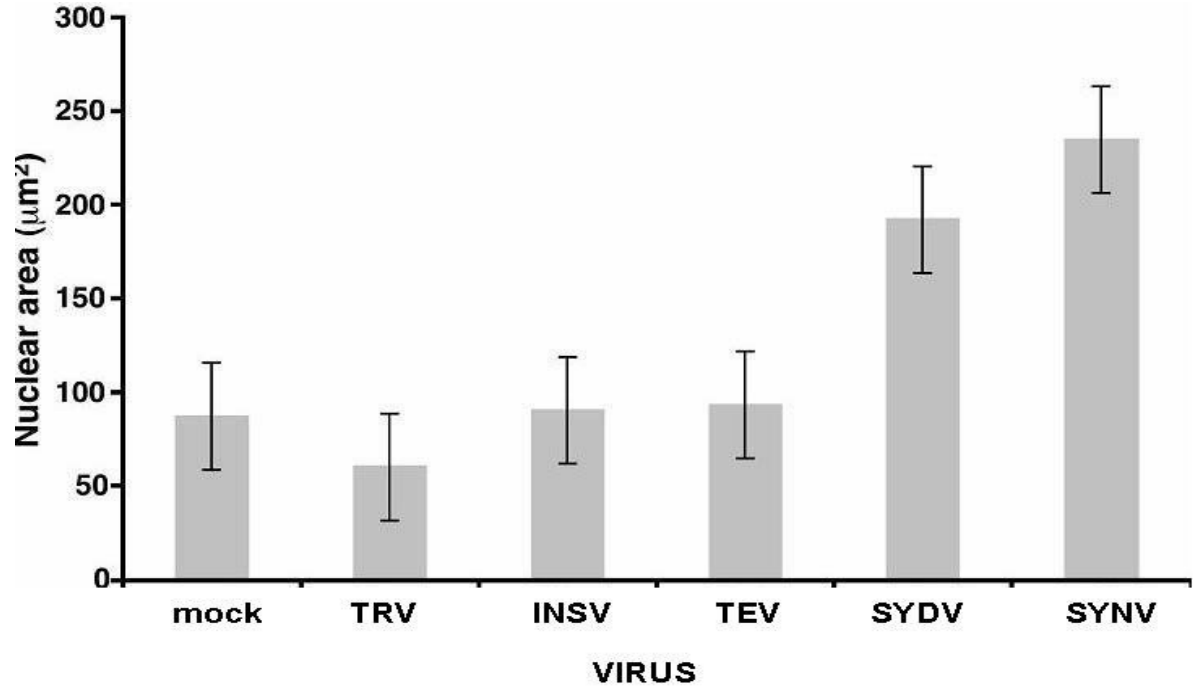


Figure 2.3: Comparison of nuclear area of *Nucleorhabdovirus*, SYDV and SYNV with cytoplasm replicating viruses e.g. TRV, TEV and INSV. Mock-inoculated cells used as control. Note that the mean area of nucleus is much larger in SYDV and SYNV infected cells when compared with mock-inoculated cells. The difference of nuclear area between TEV and INSV infected cells is not significant relative to mock-inoculated cells. (95% confidence intervals are shown for measurements of 100 nuclei in each treatment).

Chapter 3

3. Cloning and Characterization of SYDV N and P mRNA

3.1 Introduction:

Preliminary cell biology studies showed that the effect of infection of the host cell endomembrane system is completely different for SYDV and SYNIV (Figure 2.2). Infection of transgenic *Nicotiana benthamiana* plants that constitutively express GFP targeted to endomembranes with SYDV and SYNIV results in increased accumulation of GFP and membrane within nuclei. GFP accumulation was associated with the nuclear periphery in SYDV-infected cells, where as, in SYNIV infection, the GFP accumulation was more widespread within the nucleus (Figure 2.2). In order to understand cell biology difference between these two serologically distinct species of genus *Nucleorhabdovirus*, an attempt was made to characterize SYDV at the molecular level. This dissertation reports the complete cloning, characterization and subcellular localization of SYDV-N and P protein as well as reagents developed for further characterization of SYDV genome.

Materials and Methods

3.2 Virus maintenance and purification:

SYDV strains were maintained experimentally in *N. rustica* and *N. benthamiana* by serial mechanical inoculation in the greenhouse under ambient condition. Virus purification was done using the method (modified) described by Falk and Weathers (Falk & Weathers, *Phytopathology* 73:81-85, 1983) (Figure 3.1) from SYDV infected *N. benthamiana* tissues harvested approximately 4-5 weeks after inoculation. SYDV infected *N. benthamiana* leaves were harvested approximately 4-5

weeks after inoculation (50 grams) and homogenized in Solution A (0.1 M Tris-HCL, 0.01 M MgCl₂, 0.04 M Na₂SO₃, pH 8.4). The slurry was then squeezed to clarify the tissue through miracloth and centrifuged at 10,570 rpm (5719 x g) in a Beckman MLA-80 rotor for 10 minutes. The clarified supernatant was applied to a celite pad in solution A (2.5 gram of celite per 50 gram of tissue) and the filtrate was centrifuged at 35,000 rpm (63,000 x g) for 30 minutes in Beckman MLA-80 rotor. The supernatant was discarded and the pellet was resuspended in 200 µl of solution B (0.1 M Tris-HCL, 0.01 M MgCl₂, 0.04 M Na₂SO₃, and pH 7.5). The resuspended pellet was applied onto discontinuous sucrose gradients, 3 ml of 30% sucrose and 2 ml of 60% sucrose in solution B. The gradient was centrifuged again in Beckman MLA-80 rotor at 35,000 rpm for 30 minutes. The 30%-60% interface was collected, resuspended in Solution B and centrifuged in a Beckman TLA-55 rotor at 55,000 rpm (186,000 x g) for 10 minutes. The supernatant was discarded and the pellet was resuspended in 200 µl of solution B. A sucrose density gradient (5%-30% solution B) was made and the resuspended pellet was applied to the gradient. The gradient was centrifuged in Beckman MLS-50 rotor at 35,000 rpm (63,000 x g) for 30 minutes. The sucrose fractions containing the virus were collected and resuspended in solution B. The resuspended fractions were centrifuged in Beckman TLA-55 rotor at 55,000 rpm (186,000 x g) for 10 minutes. The supernatant was discarded and the viral pellet resuspended in 47.5 µl of Solution B and 2.5 µl of 2% PEG 1540 (Polyethelene Glycol). G and N proteins were detected from purified SYDV in SDS-PAGE analysis (Figure 3.2A). The purification was repeated four times and G and N protein bands were cut and sent for trypsin digested peptide sequencing analysis by Mass Spectrometry/ Edman degradation (Figure 3.2B).

3.3 SDS-PAGE analysis:

For all SDS-PAGE analyses (Coomassie and Western Immunoblot), protein extracts {purified SYDV fractions or 3 leaf discs (cut using 1 cm cork borer) from healthy as well as virus infected *N. benthamiana* leaves} were prepared by grinding the tissue sample in 2X SDS-PAGE loading buffer containing 0.5 M Tris-HCL (pH 6.8), 10% SDS, 75% Glycerol, 5% β -mercaptoethanol and 0.05 % Bromophenol Blue in a boiling water bath (100°C) for 10 minutes followed by centrifugation (Spectrafuge-240, Labnet International Inc.) at maximum speed for another 10 minutes. Protein samples were then loaded into discontinuous Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE). The gel was stained by coomassie (0.1% Brilliant Blue, 50% MeOH, 10% acetic acid) for 30 minutes followed by washing in destaining solution (30% isopropanol, 10% HAc) or by using PageBlue™ dye according to manufacturer's instructions (Fermentas Life Sciences, Hanover, MD) .

For the western Immunoblot analysis, proteins separated by PAGE were transferred to nitrocellulose membrane and the non-specific binding sites were blocked by 10 ml of non-fat dry milk in 1X TBS (0.5M Tris, 1.5M NaCl, pH 7.5) for 1 hr followed by overnight incubation with primary antibody (e.g. α -SYDV, 1:1000 dilution; GFP, 1:1000 dilution). The membrane was then washed in distilled water for 2x10 minutes and incubated with secondary antibody {Anti-rabbit conjugated with Alkaline Phosphatase, 1:20,000 dilution (for SYDV 1°Ab); Anti-chicken conjugated with Alkaline Phosphatase, 1:30,000 (for GFP 1°Ab)} for 2 hr. Membranes were developed by incubating in 12 ml of alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2 \cdot 6H_2O$, pH 9.5) containing 50 μ l each of NBT (Nitro-blue tetrazolium chloride)

and BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt). Images were scanned by Epson Scanner control software, converted into TIFF file and processed using Adobe photoshop 7.0 (Adobe, San Jose, CA).

3.4 RNA extraction and Cloning of SYDV-specific PCR product:

Total RNA was extracted from SYDV infected *N. benthamiana* leaves harvested approximately 4-5 weeks after inoculation using Qiagen's RNeasy[®] Minikit according to manufacturer's instructions. RNA was eluted in RNase free water and quantified using spectrophotometer. Then 5 µg of total RNA was used to synthesize first strand cDNA using the Superscript[™] First strand synthesis system for RT-PCR according to the manufacturer's instructions (Invitrogen life technologies, Carlsbad, CA). Briefly, total RNA {including poly (A)+ RNA} was transcribed into first strand cDNA by Superscript[™] Reverse Transcriptase (RT) at 42°C for 50 minutes using Oligo (dT) as primer. To obtain double stranded cDNA, PCR was done using Phusion high fidelity DNA polymerase (Finzyme, New England Biolab.) with the SYDV N and G-gene specific degenerate/ N and P-gene specific forward and reverse primers and first strand cDNA as template. Virus-specific amplicons were gel purified and ligated into pGEM-T (Promega Corp., Madison, WI) or GeneJET vector (Fermentas Life Sciences, Hanover, MD) and transformed into Top10 *E.coli* competent cell lines. Clones carrying inserts that hybridized with viral RNA were selected for sequence analysis.

3.5 Cloning of 5' and 3' termini of SYDV Nucleocapsid (N) and Phosphoprotein (P) mRNA:

The 5' as well as 3' end of SYDV-N and P gene were amplified by BD SMART[™] RACE (Rapid Amplification of cDNA Ends) cDNA Amplification kit

(Clontech, Takara Bio Company) according to manufacturer's instructions. Briefly, for 5'RACE, the first strand cDNA was synthesized from 2µg of total RNA (from SYDV infected *N. benthamiana* leaves) using BD Powerscript Reverse Transcriptase with BD SMART II A OligoTM (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGGG-3') and 5'-RACE CDS primer {5'-(T)₂₅VN-3'; V=A,G or C and N=A,C,G or T} at 42°C for 1.5 hr in an air incubator. For 3'RACE, first strand cDNA was made from 2µg of total RNA primed with 3'-RACE CDS primer A {5'-AAGCAGTGGTATCAACGCAGAGTAC (T)₃₀VN-3'; V=A,G or C and N=A,C,G or T} using the same reaction conditions described for 5'RACE. For 5'- and 3'-RACE, PCR was used to produce double stranded cDNA using BD TITANIUM Taq polymeraseTM with 5'RACE-Ready cDNA/3'RACE ready cDNA as template and Universal Primer A Mix (UPM, 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3') and SYDV N or P gene specific reverse/forward primer respectively. The 5'- and 3'-RACE products were cloned into pGEM-T vector (Promega Corp., Madison, WI) and positive clones that hybridized with viral RNA were selected for sequence analysis.

3.6 Peptide sequencing and Sequence analysis:

The G and N protein bands from highly purified SYDV were excised from the coomassie-stained SDS-PAGE gels and sent to the Protein Sequencing Analysis Facility, University of Kentucky Department of Biochemistry and the University of Nebraska Medical Center Protein Sequencing Core Facility for sequencing of trypsin-digestion products by mass spectrometry (MS) or Edman degradation (<http://www.unmc.edu/pscf/>-Applied Biosystems Procise protein sequencer), respectively. Two G protein- and two N protein derived peptides were partially

sequenced and degenerate oligonucleotide primers corresponding to the first six amino-acids were made (Table 3.1). During the primer design, care was taken to maintain maximum specificity at the 3' end of the primer to optimize the primer binding to template. The N protein was sequenced by conventional Edman degradation method because *de novo* protein sequencing by MS is still not regular. This also helped to compare the efficiency of the two sequencing techniques to provide useful data for the experiment.

3.7 DNA sequencing and sequence analysis:

Plasmid DNA was sent to University of Iowa and University of Florida DNA Sequencing Core Facility and the sequencing was carried out in 600-900 bp segments by primer walking (M13 forward and reverse universal primer, GeneJET vector specific primer) using a Perkin Elmer, Applied Biosystems Division (PE/ABd) 377 automated DNA sequencer and fluorescent dideoxy terminator method of cycle sequencing (University of Florida), Applied Biosystems 3730xl DNA Analyzer and BigDye Terminator Cycle Sequencing chemistry according to the supplier's instructions (Applied Biosystems, Inc., Foster City, CA) (University of Iowa). The sequences obtained were subjected to Vecscreen (National center for Biotechnology Information, NCBI) to remove any vector sequences prior to analysis; ORFs were identified with DNA Strider version1.1 and Vector NTI AdvanceTM version10 (Invitrogen Corp., Carlsbad, CA). Putative SYDV protein sequences were compared to the NCBI GenBank protein database with BLASTP search to identify sequence similarity of the same proteins of other well-characterized rhabdoviruses. Protein sequences were searched for domains and motifs, including phosphorylation motifs

(http://www.hprd.org/PhosphoMotif_finder) and hydrophathy analysis (<http://www.expasy.ch>).

3.8 Northern blot hybridization analysis:

Total RNA from healthy as well as virus infected *Nicotiana benthamiana* was (by using Qiagen's RNeasy minikit) electrophoresed in 1% denaturing Formaldehyde/Agarose gel {Agarose, 10X MOPS, Formaldehyde (37% v/v solution), water} and transferred to positively charged nylon transfer membrane (HybondTM-N+, Amersham Life Science) with 20X SSPE buffer (3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA, pH 7.7) overnight. To determine the molecular weight, RNA Ladder, High RangeTM (Fermentas Life Sciences, Hanover, MD) was used as molecular marker. Following transfer, the membrane was air dried, crosslinked by UV exposure (UV StratalinkerTM 1800, Stratagene) for 2 minutes and incubated in ULTRAhyb[®] hybridization buffer (Ambion) at 42°C for 1 hr. Probes were synthesized by gel purification of the amplified SYDV-N and P PCR product and successive addition of ³²P-dCTP by Rediprime II Random Prime Labelling System according to manufacturer's instructions (Amersham Biosciences Corp., Piscataway, NJ). Membranes were hybridized with gene-specific SYDV-N and P probes overnight at 42°C and washed two times for 10 minutes each with 2X SSPE, 0.1% SDS followed by two times for 15 minutes each with 0.1X SSPE, 0.1% SDS at 42°C. After washing, membranes were wrapped in a SaranWrap and exposed to Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA) for 12-24 hours. Images were captured using Typhoon Scanner control Software and analyzed by ImageQuant Software (Molecular Dynamics), converted to TIFF file and processed using Adobe photoshop 7.0 (Adobe, San Jose, CA).

3.9 Phylogenetic Analysis:

All comparative sequence data used in the phylogenetic analysis study was obtained from NCBI GenBank. All the amino acid sequences were analyzed and aligned using the CLUSTAL W program (Thompson et al., 1994). A phylogenetic tree was generated using the Neighbor-Joining method available in PHYLIP 3.65 (Felsenstein J, 1989). The tree was visualized by TREEVIEW program (Page, 1996). Sequences used in this study (with their GenBank accession number) are as follows;

Nucleorhabdovirus: *Maize mosaic virus* (MMV, YP_052850), *Rice yellow stunt virus* (RYSV, NP_620496), *Sonchus yellow net virus* (SYNV, NP_042281), *Maize fine streak virus* (MFSV, YP_052843), *Taro vein chlorosis virus* (TaVVCV, YP_224078), *Orchid fleck virus* (OFV, BAE93579).

Cytorhabdovirus: *Lettuce necrotic yellows virus* (LNYV, CAG34089), *Northern cereal mosaic virus* (NCMV, NP_057954).

Lyssavirus: *Rabies virus* (RABV, AAT48623), *Australian bat lyssavirus* (ABLV, NP_478339), *Mokola virus* (MV, YP_142350).

Vesiculovirus: *Vesicular stomatitis Indiana virus* (VSV; New Jersey serotype, AAA48449).

Ephemerovirus: *Bovine ephemeral fever virus* (BEFV, NP_065398).

Novirhabdovirus: *Infectious haematopoietic necrosis virus* (IHNV, CAA61495), *Snakehead rhabdovirus* (SRV, NP_050580).

3.10 Deposition of sequence data

Nucleotide sequences for the SYDV-N and P mRNAs have been deposited in GenBank as accessions EU183122 and EU183123, respectively.

3.11 Construction of pSITE expression vectors for *in planta* subcellular localization:

Construction of pSITE expression vectors (Chakrabarty et al., 2007) was carried out by Gateway[®] Technology Version E (Invitrogen Corp. Carlsbad, CA) according to manufacture's instructions. Briefly, total RNA was extracted from SYDV infected *N. benthamiana* leaves harvested approximately 4-5 weeks after inoculation using Qiagen's RNeasy[®] minikit according to manufacturer's instructions. Gateway compatible primers were designed for both putative SYDV-N and P ORF. The SYDV N and P ORFs were amplified by RT-PCR using gateway primers and PCR products were cloned into donor vector (pDONR221) to make entry clones by BP recombination reaction (using BP Clonase[™] enzyme). Following BP reaction, LR recombination reaction was done between entry clones and destination vector, pSITE4-CA and pSITE2-CA for the expression of RFP and GFP fusions, respectively (Chakrabarty, et al., 2007). The functional pSITE vectors expressing the autofluorescent protein fusions of SYDV-N and P were transformed into *Agrobacterium tumefaciens* strain CBA4604 and *A. tumefaciens* was infiltrated into leaves of *Nicotiana benthamiana* (mock-inoculated as well as SYDV infected "16c"-GFP transgenic plants) as previously described (Goodin et al. 2002; Chakrabarty et al., 2007).

3.12 Cloning of SYDV-N mRNA:

A PCR-based strategy was employed to clone and characterize the SYDV-N and P mRNA. Reverse-transcription Polymerase Chain Reaction (RT-PCR) was done using total RNA (5 µg) derived from SYDV infected *N. benthamiana* plants as template for cDNA synthesis and N-gene specific degenerate primer (N2F/ N1R) designed from partial peptide sequence of SYDV-N protein. SYDV specific amplicon

(~500 bp) that hybridized to viral RNA was cloned into pGEM-T vector (Promega, Madison WI) (Figure 3.3). The cloned cDNA for putative SYDV-N gene was subsequently used as a probe in Northern hybridization experiment. Of two clones tested in this manner, one gave the expected hybridization patterns, a 1.5 kb N mRNA transcript and a 12.6 kb SYDV genomic RNA. The consensus sequence of 465 bp PCR-amplified DNA fragment was assembled from the sequence of 10 independent clones. Sequence analysis of this 465 bp fragment showed the presence of primer sequences derived from the Forward and Reverse degenerate primers at their 5' and 3' terminal region and the two amino acid residues present immediately upstream of the A-T-P-A-A-T sequence (reverse degenerate primer), are K (lysine) and R (arginine), which is cleavage site for trypsin. The cloned sequence is a continuous open reading frame, which is expected if the amplicon really derived from the SYDV-N gene. Two sets of gene specific primers (GSPs), forward and reverse (NRF1, NRR1 and NRF2, NRR2) were synthesized based on this initial 465 bp sequence of putative SYDV-N gene for the purpose of using these primers in RACE (Rapid Amplification of cDNA Ends). RT-PCR reactions with NRF1/NRR1 primer pair resulted in amplification of a DNA fragment from cDNA synthesized from total RNA isolated from mock-inoculated *N. benthamiana* plants, in a manner similar to when degenerate N primers were used along with amplification of a fragment of predicted size (~350 bp) from SYDV infected *N. benthamiana* plants. The second primer pair (NRF2/NRR2) produced the predicted amplicon only from SYDV infected *N. benthamiana* plants. These data, together with that from Northern hybridization (Figure 3.4 A), provide validation that SYDV-N specific cDNA was cloned successfully. Completion of the N mRNA cloning was done by 5' and 3' RACE using

SMART™ technology (Clontech). This method uses the combined actions of SMART II™ Oligonucleotide and the BD-PowerScript Reverse Transcriptase (RT). The BD-PowerScript RT is a variant of *Moloney murine leukemia virus* RT that, upon reaching the end of an RNA template, initiates terminal transferase activity by adding 3–5 residues (predominantly dC) to the 3' end of the first-strand cDNA. The BD SMART-oligo contains a terminal stretch of dG residues that anneal to the dC-rich cDNA tail and serves as an extended template for RT. The PowerScript RT then switches templates from the mRNA molecule to the BD SMART oligo, generating a complete cDNA copy of the original RNA with the additional BD SMART sequence at the end. Since the dC-tailing activity of RT is most efficient if the enzyme has reached the end of the RNA template, the BD SMART sequence is typically added only to complete first-strand cDNAs. The first strand cDNA synthesis was carried out using 2 µg of total RNA extracted from SYDV infected *N. benthamiana* leaves and primed by a modified oligo dT primer. Following reverse transcription, the first strand cDNA was used directly in 5'- and 3'-RACE PCR reactions using N-gene-specific primer (NRF2/NRR2) and the BD SMART oligo. The 5' and 3' RACE reactions amplified SYDV-N specific 5' RACE fragment (~1.4 kb) and 3'RACE fragment (~ 600 bp), which were cloned into pGEM-T vector (Figure 3.5). Colonies were screened and clones carrying inserts (4 each of 5' and 3' RACE products) that hybridized with viral RNA were sequenced. All the sequences were analyzed using Vecscreen (NCBI Blast), ORF were identified using Vector NTI version 10 and DNA strider 1.1 and ~1.5 kb SYDV-N mRNA was assembled (Figure 3.6).

3.13 Cloning of SYDV-P mRNA:

To amplify the SYDV-P mRNA, RT-PCR was conducted using total RNA extracted from SYDV infected *N. benthamiana* leaves. Reverse transcription/ first strand cDNA synthesis reaction was primed by SYDV-N gene specific primer (NRF1). Following reverse transcription, conventional PCR was performed using the same SYDV-N gene specific primer (NRF1) as forward primer, which was used to prime the first strand cDNA synthesis and G-specific degenerate oligonucleotide primer (G2R) as reverse primer. A virus specific amplicon of ~2.0 kb was amplified in SYDV cDNA containing sample, but not in healthy *N. benthamiana* cDNA containing control sample (Figure 3.3 B). The amplified fragment (~ 2.0 kb) was not consistent with the predicted fragment size as G gene-specific degenerate oligonucleotide primer was failed to anneal with the G gene sequence. The PCR product was cloned into pJET-1 vector (Fermentas, MD) and subsequently used as a probe in Northern hybridization experiment for its authentic virus-specificity. Colonies were screened and 4 clones carrying the positive insert were sent off for sequencing. Sequences were analyzed as described above and an ORF of size 843 bp was identified. Gel purified putative SYDV-P PCR product was used as probe in a Northern hybridization to validate its SYDV specificity and the hybridization pattern was obtained as expected, a 0.9 kb P mRNA transcript and a 12.6 kb PYDV-S genomic RNA (Figure 3.4 B). Two sets of putative SYDV-P gene specific primers were synthesized (P5R/P3R) for use in 5' and 3' RACE reactions to amplify the entire P gene (BD SMART technology by Clontech as above). The RACE-PCR reaction amplified predicted 0.5 kb 5'RACE product and 0.7 kb 3'RACE product. Cross-hybridization of those primers with control (healthy *N. benthamiana* cDNA for RACE)

was negative (Figure 3.5). RACE products were cloned into pGEM-T vector and positive clones (4 each of 5' and 3' RACE products) were sequenced. The sequences of putative P 5'- and 3'- RACE products were aligned with the initial ~843 bp sequence and ~0.9 kb SYDV-P mRNA was assembled (Figure 3.7).

3.14 Structure, characterization and sequence analysis of SYDV-N mRNA:

The 1546 nucleotide sequence of SYDV-N protein mRNA was assembled from the initial 465 bp sequence as well as the 1.4 kb SYDV-N 5' and 600 bp 3' RACE sequence. The 5' end of SYDV-N mRNA starts with the sequence 5'-AACCA-3'. This sequence is consistent with the conserved transcription initiation sequence at the 5' end of mRNAs of other well characterized rhabdoviruses except LNYV, which has a consensus transcription start sequence GA(A/T)(A/T) for its mRNAs (Heaton et al., 1989, Wetzel et al., 1994). An 11-nucleotide (nt) 5' untranslated region (5'-UTR) preceded the AUG start codon. This 5'-UTR of SYDV-N mRNA is shorter than those of SYN-N (56 nt) and LNYV-N mRNA (78 nt), but quite similar in size when compared to RYSV-N (15 nt) and those of N protein mRNA of animal rhabdoviruses including VSV (13 nt), Rabies and Mokola virus (12 nt). The 3' untranslated region (3'-UTR) of SYDV-N mRNA has sequence length of 84 nt followed by UAG stop codon. The 3' terminus contains the sequence AACAAAA followed by a polyadenylic stretch of 30 residues. The 84 nt 3'-UTR of SYDV-N mRNA is longer than those of SYN-N (67 nt) and LNYV-N mRNA (74 nt), but considerably shorter than RYSV-N mRNA (136 nt). The N-ORF starts with the first AUG codon encodes a protein comprising of 472 amino acids with a calculated molecular weight of 51,553 Dalton, which is quite similar with the original estimates of around 50,000 Dalton obtained by sodium dodecyl sulfate –

polyacrylamide gel electrophoresis (SDS-PAGE). The SYDV-N protein is larger in size than MFSV, LNYV, VSV, and Rabies virus N proteins, which contain 462, 459, 422 and 450 amino acids respectively, but smaller than 475 amino acid long SYNV-N and 521 amino acid long RYSV-N protein. Comparison of SYDV-N protein amino acid sequence using Hitachi-Prosip and BLASTP (NCBI) program reveals significant identity with other *Nucleorhabdoviruses* including SYNV-N (26%, identity), MMV (27% identity), MFSV (22% identity), TaVVCV (27% identity), OFV (27% identity), RYSV (25% identity) and 20% identity with NCMV (a member of genus *Cytorhabdoviruses*). The bioinformatic analysis of SYDV-N protein using ProtParam algorithm (Gasteiger et al., 2005) shows the ratio of basic [Arg, Lys (total number of basic residues: 46)] and acidic [Asp, Glu (total number of acidic residues: 45)] amino acids is almost equivalent (~1.02). The N protein of SYDV contains 46 basic amino acids and 45 acidic amino acids, as compared to 53 and 51 for SYNV-N, 61 and 54 for MFSV-N and 80 and 41 for RYSV-N. The predicted theoretical isoelectric point (pI) of SYDV-N is 7.61, suggesting that it is less basic than SYNV-N (pI: 8.09), MFSV-N (pI: 8.84) and RYSV-N (pI: 10.04). Hydropathy analysis (SOAP algorithm, Kyte and Doolittle, 1982) of SYDV-N protein showed a high hydrophilic region from amino acid position 10 to 20 near the amino terminal and a stretch from position 310 to 440 located near the carboxy terminal of the protein with cumulative GRAVY (Grand average of Hydropathicity) value of -0.183 (Figure 3.8). These regions might contain the putative RNA binding domain as well as motifs for the nuclear localization signal (NLS) as typical nuclear localization signal consists of positively charged hydrophilic amino acid residues (Kalderon et al, Lusk et al., 2007). Similar hydrophilic region have been found near the carboxy terminus of SYNV, MFSV,

Rabies and RYSV-N protein, but those are less extensive in SYN and Rabies when compared to MFSV and RYSV. ProtParam analysis (Gasteiger et al., 2005) also showed that the estimated instability index and *in vivo* half-life (*E.coli*) of SYDV-N protein is 39.55 and >10 hours respectively, suggesting the protein is stable (A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable). Analysis of SYDV-N protein using the COILS algorithm (Lupas et al., 1991) predicts the presence of a distinct coiled coil region at the proximity of carboxy terminal between amino acids position 360 to 375 (Figure 3.9), which might mediate the interaction of N protein with other proteins including P protein as has been shown for other plant and animal rhabdoviruses (Goodin et al., 2001, Green et al., 2000). Similar COIL analysis reveals marked regions in other *Nucleorhabdovirus* encoding N protein including MFSV-N (amino acid region between 200-220, 275-290, and 378-400) and RYSV-N (aa region 375-400) but failed to predict any clear coiled coil region in case of SYN-N. In comparison to that, LNYV, a member of genus *Cytorhabdovirus* shows presence of distinct coiled coil regions at its amino terminus (aa position 1-25, 151-190). These predictions can be served as a preliminary data for future experiments towards the understanding and comparison of N-P interaction between the members of plant rhabdoviruses.

3.15 Structure, Characterization and sequence analysis of SYDV-P mRNA:

The 887 nucleotide sequence of SYDV-P protein mRNA was determined from 4 independent clones that corresponded to the 2.0 kb N-G amplicon as well as the 0.7 kb SYDV-N 5' and 0.9 kb 3' RACE amplicon. The 5' end of SYDV-P mRNA starts with the conserved transcription initiation sequence 5'-AACAA-3' as comparable as

SYDV-N and other rhabdoviral mRNAs except LNYV. An 18-nt 5' untranslated region (5'-UTR) preceded the AUG start codon. This 5'-UTR of SYDV-P mRNA is shorter than that of SYNV-P (50 nt). The 3' untranslated region (3'-UTR) of SYDV-P mRNA is 126 nt long and is followed by UGA stop codon. It is longer than the 3'-UTR of SYNV-P mRNA (45 nt). The 3' terminus contains the sequence AAUAAA followed by a polyadenylic stretch of 15 residues. The P-ORF starts with the first AUG codon encodes a protein comprising of 280 amino acids with a calculated molecular weight of 31,366 Dalton, which is almost similar with the original estimates of around 32,000 Dalton obtained by SDS-PAGE analysis. The 280 amino acid SYDV-P protein is shorter in size than MFSV, RYSV, SYNV, LNYV and Rabies virus P protein, which contain 338, 322, 286, 300 and 297 amino acids respectively, but larger than 265 amino acid long VSV-P protein. Comparison of SYDV-P protein amino acid sequence using BLASTP (NCBI) program did not reveal any identity with the P protein of other well characterized rhabdoviruses.

Bioinformatic analysis of SYDV-P protein using ProtParam algorithm (Gasteiger et al., 2005) shows the ratio of basic (Arg, Lys) and acidic (Asp, Glu) amino acids is almost equivalent. The P protein of SYDV contains 32 basic amino acids and 31 acidic amino acids, as compared to 41 and 40 for SYNV-P, 46 and 56 for MFSV-P and 46 and 39 for RYSV-P. The predicted theoretical isoelectric point (pI) of SYDV-P is 7.72, suggesting that it is more basic than SYNV-P (pI: 7.67), MFSV-P (pI: 5.14), LNYV-P (pI: 4.59), VSV-P (pI: 4.64) and Rabies virus-P (pI: 4.99) but less basic when compared to RYSV-P (pI: 9.23). Hydropathy analysis (SOAP algorithm, Kyte and Doolittle, 1982) of SYDV-P protein predicts an extensive hydrophilic region from amino

acid position 1-61, 100-118 close to amino terminal and a moderately hydrophilic region from amino acid position 190- 205 near the carboxy terminal (Figure 3.8) with cumulative GRAVY (Grand average of Hydropathicity) value of -0.615. Similar but highly extensive hydrophilic region was found (aa position 175-235) near the C-terminus of SYNV-P protein. This region might possess the putative RNA binding domain as well as motifs for the nuclear localization signal. The estimated instability index of SYDV-P protein is 48.64, which suggests that the protein is chemically unstable though the *in vivo* half-life (*E.coli*) of SYDV-P protein is >10 hours. Coiled coil analysis of SYDV-P protein using COILS algorithm (Lupas et al., 1991) predicts the presence of minor but distinct coiled coil regions at the amino- (aa position 32-50) as well as central proximity of the protein (aa position between 125-145 and 150-175) and an extensive coiled region at the carboxy terminal between amino acids position 250 to 278 (Figure 3.9). Similar central and terminal coiled coil regions are also present in other *Nucleorhabdovirus* encoding P protein including MFSV-P (aa position 83-100, 200-223 and 226-247), RYSV-P, SYNV-P, LNYV-P and NCMV-P (Goodin et al., 2001). In contrast to that, animal infecting VSV and Rabies virus P protein show presence of a distinct and extensive coiled coil region exclusively at their amino terminal and a very weak region at their C-terminal (unpublished data). In this regard, the SYDV-P protein is more similar to the P protein of plant infecting rhabdoviruses than their animal counterpart though there is no significant sequence identity between them.

3.16 Phylogenetic relationship of SYDV with other *Rhabdoviruses* based on consensus sequences of the N ORF:

Taxonomic relationships of Rhabdoviruses are primarily based on structural properties (genome structure), biological properties (host specificity, mode of transmission) and serological relationships between the members (Bourhy et al., 2005). Although serological and other biological data were widely used to establish evolutionary relationships among closely related members of viruses (Calisher et al., 1989; Shope, 1995; Wang et al., 1995), molecular sequence analysis/ genome structure and its properties became more important since the development of modern molecular biological techniques. Molecular sequence analysis of specific genes has been used to examine the phylogenetic relationship among rhabdoviruses (Bourhy et al., 2005, Kondo et al. 2006, Dietzgen et al. 1994). For example, sequence similarity of the Nucleocapsid gene and conserved block III of the L polymerase gene was used to examine taxonomic relationships between different genotypes of *Lyssaviruses* and among members of the *Rhabdoviridae* family, respectively. (Arai et al., 2003; Bourhy et al., 1993; Kuzmin et al., 2003 and Bourhy et al., 2005). Similarly, phylogenetic relationship analysis of SYDV and other well-characterized members of the family *Rhabdoviridae* using the consensus region of N-ORF support the previous taxonomic characterization of SYDV based on its EM, serological and other biological data (Black, 1938, 1970; Falk et al., 1981, 1983) into genus *Nucleorhabdoviruses*. An unrooted tree was chosen in order to address the evolutionary relationship between the members of this group of viruses because the sequence alignment alone does not provide sufficient information to determine the node that represents the ancestor, the root. The phylogenetic analysis using the deduced amino

acid sequence of the N gene places members of the plant rhabdoviruses into two separate groups, genus *Nucleo-* and *Cytorhabdoviruses*. This analysis predicts that plant rhabdoviruses are more closely allied with members of the genus *Novirhabdoviruses* than members of the other animal rhabdovirus genus (*Vesiculoviruses*, *Ephemeroviruses* and *Lyssaviruses*). Similar phylogenetic relationship among plant and animal rhabdoviruses was determined based on conserved motifs of pre-A-E of L polymerase gene (Dietzgen et al 1994). The six sequenced members of the genus *Nucleorhabdoviruses* forms two sub-groups, SYNV, MFSV and OFV fall into one sub-groups and TaVCV, MMV and RYSV form the other. SYDV is clustered with members of the genus *Nucleorhabdoviruses*, but seems more closely related to TaVCV, MMV and RYSV based on the phylogenetic analysis of consensus N-ORF (Figure 3.10).

3.17 Subcellular localization studies of SYDV-N and P protein:

In order to study the subcellular localization of SYDV-N and P protein in plant cell, *in planta* localization and co-localization experiments were performed by cloning the full length ORFs of N and P protein into pSITE expression vectors for *Agrobacterium tumefaciens* mediated infiltration into wild type as well as “16c” (GFP-transgenic) *Nicotiana benthamiana* leaves.

Agroinfiltration of fluorescent protein fusions of SYDV-N and P protein in non-“16c” (Figure 3.11) mock and SYDV infected “16c” *N. benthamiana* cells (Figure 3.12) showed the nuclear localization of RFP: N, GFP: P and RFP: P protein in the cells. GFP: P localized as distinct punctuate loci in the nucleus, where as RFP: N diffused throughout the nucleus. In contrast to its localization in mock-inoculated plants, RFP: N partially colocalized with intranuclear membranes in the case of SYDV-infected plants

(Figure 3.12 P). These localization results were consistent with the predicted hydrophilic region found in SYDV-N and P protein, which might possess the putative karyophilic region required for nuclear localization, because no putative NLS was found either in N or P protein using the pSORT algorithm (algorithm for the prediction of putative NLS). Similar results were observed for the localization of N protein fusion of two other members of the genus *Nucleorhabdoviruses*, SYN-V and MFSV, though the patterns of localization for P (ORF2 for MFSV) fusion protein were different in each case as compared to SYDV-P. SYN-V-P (GFP: P) was localized in the nucleus along with some cytoplasmic fluorescence also observed around the periphery of the cell (Goodin et al., 2001) and MFSV-ORF2 (YFP: ORF2) spread throughout the cell (Tsai et al., 2005).

Co-infiltration of SYDV-N and P autofluorescent protein fusions, RFP: N and GFP: P results in a unique localization pattern when compared to their independent localization in *N. benthamiana* cells. When co-expressed, both SYDV-N and P protein fusions were colocalized in a distinct subnuclear locale (Figure 3.11). This result is consistent with the localization pattern of co-expressed fluorescent protein fusion of SYN-V-N and P protein, where subnuclear localization was evident for both protein fusions (Goodin et al., 2001). Taken together, this data suggests an exclusive nuclear localization pattern for SYDV-N and P protein in plant cells. Further experiments are required to determine the presence of any motifs for a putative nuclear localization signal (as evident for SYN-V-N) or a specific karyophilic region (as evident for SYN-V-P) in SYDV-N and P protein.

3.18 Time course systemic infection pattern of SYDV in *Nicotiana benthamiana*:

Given the fact that infection of SYDV induces increased accumulation of GFP in the nuclei of 16c *Nicotiana benthamiana* cells, a time course infection study of SYDV in *N. benthamiana* plants was conducted in an attempt to correlate the accumulation of GFP upon SYDV infection with an increase in virus titer and infection intensity (Figure 3.13). 16c *Nicotiana benthamiana* plants with 4-6 fully expanded leaves were inoculated with SYDV for a 5-week time period and leaf tissue samples were collected over the entire time course. Marked symptoms of SYDV infection were first observed in 3rd week post inoculation (wpi) plants which include vein clearing and yellowing of infected leaves and become very prominent in the 5th wpi as an indication of increasing virus infection, although leaf malformation started around the 2nd wpi (Figure 3.13 A, B). Both *N. rustica* and *N. benthamiana* were killed after 6-7 weeks post inoculation with SYDV (data not shown). This appearance of symptoms in the infected plant was coincident with increased accumulation of GFP in the nuclei of infected plant cells. Western immunoblot analysis with equivalent amount of total protein isolated from the 2nd, 3rd, 4th and 5th wpi infected leaves was consistent with the pattern of appearance of visual symptoms. Virus titers (as determined using anti-SYDV antibody) accumulated in the infected leaves at 3rd wpi (Figure 3.13 C). The immunoblot also showed that the virus titer reached to an elevated level during the 5th wpi (Figure 3.13 C). Northern hybridization experiment with total RNA isolated from same source of SYDV infected leaves probed with SYDV-N was coherent with the visual symptoms and western immunoblot data where differential increase of N-mRNA and genomic RNA was observed over the course of the time period (Figure 3.13 E). The increasing pattern of

viral protein and RNA from western immunoblot and northern hybridization data supports the initial observation of SYDV causing GFP accumulation in the infected nuclei of 16c *Nicotiana benthamiana* cells, which is believed to be consistent with the site of virus accumulation. Finally, the time course data suggests that the infection intensity is at its highest level between the 4th-5th wpi; accordingly, this is the best time for harvesting infected tissue.

3.19 Primers used in this study:

SYDV-N gene specific degenerate primer

1. N1F: GCNATNGARATNCCNGAY
2. N1R: NGTNGCNGCNGGGNGTNGC
3. N2F: GCNACNCCNGCNGCNACN
4. N2R: RTCNCGNATYTCNATNGC

SYDV-G gene specific degenerate primer

5. G1F: TTYCCNGCNGGNYTNAAR
6. G1R: YTTNARNCCNGCNGGRAA
7. G2F: TTYGAYCAYATGGAYCCN
8. G2R: NGGRTCCATRTGRTCRAA
9. G-RR1: CCGTTTATACCTTTGCTAC

SYDV-N gene specific primer for RACE (Rapid Amplification of cDNA Ends)

10. NRR2: CTCTCTTCTGTGCTGCTGGTGG
11. NRF2: ATCTAATGGCATGCATCCT
12. NRR1: GTGGCGGCGGGGGTGGCTCTC
13. NRF1: ACAAAGTTTGCCTATCTAATGGC

SYDV-P gene specific primer for Gateway recombination

14. (PF) att_SYDV_PF: AAAAAGCAGGCTTAATGTCAGGGCATGATATCAGTCC
15. (PR) att_SYDV_PR: AGAAAGCTGGGTTTCAGTTCTCCGTCATTC

SYDV-P gene specific primer for RACE (Rapid Amplification of cDNA Ends)

16. P5R: TGGTAACTCTTTTCAAGACTAGGCAACA
17. P3R: TATCTGATAGCGTGATCAGACCTCTGTGGG

SYDV-N gene specific primer for Gateway recombination

18. (NF) att_SYDV_NF: AAAAAGCAGGCTTAATGAATAACGCTAACAC
CGCTCAGTTCGTGC

19. (NR) att_SYDV_NR: AGAAAGCTGGGTCTAGGGCAGCTGGTCCAGC
ACTCCGGATTGTAG

SYDV-N gene specific primer to amplify N-P intergenic region

20. NPF1: TGAATGCTGCTCTACAATCCGGAGTGCTGG

21. NPF2: GGGACATCCGGAACAGCATCAGCCACTCCG

SYDV-P gene specific primer to amplify N-P intergenic region

22. NPR1: TCTCTAAGCTTTCTCGAAGGACTGA

23. NPR2: GCTTGTTTTACTGGGTCATAAGGGGCT

L gene specific degenerate primer (common to animal and plant rhabdovirus)

24. LCF: GAAGGTAGATTTTTCTCATTAATG

25. LCR: CCATCCCTTTTGCCGTAGACCTTA

L gene specific degenerate primer (SYNV specific)

26. LSF: AAAGCCAGATTTTTCTCATTGATG

27. LSR: CCAACCCTTATGACATACACCTTC

Table 3.1: Degenerate primer sequence of SYDV-N and G peptide fragments.

Gene	Peptide sequence	Degenerate Oligo sequence	Forward/ Reverse Sequence
SYDV-N	AIEIPDPVTA	GCNATNGARATNCCNGAY	Forward
		RTCNGGNATYTCNATNGC	Reverse
	ATPAATQQLQ	GCNACNCCNGCNGCNACN	Forward
		NGTNGCNGCNGGNGTNGC	Reverse
SYDV-G	FPAGLK	TTYCCNGCNGGNYTNAAR	Forward
		YTTNARNCCNGCNGGAA	Reverse
	FDHMDPGLAAYK	TTYGAYCAYATGGAYCCN	Forward
		NGGRTCCATRTGRTCRAA	Reverse

Coomassie stained SYDV-N and G gel bands were cut and sequenced by Mass Spectrometry and Edman Degradation. Two each of N and G-specific degenerate oligonucleotide primers (Forward and Reverse) were designed based on their peptide sequences. The degenerate primers were used in RT-PCR reaction.

(Y = C, T; R = A, G; N = A, G, T, C)

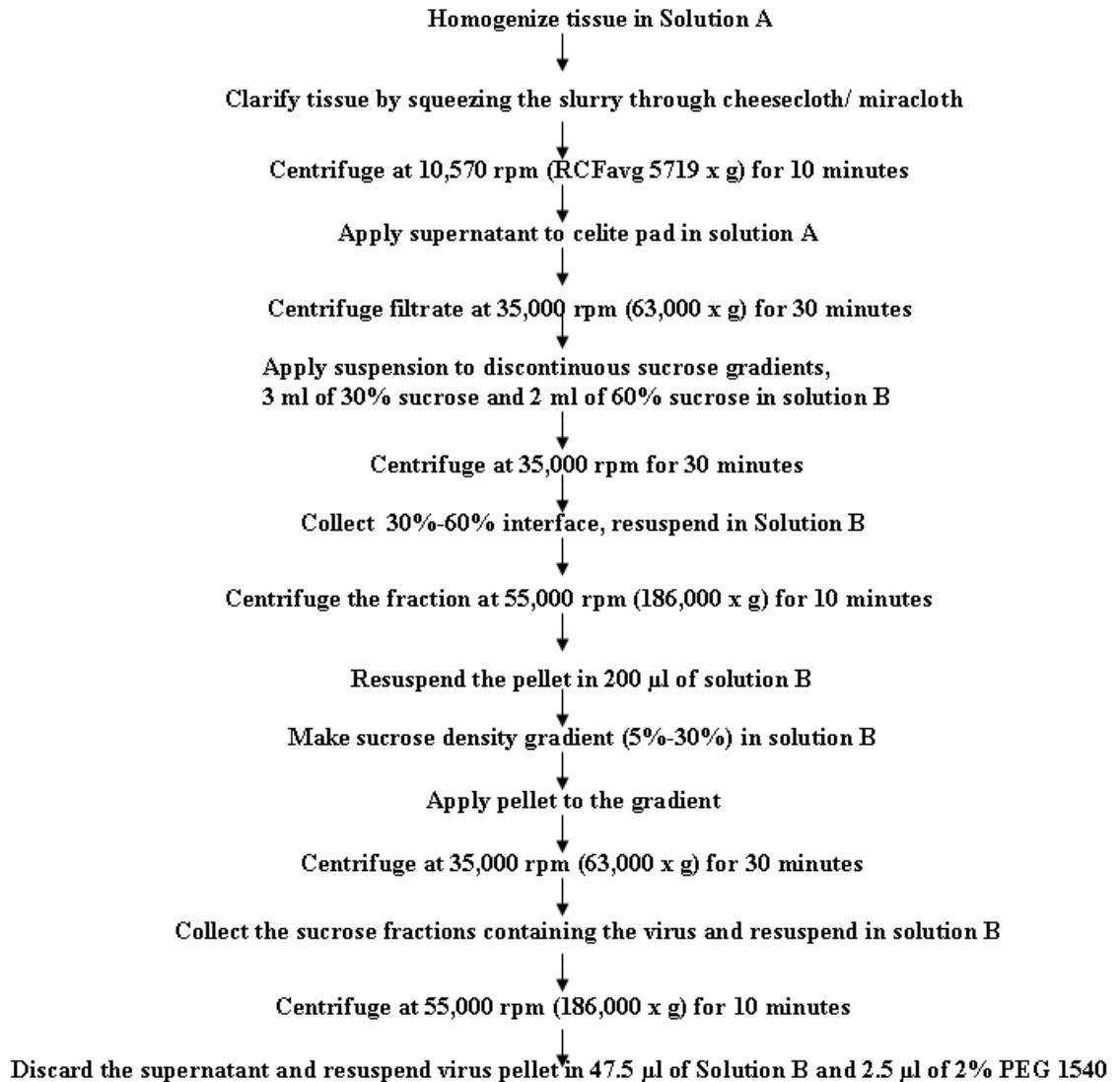


Figure 3.1: Flow chart of SYDV purification (modified Falk and Weather's method, 1983)

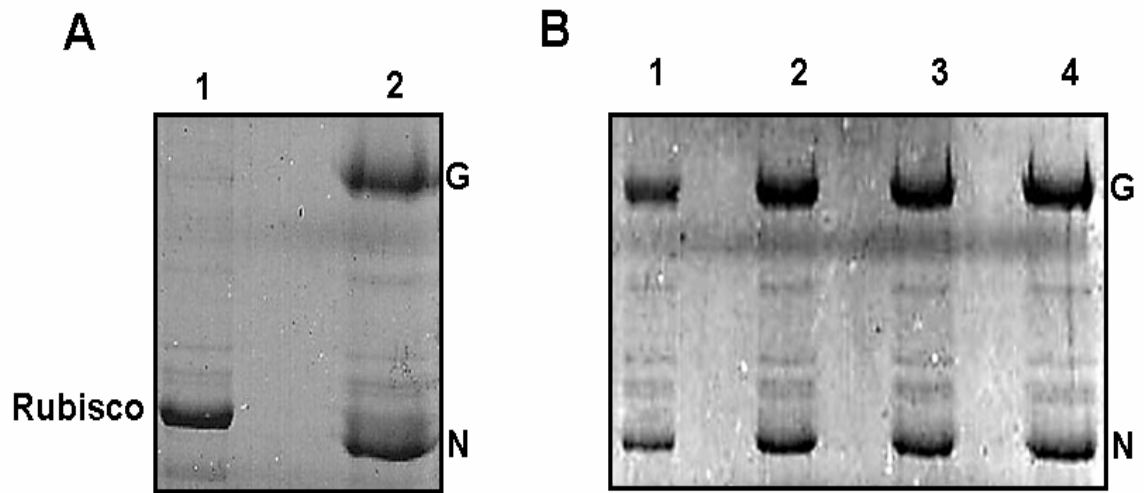


Figure 3.2: Detection of purified SYDV from *Nicotiana benthamiana* leaf tissue. A. Coomassie brilliant blue gel (CBB) image showing the G and N protein from purified SYDV by sucrose density gradient centrifugation (lane 2). Similar purification from healthy *Nicotiana benthamiana* leaves (lane 1) was used as negative control, which shows abundant Rubisco protein. **B.** CBB image of four independent purification of SYDV from *Nicotiana benthamiana* leaves (lane 1, 2, 3 and 4). Gel bands of G and N protein were cut and provided for peptide sequencing by LC/MS or Edman degradation analysis.

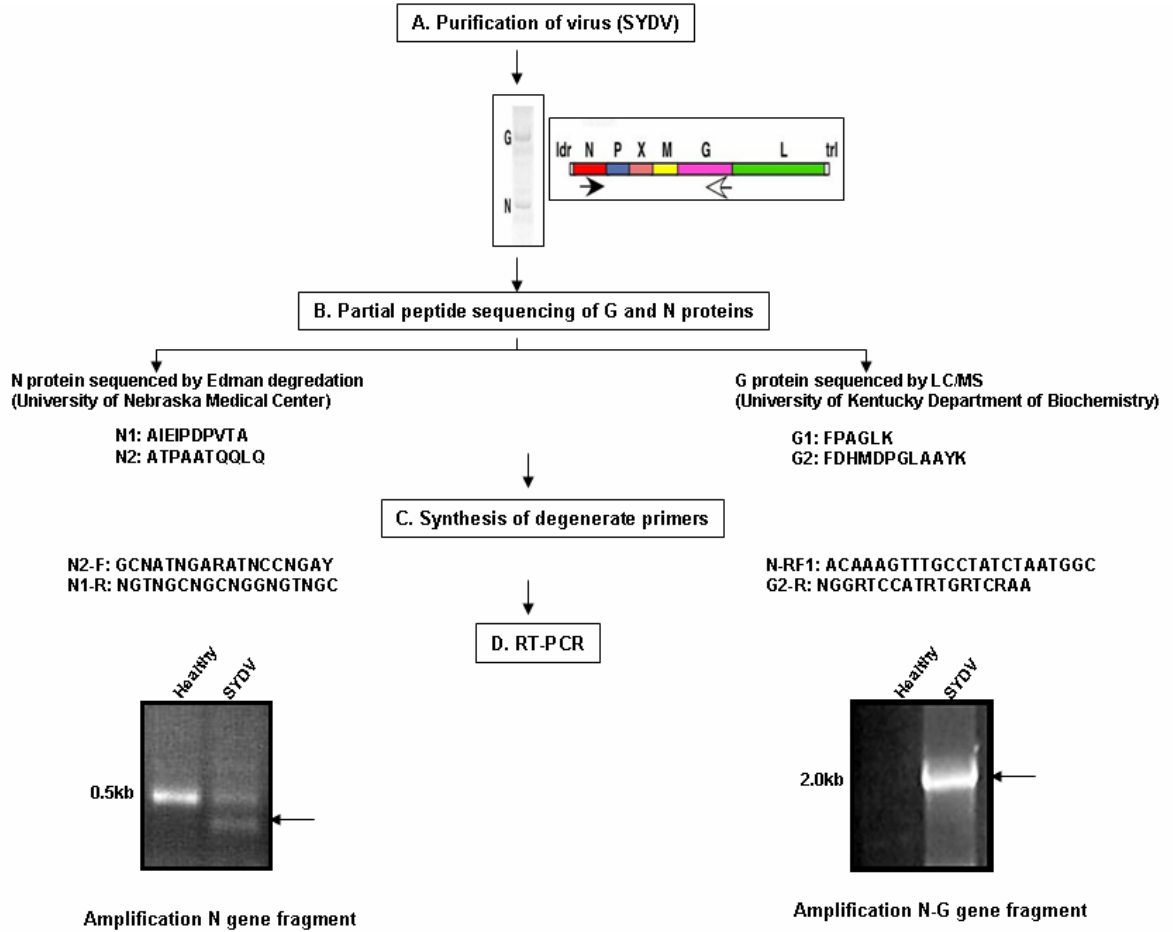


Figure 3.3: Strategy for cloning SYDV-N and P mRNA using gene specific degenerate primers. **A.** SYDV was purified from *N. benthamiana* plants. Coomassie-stained bands containing the N and G proteins were excised from 10% SDS-PAGE gels. Gels-slices were digested with trypsin and eluted peptides were purified by liquid-chromatography and partially-sequenced by Edman degradation and mass-spectrometry respectively. **B.** Two peptide sequences were synthesized for N and three for G protein. **C.** Degenerate primers were designed based upon the first six residues of two peptides. Both forward and reverse primers were designed for the specific peptide sequences as the

relative positions of the peptides in the cognate protein sequences was not known and they were subjected to RT-PCR reaction with all possible combinations using template cDNA derived from total RNA isolated from mock-inoculated or SYDV-infected plants.

D. Ethidium bromide gel image of the RT-PCR reaction showing the amplification of SYDV-specific amplicon. A 0.45 kb SYDV-N fragment were amplified using primer combination N1-F/ N2-R and 2.0kb N-G specific amplicon were amplified using N-RF2/ G2R combination (*see chapter 3 for primer sequences*). The SYDV-N and N-G amplicon were cloned into pGEM-T and pJET-1 by T/A cloning.

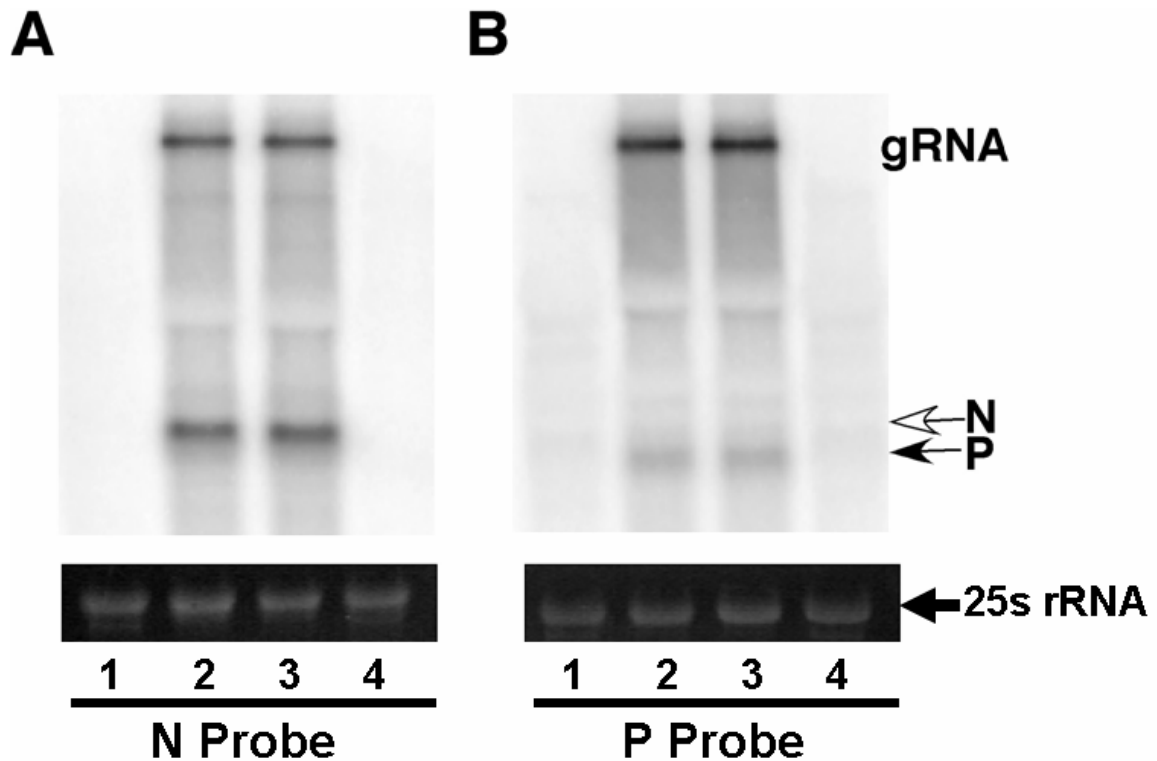


Figure 3.4: Detection of N and P mRNA transcript in SYDV infected *Nicotiana benthamiana* by Northern Hybridization. Equivalent amounts of total RNA isolated from SYDV and CYDV infected *N. benthamiana* leaves (lane 2, 3 respectively) hybridized to genomic RNA (gRNA) and **A.** SYDV-N mRNA (white arrow). **B.** SYDV-P mRNA (black arrow). Cross-hybridization with the RNA transcripts from total RNA isolated from healthy *N. benthamiana* (lane 1) and SYNIV infected *N. benthamiana* leaves (lane 4) was not observed. Ethidium bromide (EtBr) gel images of 25s ribosomal RNA were used as loading controls.

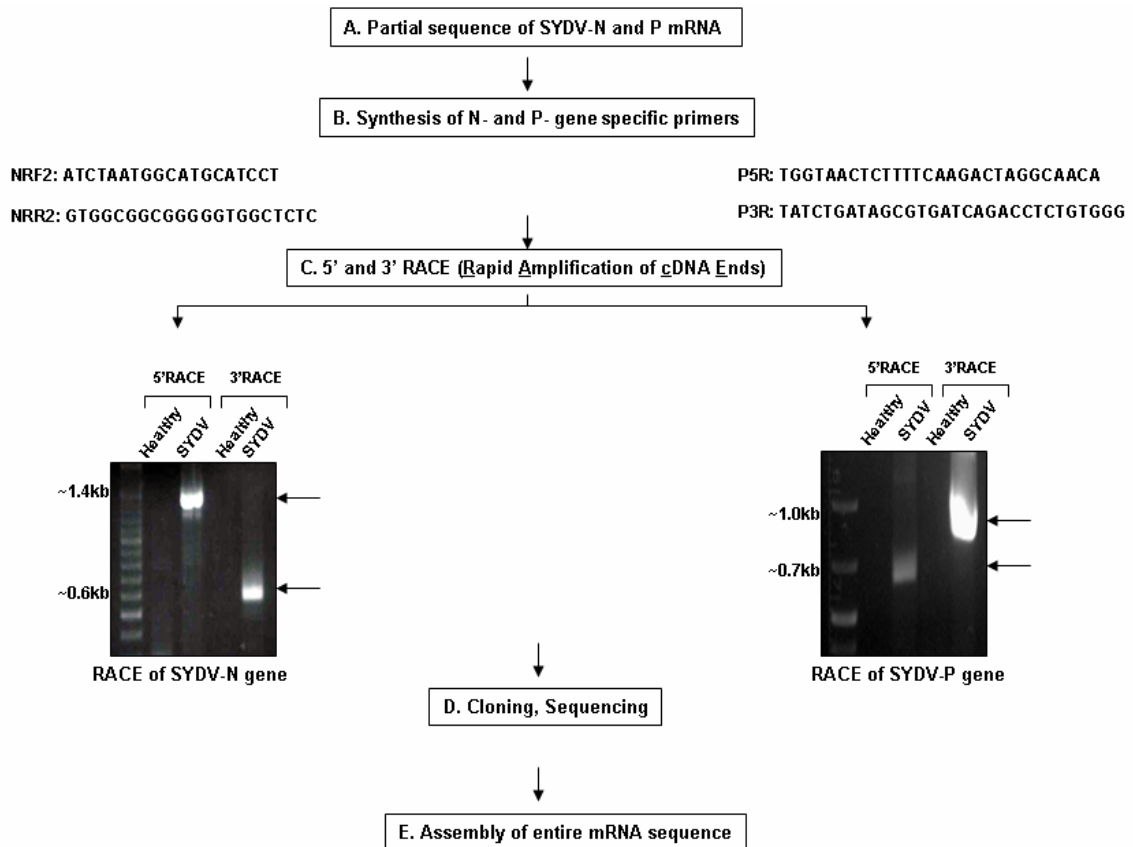


Figure 3.5: Strategy for cloning 5' and 3' ends of SYDV-N and P mRNA. **A, B.** A set of gene specific forward and reverse primers were designed based on the initial partial sequence of SYDV-N and P mRNA. **C.** Ethidium bromide (EtBr) gel picture of 5' and 3' RACE-PCR reaction of cDNA obtained from total RNA isolated from SYDV infected *N. benthamiana* tissue using universal primer mix (UPM) and N/ P gene specific primer. Black arrow indicates 1.4 kb 5'RACE and 0.6 kb 3'RACE amplification for SYDV-N mRNA as well as 0.7 kb 5'RACE and 1.0 kb 3'RACE amplification of SYDV-P mRNA. Cross-hybridization with the cDNA obtained from total RNA isolated from healthy *N. benthamiana* was not observed. **D.** N and P 5' and 3' RACE products were cloned into pGEM-T vector by T/A cloning and clones carrying the positive inserts were sent for sequencing.

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001 AUG AAU AAC GCU AAC ACC GCU CAG UUC GUG CAG UUG UUG AAG GGG GGG 5' AAACA CACAAUC 060
Met Asn Asn Ala Asn Thr Ala Gln Phe Val Gln Leu Leu Lys Gly Gly Lys Glu Tyr Glu
061 GAU UGG AAU UCG CGU GAU GAU AUA CCC GCA GCA GAU GGA AAG UUA GUU CAG AUA GAG UAU 120
Asp Trp Asn Ser Arg Asp Asp Ile Pro Ala Ala Asp Gly Lys Leu Val Gln Ile Glu Tyr
121 UCU GAU GCG GAU UUU UGG AGU AAG UUG ACC ACG AUA UAC AGU CUC GAU GAU CUU GGA CCG 180
Ser Asp Ala Asp Phe Trp Ser Lys Leu Thr Thr Ile Tyr Ser Leu Asp Asp Leu Gly Pro
181 AGU GAU UUG GUU GCA GCG UGG ACU AGA AUC AGA GAA AGU AUC GAU AAU AGU ACU AUC UCU 240
Ser Asp Leu Val Ala Ala Trp Thr Arg Ile Arg Glu Ser Ile Asp Asn Ser Thr Ile Ser
241 GAA UCU AUA AUC GGA GAG AUU UUC CGU GUG GCA GCA CAC AUU AAA AAG GUA GAG GAU AAG 300
Glu Ser Ile Ile Gly Glu Ile Phe Arg Val Ala Ala His Ile Lys Lys Val Glu Asp Lys
301 ACC AGC AGA AUU AUC GGU AGC UUU GCU CCU CCC ACA GCG CGA GGC GUC GUC ACC AAG ACC 360
Thr Ser Arg Ile Ile Gly Ser Phe Ala Pro Pro Thr Ala Arg Gly Val Val Thr Lys Thr
361 GGA CUA GAC ACC GCU CCC AUC GUA UUC GGA CAG UCG GUU ACA GUA GGU GCA GAU AAU ACU 420
Gly Leu Asp Thr Thr Ala Pro Ile Val Phe Gly Gln Ser Val Thr Val Gly Ala Asp Ile Thr
421 GUU CAG AAC ACC GCU CCC ACC GUC CGC ACA ACA GAC GCA GGA GAC GCU GUG GUA UCU GCA 480
Val Gln Asn Thr Ala Pro Thr Val Arg Thr Thr Asp Ala Gly Asp Ala Val Val Ser Ala
481 CCU UAC CUG UGC AUG GCC UUG CUA CGC CUG AUG ACU AAA CCG GUG GAA UCC UUU AAC CCG 540
Pro Tyr Leu Cys Met Ala Leu Leu Arg Leu Met Thr Lys Pro Val Glu Ser Phe Asn Arg
541 UCU UUA ACU ACC AUU AGA ACA AGU UAC GGG AGA UUC UAC GGG AUG CAG UCA GCG GAA GUC 600
Ser Leu Thr Thr Ile Arg Thr Ser Tyr Gly Arg Phe Tyr Gly Met Gln Ser Ala Glu Val
601 ACC AAU UUU UCG GCA CCG CUU AAC UCA CUU CAA CAG UUA UCC ACC GGG CUA GAC ACA UAU 660
Thr Asn Phe Ser Ala Pro Leu Asn Ser Leu Gln Gln Leu Ser Thr Gly Leu Asp Thr Tyr
661 CCG ACN UGC AAU UCG ACA AUG GCA UGG AUG AUG GGG UUU GCU GAA GGG AUG AUC CUC AGA 720
Pro Thr Cys Asn Ser Thr Met Ala Trp Met Met Gly Phe Ala Glu Gly Ser Ile Leu Arg
721 AAC AAC AAA AAU CAU GGU UUC AUG AGG UUU CUG AUC UUU CAG CAU GAG AUG AGA GGU 780
Asn Asn Lys Asn His Gly Phe Met Arg Phe Leu Ile Phe Gln His Ala Glu Met Arg Gly
781 AUG CAG AUC UAU AAA AUG AUC CUG ACA GCU CUA GCU GGA CUG CCA GCC AUU ACC CCU GCU 840
Met Gln Ile Tyr Lys Met Ile Leu Thr Ala Leu Ala Gly Leu Pro Ala Ile Thr Pro Ala
841 CAA UUU CUG AGA GCU AUU GAA AUA CCU GAU GCU GUA AAG GCA AUC AAG ACA GUG AUA AAG 900
Gln Phe Leu Arg Ala Ile Glu Ile Pro Asp Ala Val Lys Ala Ile Lys Thr Val Met Lys
901 AUU GCC ACC ACC CUG GAU AAA CCG GGG AGA CAA GAC CCC ACC UAC UGG UGG AAA UAU GGG 960
Ile Ala Thr Thr Leu Asp Lys Pro Gly Arg Gln Asp Thr Thr Tyr Trp Trp Lys Tyr Gly
961 AAG UAC AUA GAA CCA UCC UAC UUU GUA GAU CUA AGU GUG GGU AGG AAU ACA AAG UUU GCC 1020
Lys Tyr Ile Glu Pro Ser Tyr Phe Val Asp Leu Ser Val Gly Arg Asn Thr Lys Phe Ala
1021 UAU CUA AUG GCA UGC AUC CUC AAU GAG AUG AGC UUA AUC AAU GGU CCU GAA UAU GCC AAC 1080
Tyr Leu Met Ala Cys Ile Leu Asn Glu Met Ser Leu Ile Asn Gly Pro Glu Tyr Ala Asn
1081 CCC AAA AAC AUA AAG GCU CUU GAG UCC AUU AAA AAC AAC AUA GAG CUC ACA AAC UAC UAC 1140
Pro Lys Asn Ile Lys Ala Leu Glu Ser Ile Lys Asn Asn Ile Glu Leu Thr Asn Tyr Tyr
1141 GAA GGA CUU UCA AGG AAC UUC UCU AUC CUC UAC AGG UCC UUG GAG ACA GAG AGU GGC AUU 1200
Glu Gly Leu Ser Arg Asn Phe Ser Ile Leu Tyr Arg Ser Leu Glu Thr Glu Ser Gly Ile
1201 GGA AUA GGU AUA GCA AUG CAG AUG GGA GGA GCC CCC GCU CCC AAG GCA AGA GCA CAG AAA 1260
Gly Ile Gly Ile Ala Met Gln Met Gly Gly Ala Pro Ala Pro Lys Ala Arg Ala Gln Lys
1261 CGU GCC AAU GAA GAA GCA CCA CCA GCA GCA CAG AAG AGA GCC ACA CCA GCA GCC CAG CAG 1320
Arg Ala Asn Glu Glu Ala Pro Pro Ala Ala Gln Lys Arg Ala Thr Pro Ala Ala Gln Gln
1321 GAC CAG CAA GCA GCG GGG ACA UCC GGA ACA GCA UCA GCC ACU CCG GUG GAU GCA AUG AAU 1380
Asp Gln Gln Ala Ala Gly Thr Ser Gly Thr Ala Ser Ala Thr Pro Val Asp Ala Met Asn
1381 GCU GCU CUA CAA UCC GGA GUG CUG GAC CAG CUG UAG UCGAGCUAGAUGGUAUUUGCCUUUGU
Ala Ala Leu Gln Ser Gly Val Leu Asp Gln Leu Pro stop
UUUAUGUUGUUCGUGUUAAAUAUCUAUUGUGUAUAUGUAUGUGUGUGCCUUACAAAAA-3'

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Figure 3.6: Nucleotide and deduced amino acid sequence of the SYDV-N mRNA.

The nucleotide sequence is presented as messenger RNA sequence. The 5' and 3' untranslated region of the mRNA is not taken into consideration for numbering. The conserved transcription initiation region found is boxed. The peptide sequences used for designing degenerate primers are underlined. Black arrow indicates the position of N-specific forward and reverse primers used for RACE.

```

5' AAACA ACCACACAUGAAGC
001 AUG UCA GGG CAU GAU AUC AGU CCU UCG AGA AAG CUU AGA GAU AGA CAC CCC AGC AAG CCA 060
    Met Ser Gly His Asp Ile Ser Pro Ser Arg Lys Leu Arg Asp Arg His Pro Ser Lys Pro
061 CUU GCC CGG UCA GCC CCU UAU GAC CCA GUA AAA CAA GCA AAA UAC AAA AAA CAA GUA UUA 120
    Leu Ala Arg Ser Ala Pro Tyr Asp Pro Val Lys Gln Ala Lys Tyr Lys Lys Gln Val Leu
121 GAG GGA AAC AAA UAC GAG GAC UUU AAU CCG CAA UCU CUA ACA GAC AUU AAG AAC GAU AGC 180
    Glu Gly Asn Lys Tyr Glu Asp Phe Asn Pro Gln Ser Leu Thr Asp Ile Lys Asn Asp Ser
181 AAU CCC CCU AUC AUG GCU CCU GUA AAC CCA GAA GGG ACA AAA UUC UCU GAG UUA UGU CAA 240
    Asn Pro Pro Ile Met Ala Pro Val Asn Pro Glu Gly Thr Lys Phe Ser Glu Leu Cys Gln
241 AUG UUA AAA GAG CAG GGA AAC ACG GUA UCU GAU AGC GUG AUC AGA CCU CUG UGG GAC GCC 300
    Met Leu Lys Glu Gln Gly Asn Thr Val Ser Asp Ser Val Ile Arg Pro Leu Trp Asp Ala
301 ACA CCA AAA GGA CAG ACC ACA GAG GAA AAU GAU GAC GCA GUC AAA GCU GCA GUA GGA UGG 360
    Thr Pro Lys Gly Gln Thr Thr Glu Glu Asn Asp Asp Ala Val Lys Ala Ala Val Gly Trp
361 UUU AAU CUU GGU CAG ACG AAC UUA GAG ACU CAA AUA AAC CUU CAG AAU AUG AGA UAU GCU 420
    Phe Asn Leu Gly Gln Thr Asn Leu Glu Thr Gln Ile Asn Leu Gln Asn Met Arg Tyr Ala
421 CAA ACC AGU CUG CCC AAC UUU GUA UCG GGA CUA GCC AAU ACU GCU UCA GCA CUG ACU UCU 480
    Gln Thr Ser Leu Pro Asn Phe Val Ser Gly Leu Ala Asn Thr Ala Ser Ala Leu Thr Ser
481 GUA GUG CAA AAA UUA GAG AAC GUG UUG CCU AGU CUU GAA AAG AGU UAC CAU AUC AAC AGU 540
    Val Val Gln Lys Leu Glu Asn Val Leu Pro Ser Leu Glu Lys Ser Tyr His Ile Asn Ser
541 AUG AGU GAA GCU GAC AAA AUC AAU CUA GCU UUG UCU AUU UAC AAG AAC AAG AAC CAG GGG 600
    Met Ser Glu Ala Asp Lys Ile Asn Leu Ala Leu Ser Ile Tyr Lys Asn Lys Asn Gln Gly
601 GAG AAA UAU CGU AUG AUU UCA GAC UUU CUA GUG AAU GAG UUA GGC UAC UCU GAG UUU UAC 660
    Glu Lys Tyr Arg Met Ile Ser Asp Phe Leu Val Asn Glu Leu Gly Tyr Ser Glu Phe Tyr
661 AAU GAU AUG AUG UCC CCU CAU UAU AAG AAC UUU GCU CUA CAG CAA CUC AGA ACU GUC ACG 720
    Asn Asp Met Met Ser Pro His Tyr Lys Asn Phe Ala Leu Gln Gln Leu Arg Thr Val Thr
721 CCG GAG AUU GUC GCA GCA GUA UCG UGU UUU GGA CUA CAC AAU UUC CCU AGU CUC AAA GAU 780
    Pro Glu Ile Val Ala Ala Val Ser Cys Phe Gly Leu His Asn Phe Pro Ser Leu Lys Asp
781 AGG AUG UUG CAG GCA CAA AAG ACC CUA AGA GAU GCA AUA UCU GGG AGA AUG ACG GAG AAC 840
    Arg Met Leu Gln Ala Gln Lys Thr Leu Arg Asp Ala Ile Ser Gly Arg Met Thr Glu Asn
841 UGA CAACGGUGCUGCAUCAAGAUGAUGACCCGCCCAAUACCCUCGGAAGCACUCCGCUACCAUCUAUAAGACUA
    stop
    UCCCAUUGUAUCCCUAGUAUUUUUCAGUAUUUUCGUGUUCUGCUUAAUAAAAAAAAAAAAAAAA-3'

```

Figure 3.7: Nucleotide and deduced amino acid sequence of the SYDV-P mRNA.

The nucleotide sequence is presented as messenger RNA sequence. The 5' and 3' untranslated region of the mRNA is not taken into consideration for numbering. The conserved transcription initiation region found is boxed. Black arrow indicates the position of P-specific forward and reverse primers used for RACE.

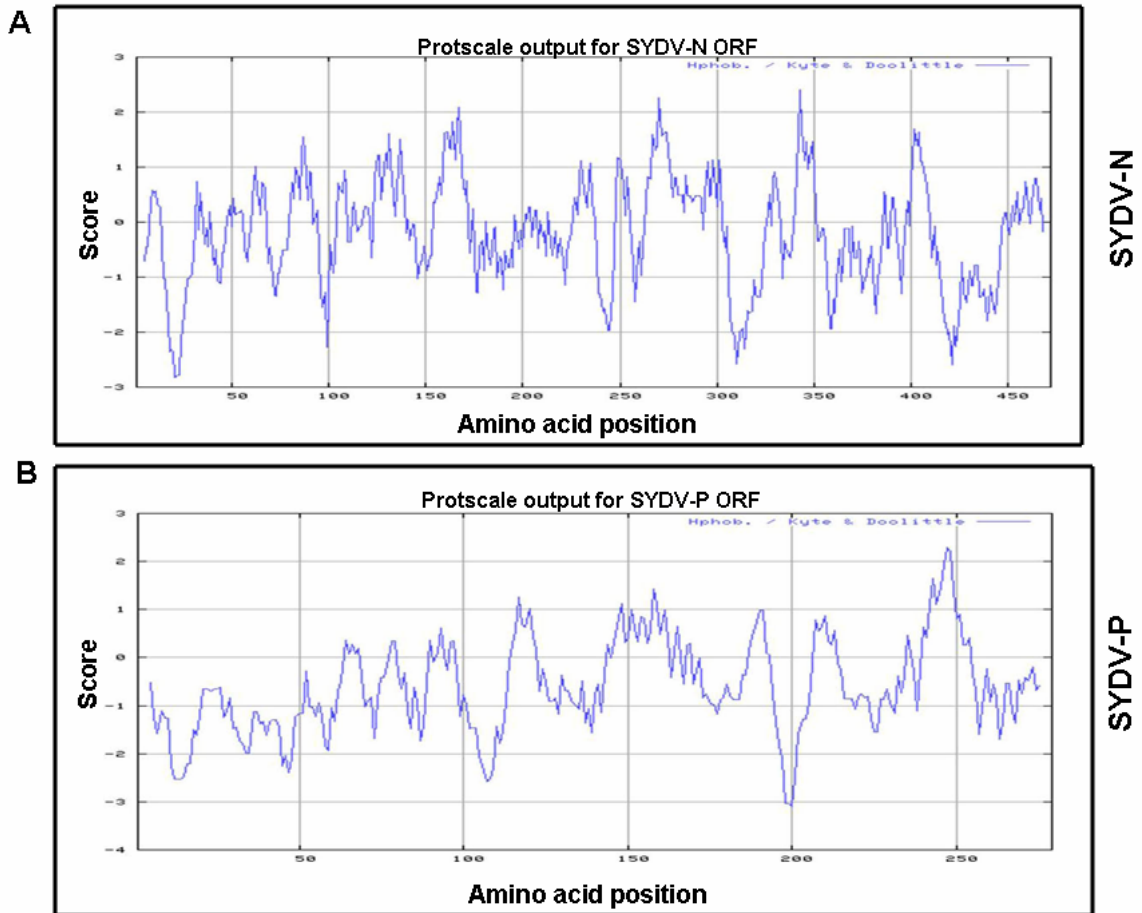


Figure 3.8: Hydropathy profile of A. SYDV-N and B. P protein. The plots were generated by ProtScale using SOAP algorithm (Kyte and Doolittle, 1982). Lines above and below the center of the scale predicts the relative hydrophobicity and hydrophilicity respectively. SYDV-N protein showed a highly hydrophilic region from amino acid position 10-20 near the amino terminal and a stretch from position 310-440 located near the carboxy terminal and SYDV-P protein showed hydrophilic region from amino acid position 1-61, 100-118 close to amino terminal and a weak region from amino acid position 190-205 near the carboxy terminal.

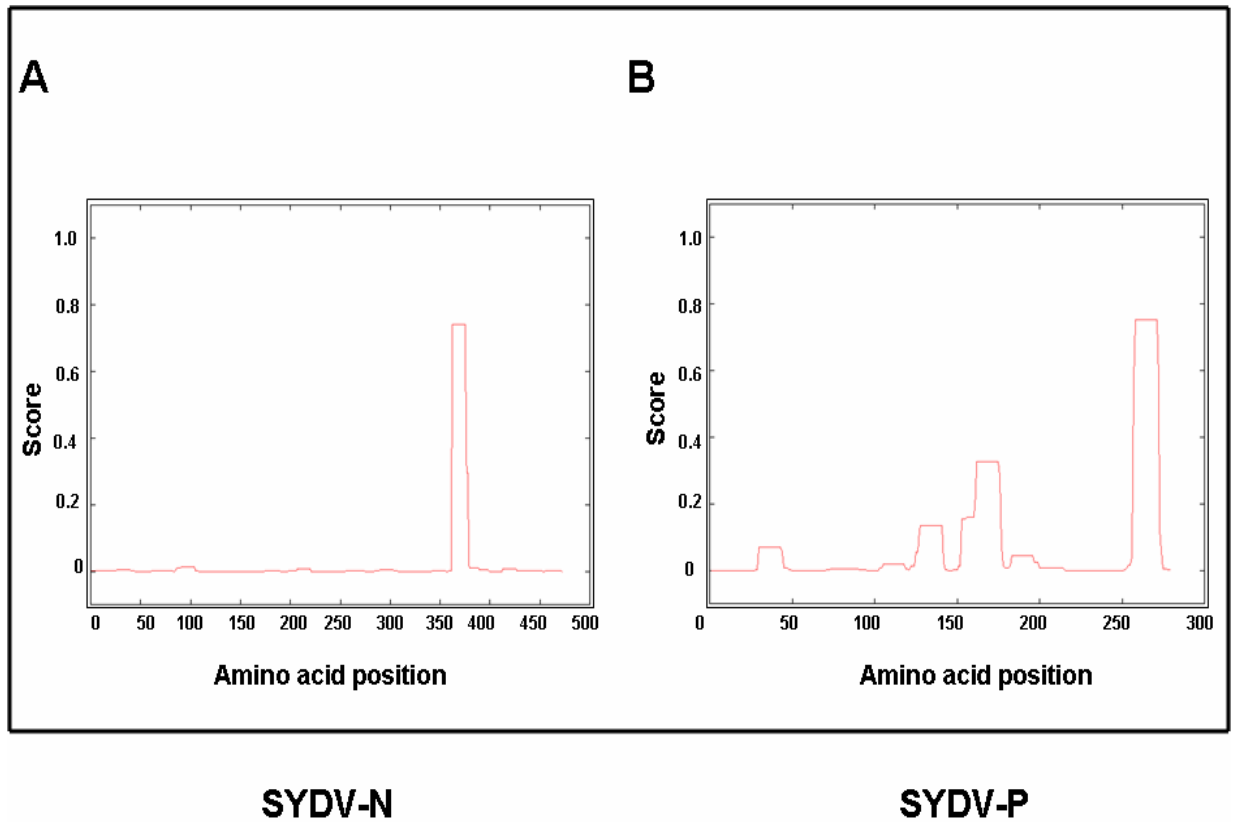


Figure 3.9: Prediction of coiled coil region in SYDV-N and P protein. Analysis of **A.** SYDV-N and **B.** SYDV-P protein using COIL algorithm predicts putative distinct coiled coil region at amino acids position 360 to 375 for SYDV-N and at the amino acid position 32-50, 125-145, 150-175 along with an extensive coiled region at amino acids position 250 to 278 for SYDV-P (using the window matrix of 14 amino acid residues). (Y-axis denotes the matrix of amino acid score for COIL algorithm; X-axis denotes the no. of amino acids/ amino acid position in the sequence)

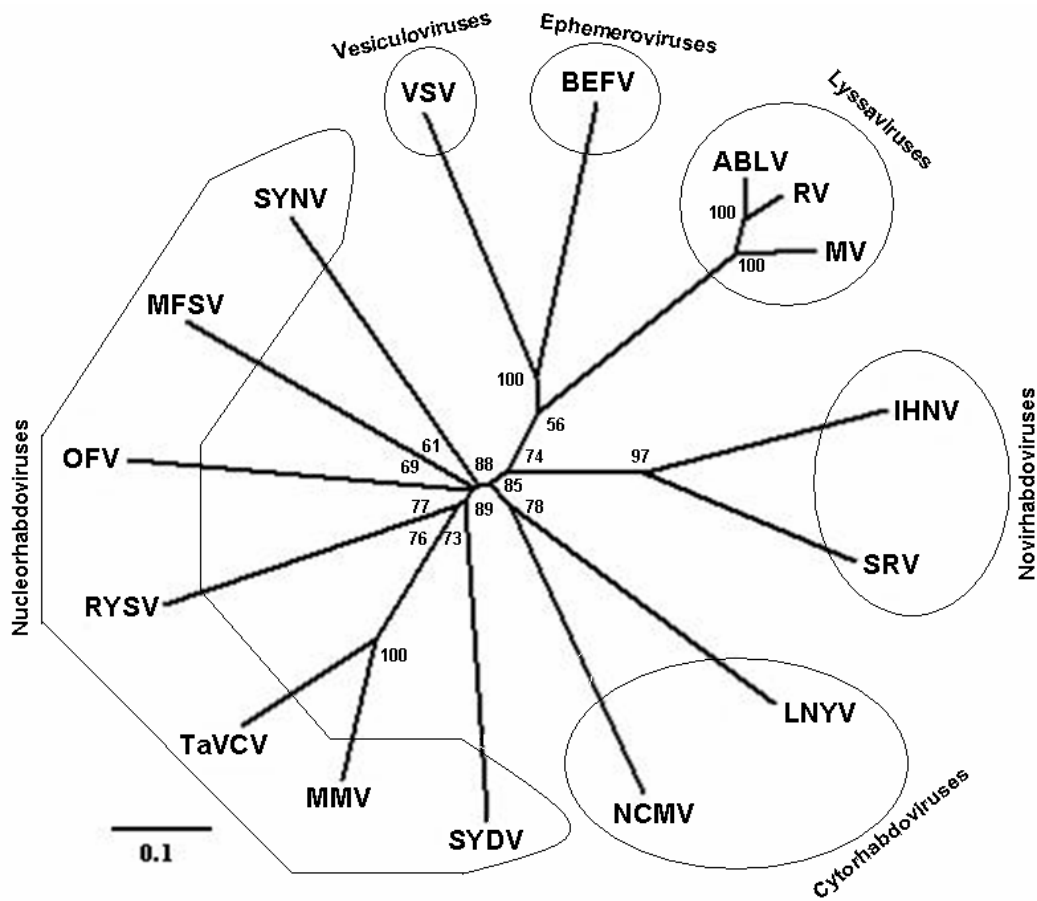


Figure 3.10: Neighbor-joining tree constructed using N protein amino acid sequences and orthologs from selected plant and animal rhabdoviruses. Branch lengths are proportional to the differences between pairs of neighboring nodes. The scale bar represents the number of aa replacements per site. Figures on the branches represent the percentage of trees containing each cluster out of 1000 bootstrap replicates. The virus names and GenBank accession numbers of sequences used in the analysis are listed in the materials and method section.

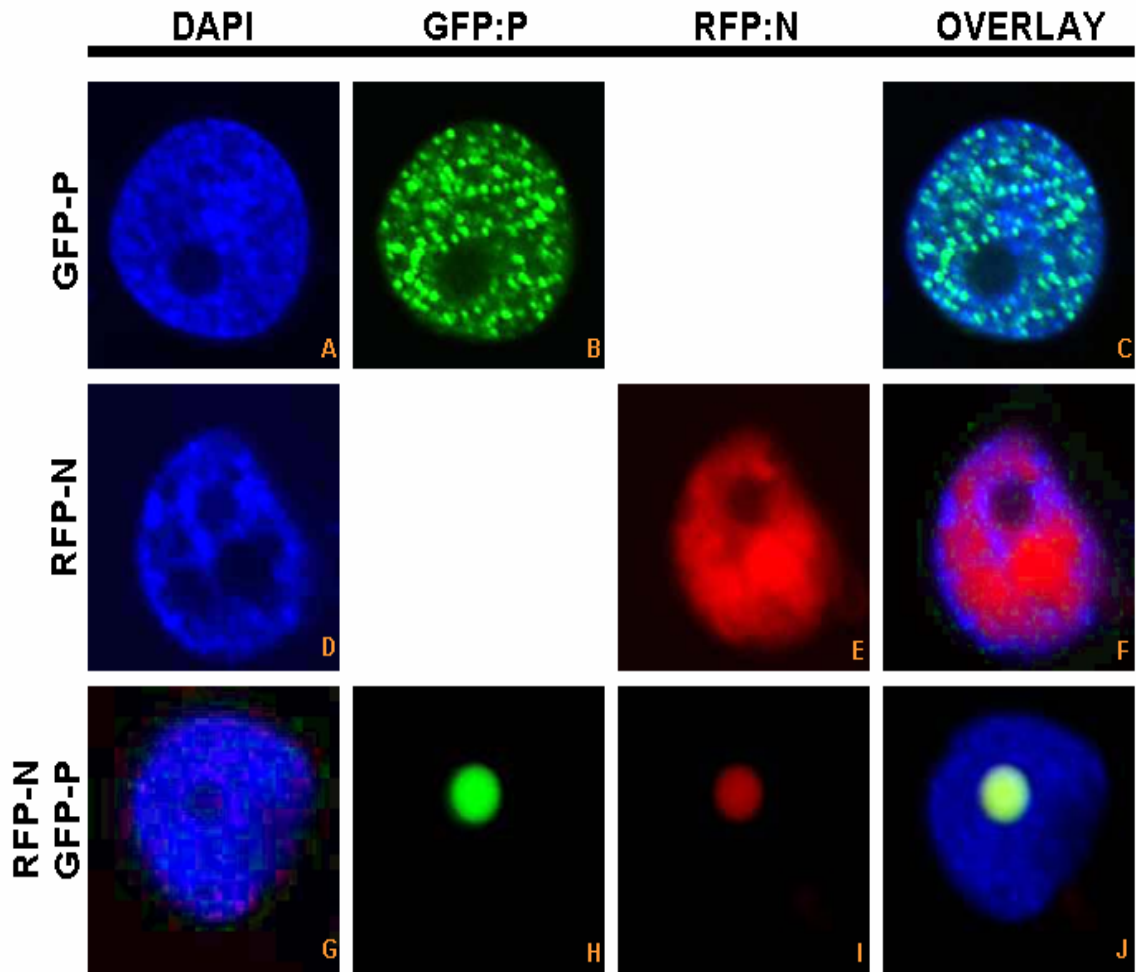


Figure 3.11: Confocal micrographs showing the subcellular localization of SYDV-N and P protein in *Nicotiana benthamiana* cells. A-C. Confocal micrographs of GFP:P protein fusion showing the localization of SYDV-P protein in the nucleus. **D-F.** Confocal micrographs of RFP:N protein fusion showing the nuclear localization of SYDV-N protein. **G-J.** Co-expression of SYDV-N (RFP:N) and SYDV-P (GFP-P) results in subnuclear localization of N and P protein. The DNA selective dye DAPI was used to validate the nuclear localization in the plant cells.

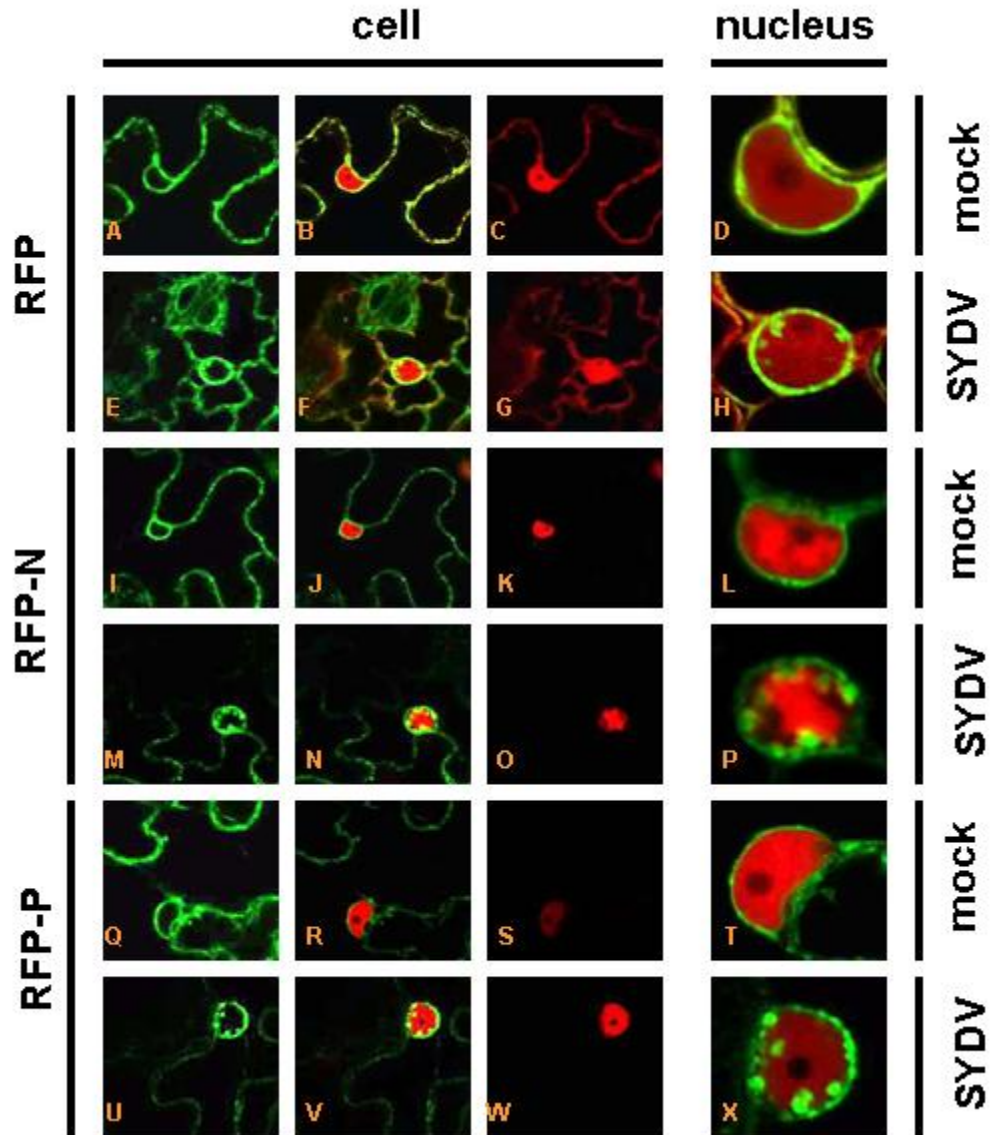


Figure 3.12: Confocal micrographs showing the subcellular localization of SYDV-N and P protein in mock and SYDV-infected “16c” *Nicotiana benthamiana* cells. A-H. Confocal micrographs of mock and SYDV-infected “16c” *Nicotiana benthamiana* cells showing the detection of RFP fluorescence (RFP alone) in the nucleus and cytoplasm. **I-P.** Confocal micrographs of RFP:N protein fusion in mock and SYDV-infected “16c” *Nicotiana benthamiana* cells showing that nuclear localization of SYDV-N protein and RFP fluorescence is detected only from nucleus. **Q-X.** Confocal micrographs of RFP:P

protein fusion showing SYDV-P protein localized in the nucleus of mock and SYDV-infected “16c” *Nicotiana benthamiana* cells with nuclear detection of RFP fluorescence .

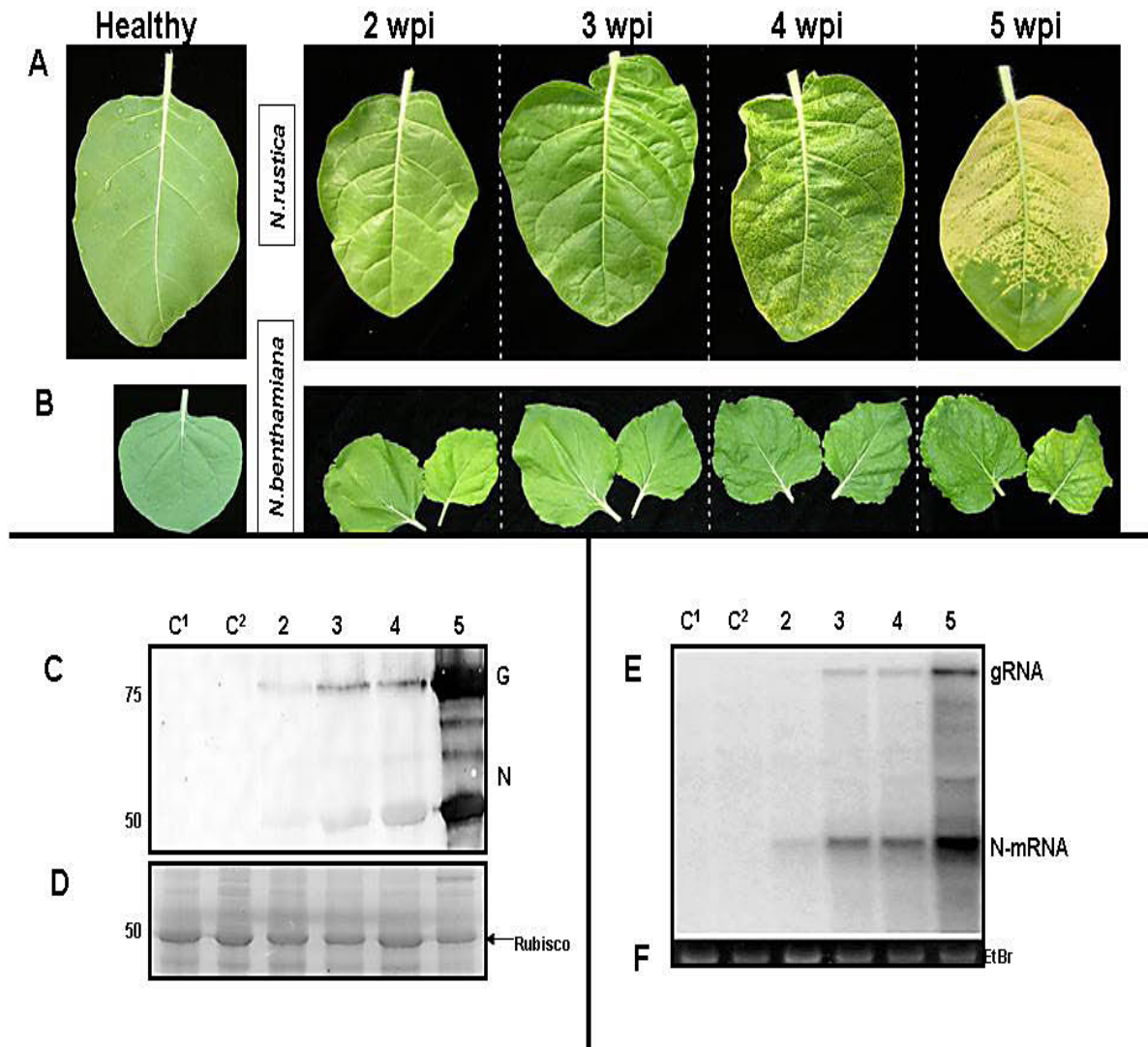


Figure 3.13: Time course virus infection in SYDV infected *Nicotiana* leaves. A, B. Symptoms of SYDV infection in *Nicotiana rustica* and *Nicotiana benthamiana* leaves after 2nd, 3rd, 4th and 5th week post inoculation (wpi). Virus-induced symptoms are observed as venial chlorosis of leaves, and are severe at 4th and 5th wpi in *N. rustica* leaves, and at 5th wpi in *N. benthamiana* leaves. **C.** Western immunoblot analysis showing the gradual increase of SYDV encoded protein level as an indication of

increasing virus infection at 2nd, 3rd, 4th and 5th wpi respectively. Following separation on 10% SDS-PAGE gel, equivalent amounts of protein from healthy *N. benthamiana* (lane C¹), SYDV (lane 2,3,4 and 5) and SYN^V-infected *N. benthamiana* (lane C²) leaves were transferred to nitrocellulose membrane and probed with SYDV-specific polyclonal antibodies raised in rabbits to disrupted virions. Cross-reaction with protein isolated from healthy *N. benthamiana* (C¹) and SYN^V infected *N. benthamiana* (C²) leaves was not observed. **D.** A coomassie stained gel image showing rubisco (50 kD) were used as loading controls. **E.** Northern hybridization showing the gradual increase of SYDV specific RNA as an indication of increasing virus infection at 2nd, 3rd, 4th and 5th wpi respectively. Equivalent amounts of total RNA, from healthy *N. benthamiana* (lane C¹), SYDV (lane 2, 3, 4 and 5) and SYN^V-infected *N. benthamiana* (lane C²) leaves were separated on denaturing 1.2% formaldehyde-agarose gel, transferred to positively charged nylon membrane and probed with SYDV-N. Cross-hybridization with total RNA isolated from healthy *N. benthamiana* (C¹) and SYN^V infected *N. benthamiana* (C²) leaves was not observed. **F.** Ethidium bromide gel images of 25s ribosomal RNA were used as loading controls.

Chapter 4

4.1 Discussion

This dissertation reports (1) a detailed analysis of SYDV infection pattern on host cell endomembrane system and its comparative study with SYNVDV infection, (2) cloning and characterization of SYDV-N and P protein is reported towards the aim of completion of whole genome sequencing of SYDV, (3) subcellular localization of SYDV-N and P protein in mock-inoculated and virus-infected plants, (4) a comprehensive study of the symptoms of SYDV on experimental hosts, and (5) a phylogenetic relationship analysis of SYDV with other well-characterized plant and animal rhabdoviruses based on their consensus N-ORF. Currently the biology of plant-adopted rhabdovirus-host cell interaction, especially the infection process and how this group of viruses alters the host cell biology and cause disease is not clearly understood. In recent years, due to their increasing threat to agricultural production for many crops, sequencing of a number of plant rhabdovirus genomes has been completed (Huang et al 2003; Dietzgen et al 2005; Reed et al., 2005; Revill et al 2005; Tsai et al 2005) but very little is known about the cell biology of these newly sequenced viruses. PYDV is the type member of genus *Nucleorhabdovirus* and due to the elegant and pioneering work of Lindsey Black and colleagues, PYDV has long served as the paradigm for plant rhabdovirus research particularly in the area of virus:vector relationships (Black 1938, 1943; McLeod 1966,1968). However, until now, no molecular data were available for PYDV prior to this study. In addition, two serologically distinguishable strains, SYDV and CYDV are now available for research. Further, the finding that *N. benthamiana* can serve as the experimental host for both SYDV and SYNVDV, is an excellent tool for

comparative virology for plant *nucleorhabdoviruses*. The live cell imaging study of SYDV and SYNVD infection in GFP transgenic *N. benthamiana* revealed different infection pattern on the host cell nuclei upon virus infection. The GFP accumulation was found mainly in the external and internal loci of the infected nucleus in SYDV infected cells. In contrast, GFP accumulated throughout the nucleus in the case of SYNVD infection. It is assumed that the differences in the patterns of increased GFP accumulation depend on the mode of morphogenesis adopted by SYDV and SYNVD. Invaginations of the inner nuclear membrane upon SYNVD infection result in nuclear accumulation of GFP. On the other hand, infection of SYDV induces expansion of the outer nuclear membrane with an apparent accumulation of GFP on the “outside” of the nucleus (Goodin et al., 2005). Additionally, the time course infection study of SYDV on *N. benthamiana* showed that the infection intensity is high around the 3rd wpi, which was followed by plant death at approximately six to seven weeks post inoculation (data not shown). Similar time course infection studies of SYNVD in *N. benthamiana* conducted in our lab showed marked recovery phenotype in SYNVD infected plants at 4 wpi as depicted by loss of viral antigens and a return of newly emergent leaves to near wild-type morphology (Ghosh et al, manuscript submitted). In contrast, SYDV infected plants did not recover, and the protein sample from the 5th wpi contained a ~90 kD protein band in the Coomassie-stained gel that may correspond to the PYDV-G protein (glycoprotein) (Figure 3.12). Cloning of the SYDV genome and characterization of SYDV-encoded proteins are required to characterize this 90 kD protein, and to understand the difference in infection patterns as well as the recovery phenotype of SYDV and SYNVD in *N. benthamiana*. In general, there are three genes in between N (Nucleocapsid) and G gene

in plant rhabdoviruses, namely P (Phosphoprotein), putative matrix protein and M (Matrix protein) gene (Jackson et al., 2005) (Figure 1.2). Attempt has been made to amplify and clone all five genes by designing degenerate primers based on partial peptide sequence of N and G ORF. Unfortunately, the G degenerate primer did not hybridize to the G gene sequence as expected. But when used in combination with an N-gene specific forward primer, one of the G degenerate reverse primers amplified a 2.0 kb SYDV-specific fragment (Figure 5.6). An ORF was identified upon successful cloning and sequencing of that 2.0 kb SYDV-specific fragment, which showed characteristics of rhabdoviral P proteins, mainly based on its position next to N-ORF and its ability to change the localization pattern of N protein from the nucleoplasm to a sub-nuclear local when both proteins were co-expressed as autofluorescent protein fusion (Figure 3.11). Similar change in the localization pattern has also been observed for the N and P proteins of both SYNV and MFSV (Goodin et al 2001, Tsai et al 2005).

Complete sequence and characterization of SYDV-N and P protein will help to understand the biology and role of these proteins in the SYDV life cycle. Prediction of coiled coil region in SYDV-N and P protein suggests a possible interaction between N and P protein, and between P protein itself similar to other well characterized animal and plant rhabdoviruses, because the presence of coiled coil region often mediates protein-protein interaction (Curran et al., 1995; Goodin et al., 2001). Studies with SYNV-N have shown that Tyr40 in a helix-loop-helix region near the amino terminus of the N protein is required for efficient N-N and N-P interaction (Deng et al., 2007). In addition, homologous interactions mediated by the same helix-loop-helix region near the amino terminus of SYNV-N protein are required for formation of the viroplasm-like foci (Deng

et al., 2007). Yeast two-hybrid assay/ co-purification of proteins using GST or His tag can be done to examine the prediction of possible interaction between SYDV-N and P protein, as has been done for VSV (Takacs et al., 1993) and SYNV (Goodin et al., 2001). Phosphorylation studies of SYDV-P protein will demonstrate the potential domain responsible for P protein phosphorylation as 123 putative motifs for serine kinase and 12 putative motifs for tyrosine kinase were predicted for SYDV-P ORF using Phosphofinder algorithm (Peri et al., 2003; Mishra et al., 2006). Although no putative NLS's were predicted for SYDV-N and P protein by the pSORT algorithm, presence of a distinct hydrophilic region in both the ORFs indicates there might be a specific karyophilic domain that guides these two proteins to be in the nucleus. The subcellular localization studies of autofluorescent protein fusions of SYDV-N and P protein showed that they are exclusively localized to the nucleus. At this point it is unknown which specific domains of SYDV-N and P protein and/or which precise host nuclear import system are responsible for the nuclear localization of these proteins. In the case of SYNV, importin- α is thought to be required for the nuclear localization of the N protein and P protein contains a karyophilic region between amino acid 40-124 (Goodin et al 2001; Deng et al 2007). Like SYNV, SYDV-N fusion protein colocalizes with the intranuclear membrane in SYDV infected cells, suggesting that N protein must be directed to intranuclear membrane during viral morphogenesis (Jackson et al., 2005). However, SYDV-P did not colocalize with the intranuclear membrane in SYDV infected cells. These data are entirely consistent with the proposed model for the spatial relationship between the sites of nucleorhabdovirus (SYNV) replication and morphogenesis (Goodin et al, 2007; Green et al, 2000), according to which, P protein delivers N protein as N/P complex to the

nascent strands of viral RNA and is then removed from N/P/RNA complex during the maturation of nucleocapsid.

Taken together, nucleotide and deduced amino acid sequences of SYDV-N and P gene along with their bioinformatic characterization will serve as excellent tools for future work on biochemistry and cell biology of N and P protein of SYDV. The nucleotide sequence of SYDV-N and P gene will not only prove beneficial to complete the sequencing of the entire SYDV genome, but it can be used as an efficient reagent to start the cloning and sequencing of another PYDV strain, CYDV (*constricta* strain). Subcellular localization data of SYDV-N and P protein will be helpful to understand the virus-host interaction and multiplication cycle of SYDV in host cell. Furthermore, complete sequence data and cell biology of these two strains of PYDV, which is the type species of genus *Nucleorhabdoviruses*, will enrich our understanding of rhabdoviruses especially their comparative genomics, cell biology and virus-host interaction

4.2 Proposed strategy to complete the sequencing of SYDV genome:

Given the set of primers already designed based on the SYDV gene sequences, completion of cloning and sequencing of the entire SYDV genome (~12.6 kb) should be accomplishable using primer walking method as has been done for several plant rhabdoviruses (Tsai et al., Revill et al., Wetzell et al). The complete sequence data of SYDV-N and P mRNA discussed in this dissertation as well as successful sequencing of already cloned PCR products (Table 5.1 Appendix) and cloning and sequencing of amplified 5.0 kb P-L and 7.0 kb N-L fragment (Figure 5.18 Appendix) will facilitate the characterization of more than half of the SYDV genome, that includes the matrix protein (M) gene, glycoprotein (G) gene, putative movement protein gene (s), 5' fragment of

polymerase (L) protein along with conserved “gene junction” sequences unique for each rhabdoviruses (Heaton et al., 1989, Jackson et al., 2005). Cloning of rest of the L gene could be done by conventional RT-PCR or 3’ RACE using the L gene-specific degenerate primers based on conserved block III region of rhabdoviral L gene (Bouhry et al., 2005). Completion of entire L polymerase gene sequencing should provide the necessary information to clone and sequence the trailer sequence at the 5’ end of SYDV genome. 5’ and 3’ RACE using gene specific primers (GSPs) located at the 5’ end of N gene and 3’ end of L gene should amplify the remaining 3’-leader as well as 5’-trailer sequences of SYDV genome respectively. Alternatively, a ligation-anchored PCR method can be applied to characterize the 5’ and 3’ terminus of SYDV genome as have been done for LNYV (Wetzel et al., 1994) and/ or OFV (Kondo et al., 2006).

The cloning strategy described above should facilitate an efficient means to clone and sequence the complete SYDV genome. In addition to that, the random shotgun sequencing method could also be applied to characterize the SYDV genome, as has been done successfully for another member of genus *Nucleorhabdovirus*, *Maize mosaic virus* (MMV) (Reed et al., 2005) as well as several other bacterial and eukaryotic genomes (Venter et al., 2004; Perna et al., 2001). This strategy employs the construction of a cDNA library from genomic RNA isolated from purified SYDV by random priming (SuperScript Choice system for cDNA synthesis Invitrogen Corp, Carlsbad, CA). After successful cloning of the cDNA into an appropriate vector, screening of positive colonies and sequencing can provide sufficient depth of genome coverage to assemble the SYDV genome sequence.

APPENDICES:

5. Complete organization of all SYDV cDNAs, PCR products and northern hybridization data

A PCR-based strategy using degenerate primers based on partial peptide sequence of N and G gene has been employed to initiate the cloning and characterization of entire 12600 nt (~12.6 kb) long SYDV genome. The reason being, in addition to clone the SYDV-N and G genes, these primers should permit amplification of approximately half of the SYDV genome. The advantage of this cloning strategy is, it also permit unambiguous assignment of the gene order between the N and G genes.

5.1 Amplification of SYDV-N mRNA fragment:

SYDV-N mRNA was amplified by RT-PCR (Superscript™ First strand cDNA synthesis system for RT-PCR, Invitrogen, Carlsbad, CA) using total RNA (5 ug) derived from SYDV infected *N. benthamiana* plants as template for first strand cDNA synthesis and oligo(dT) primer. The first strand cDNA synthesis reaction was catalyzed by Superscript™ II reverse transcriptase enzyme (modified *Moloney murine leukemia virus* RT). The first strand cDNA obtained was amplified by PCR reaction using Dynazyme DNA polymerase and SYDV-N gene specific degenerate primer combination (N2F/ N1R). A 500 bp SYDV specific DNA fragment was amplified (Figure 5.1), which was gel purified and used as probe in a Northern hybridization for its authenticity (Figure 3.4 A). The 500 bp PCR product was cloned into pGEM-T vector (Promega, Madison, WI) by T/A cloning. Cloned cDNA was subsequently used as a probe in Northern hybridization experiment. Of two clones tested in this manner, one gave the expected hybridization patterns, a 1.5 kb N mRNA transcript and a 12.6 kb SYDV genomic RNA

(Figure 5.2 B). A total of 56 clones were tested and the consensus sequence of 465 bp PCR-amplified DNA fragment was assembled from the sequence of 10 independent clones.

PCR condition for amplification of SYDV-N mRNA fragment (annealing temperature: 45°C):

94°C – 2 min

94°C – 1 min

45°C – 1 min

72°C – 1 min

72°C – 10 min

X 35

5.2 5' and 3' RACE of SYDV-N mRNA:

Two sets of N-mRNA specific primers (GSPs), forward and reverse (NRF1, NRR1 and NRF2, NRR2) were designed based on this initial 465 bp sequence of putative SYDV-N mRNA for the purpose of using these primers in RACE (Rapid Amplification of cDNA Ends). RT-PCR reactions with oligo(dT) primed first strand cDNA and NRF1/NRR1 primer pair resulted in amplification of a DNA fragment from cDNA synthesized from total RNA isolated from mock-inoculated *N. benthamiana* plants, in a manner similar to when degenerate N primers were used along with amplification of a fragment of predicted size (~350 bp) from SYDV infected *N. benthamiana* plants (PCR condition was same as Figure 4.1). The second primer pair (NRF2/NRR2) produced the predicted amplicon only from SYDV infected *N. benthamiana* plants (Figure 5.3). The primer pair NRF2/ NRR2 was used for 5' and 3' RACE reaction to amplify the 5' as well as 3' end of N-mRNA using SMART™

technology (Clontech). This method uses the combined actions of SMART II™ Oligonucleotide and the BD-PowerScript Reverse Transcriptase (RT). The first strand cDNA synthesis was carried out using 2 ug of total RNA extracted from SYDV infected *N. benthamiana* leaves and primed by a modified oligo dT primer. Following reverse transcription, the first strand cDNA was used directly in 5'- and 3'-RACE PCR reactions using N-gene-specific primer (NRF2/NRR2) and the BD SMART oligo (UPM, Universal primer mix A). The 5' and 3' RACE reactions amplified SYDV-N specific 5' RACE fragment (~1.4 kb) and 3'RACE fragment (~ 600 bp) (Figure 5.4.), which were cloned into pGEM-T vector. The RACE fragments were gel purified and subsequently used as probe in Northern hybridization experiment with total RNA isolated from SYDV and CYDV along with mock and SYNIV infected *N. benthamiana* leaves (negative control) for its SYDV specific authenticity (Figure 5.5).

PCR condition for SYDV-N 5' and 3' RACE-PCR (annealing temperature: 68°C):

<u>5'RACE</u>		<u>3'RACE</u>	
94°C – 30 sec	X 5	94°C – 30 sec	X 20
72°C – 3 min		68°C – 30 sec	
94°C – 30 sec	X 5	72°C – 3 min	
70°C – 30 sec			
72°C – 3 min			
94°C – 30 sec	X 25		
68°C – 30 sec			
72°C – 3 min			

5.3 Amplification of SYDV-N-G fragment:

SYDV-N-G fragment was amplified by RT-PCR using total RNA extracted from SYDV infected *N. benthamiana* leaves. Reverse transcription/ first strand cDNA synthesis reaction was primed by SYDV-N gene specific primer (NRF1). Following reverse transcription, conventional PCR was done by the same SYDV-N gene specific primer (NRF1) as forward primer, which was used to prime the first strand cDNA synthesis and G-specific degenerate oligonucleotide primer (G2R) as reverse primer. A virus specific amplicon of ~2.0 kb was amplified in SYDV cDNA containing sample, but not in healthy *N.benthamiana* cDNA containing control sample (Figure 5.6A.). The PCR product was cloned into pJET-1 vector (Fermentas, MD) and subsequently used as a probe in Northern hybridization experiment for its virus-specific authenticity (Figure 5.6B).

PCR condition to amplify the SYDV-N-G fragment (annealing temperature: 50°C):

94°C – 2 min

94°C – 30 sec		X 35
50°C – 30 sec		
72°C – 3 min		

72°C – 10 min

5.4 Amplification of SYDV-P mRNA:

SYDV-P mRNA was amplified by RT-PCR (Superscript™ First strand cDNA synthesis system for RT-PCR, Invitrogen, Carlsbad, CA) using total RNA (5 ug) derived from SYDV infected *N. benthamiana* plants as template for first strand cDNA synthesis and oligo(dT) primer. The first strand cDNA synthesis reaction was catalyzed

by SuperscriptTM II reverse transcriptase enzyme (modified *Moloney murine leukemia virus* RT). The first strand cDNA obtained was amplified by PCR reaction using “Phusion” proofreading DNA polymerase (Finzymes, New England Biolabs) and SYDV-P gene specific primer combination (PF/ PR). The same primer combination was used to clone the P ORF into pSITE expression vector by Gateway recombination reaction for subcellular localization studies (Invitrogen, Carlsbad, CA). A ~0.9 kb SYDV specific DNA fragment was amplified (Figure 5.7), which was gel purified and used as probe in a northern hybridization for its authenticity (Figure 3.4 B).

PCR condition to amplify SYDV-P mRNA (annealing temperature: 55°C):

98°C – 30 sec	
98°C – 10 sec	X 35
55°C – 30 sec	
72°C – 4 min	
72°C – 10 min	

5.5 5’ and 3’ RACE of SYDV-P mRNA:

A set of P-mRNA specific forward and reverse primers (P5R/P3R) were designed based on this initial 843 bp sequence of putative SYDV-P mRNA for the purpose of using these primers in RACE (Rapid Amplification of cDNA Ends). Completion of the entire P mRNA cloning was done by 5’ and 3’ RACE using SMARTTM technology as described for SYDV-N mRNA (Clontech). The first strand cDNA synthesis was carried out using 2 ug of total RNA extracted from SYDV infected *N. benthamiana* leaves and primed by a modified oligo dT primer. The first strand cDNA obtained, was used directly in 5’- and 3’-RACE PCR reactions using P-gene-specific

primer (P5R/P3R) and the BD SMART oligo (UPM, Universal primer mix A). The 5' and 3' RACE reactions amplified SYDV-P specific 5' RACE fragment (~0.5 kb) and 3'RACE fragment (~0.7 bp) (Figure 5.8), which were cloned into pGEM-T vector. The RACE fragments were gel purified and subsequently used as probe in Northern hybridization experiment with total RNA isolated from SYDV and CYDV along with mock and SYNIV infected *N. benthamiana* leaves (negative control) for its SYDV specific authenticity.

PCR condition for SYDV-P 5' and 3' RACE-PCR (annealing temperature: 68°C):

<u>5'RACE</u>		<u>3'RACE</u>	
94°C – 30 sec	X 20	94°C – 30 sec	X 5
68°C – 30 sec		72°C – 3 min	
72°C – 3 min		94°C – 30 sec	X 5
	70°C – 30 sec		
	72°C – 3 min		
		94°C – 30 sec	X 25
		68°C – 30 sec	
		72°C – 3 min	

5.6 Amplification of the region between N and P gene (Intergenic region):

Two sets of SYDV-N (forward) and P gene (reverse) specific primers were designed in order to amplify the region between N and P gene. The positions of the primers were determined in such, so that it will amplify the region between N and P gene along with flanking 3' end of N gene as well as 5' end of P gene at the 5' and 3' end of the predicted amplifying fragment, respectively. Reverse transcription reaction was

conducted using 5 ug of total RNA isolated from SYDV infected *N. benthamiana* leaves by SuperscriptTM First strand cDNA synthesis system for RT-PCR according to manufacturer's instruction (Invitrogen, Carlsbad, CA). The first strand cDNA was used as template in the PCR reaction using "Phusion" high fidelity DNA polymerase (Finzymes, New England Biolabs) and combination of two different primer sets, NPF1/NPR1 and NPF2/ NPR2 respectively. The PCR reaction results in predicted 0.5 kb and 0.6 kb SYDV specific DNA fragment (Figure 5.9). Cross hybridization with the cDNA obtained from total RNA isolated from healthy *N. benthamiana* leaves was not observed using the same primer combination. The PCR products were cloned into pGEM-T vector (Promega, Madison, WI) by T/A cloning. Colonies were screened and 4 independent clones carrying the positive insert for each DNA fragment were sent off for sequencing. Analysis of the sequences revealed 327 nt long region between SYDV-N and P gene (Figure 5.10). This 327 nt long putative region between SYDV-N and P gene is longer when compared to 14-16 nt long "gene junction" of other plant or animal rhabdoviruses (SYNV, MFSV, VSV, Rabies) (Heaton et al., 1989, Tsai et al., 2005), although 400-500 nt long intergenic region was evident between G and L gene in case of some fish rhabdoviruses (Thoulouze et al., 2004). Further experiments and analysis are required to validate this data.

PCR condition to amplify the region between SYDV-N and P gene (annealing temperature: 60°C):

98°C – 30 sec

98°C – 10 sec		X 35
60°C – 30 sec		
72°C – 2 min		

72°C – 10 min

5.7 Amplification of SYDV-G gene:

To amplify the SYDV-G gene, RT-PCR was conducted (Superscript™ First strand cDNA synthesis system for RT-PCR, Invitrogen, Carlsbad, CA) using total RNA (5 ug) derived from SYDV infected *N. benthamiana* leaves as template for first strand cDNA synthesis and random hexamer as primer. The first strand cDNA obtained was amplified by PCR reaction using “Dynazyme” proofreading DNA polymerase and SYDV-G gene specific degenerate primer combination (G1F/G2R). A 1.0 kb SYDV specific DNA fragment was amplified (Figure 5.11). Cross hybridization with the cDNA obtained from total RNA isolated from healthy *N. benthamiana* leaves was not observed using the same primer combination. The 1.0 kb PCR product was gel purified and used as probe in a Northern hybridization experiment using total RNA isolated from SYDV infected *N. benthamiana* leaves. The northern hybridization failed to reveal the authenticity of this SYDV-G gene specific 1.0 kb PCR product. Further experiments are necessary using different combination of G gene specific degenerate primers and PCR annealing temperature to validate this amplification.

PCR condition to amplify SYDV-G gene fragment (annealing temperature: 45°C):

94°C – 2 min

94°C – 1 min

45°C – 1 min

72°C – 1 min

72°C – 10 min

X 35

5.8 Amplification of SYDV-L gene fragment:

Given the fact that there are three highly conserved regions (pre motif A, motif A and motif B) present in the block III segment of RNA dependent RNA polymerase (L) gene of all plant and animal rhabdoviruses characterized so far (Bourhy et al., 2005), two sets of forward and reverse degenerate primers were designed (LCF/LCR, LSF/LSR) based on the conservative pre motif A and motif B region. All the primer sets were tested by *in silico* PCR (AmplifyTM Software) using the L gene sequence of SYNV, VSV and Rabies virus as well as other plant *nucleo-* and *cytorhabdoviruses* (MFSV, MMV, TaVCV, OFV, RYSV, LNYV), that were not included in the phylogenetic analysis study conducted by Bourhy et al. SYDV-L gene fragment was amplified by RT-PCR using total RNA extracted from SYDV infected *N. benthamiana* leaves. Reverse transcription/ first strand cDNA synthesis reaction was primed by SYDV-N gene specific primer (NRF1). Following reverse transcriptions, conventional PCR was done by using “Phusion” proofreading DNA polymerase (Finzymes, New England Biolabs) and L gene specific degenerate forward and reverse primer (LCF/LCR). Two virus specific amplicon, predicted 0.45 kb and an authentic SYDV specific 2.0 kb were amplified in SYDV cDNA containing sample using different PCR conditions, but

not in healthy *N.benthamiana* cDNA containing control sample (Figure 5.12). The PCR products were gel purified and subsequently used as a probe in Northern hybridization experiment for its virus-specific authenticity (Figure 5.13).

PCR condition of SYDV-L amplification {annealing temp.: 50°C (0.45 kb)/ 55°C (2.0 kb)}:

<u>0.45 kb fragment</u>		<u>2.0 kb fragment</u>
98°C – 30 sec		98°C – 30 sec
98°C – 10 sec	X 35	98°C – 10 sec
50°C – 30 sec		55°C – 30 sec
72°C – 2 min		72°C – 3 min
72°C – 10 min		72°C – 10 min

5.9 Amplification of SYDV-N-L fragment:

SYDV-N-L fragment was amplified by RT-PCR (Superscript™ First strand cDNA synthesis system for RT-PCR, Invitrogen, Carlsbad, CA) using total RNA (5 ug) derived from SYDV infected *N. benthamiana* plants as template for first strand cDNA synthesis and SYDV-N gene specific primer (NRF2). The first strand cDNA obtained was amplified by PCR reaction using “Phusion” proofreading DNA polymerase (Finzymes, New England Biolabs) and SYDV-N gene specific forward (NRF2) and L-gene specific degenerate primer (LCR). A ~7.0 kb SYDV specific DNA fragment was amplified (Figure 5.14), which was gel purified and used as probe in a Northern hybridization for its authenticity (Figure 5.15). Cross hybridization with the cDNA obtained from total RNA isolated from healthy *N. benthamiana* leaves was not observed using the same primer combination.

PCR condition to amplify SYDV-N-L fragment (annealing temperature: 55°C):

98°C – 30 sec

98°C – 10 sec

55°C – 30 sec

X 35

72°C – 6 min

72°C – 10 min

5.10 Amplification of SYDV-specific 5.0 kb fragment:

In order to amplify the region between SYDV-P and L gene, RT-PCR reaction was conducted using total RNA extracted from SYDV infected *N. benthamiana* leaves. Reverse transcription/ first strand cDNA synthesis reaction was primed by SYDV-N gene specific primer (NRF1). Following reverse transcription, PCR was done by using Phusion high fidelity DNA polymerase (Finzymes, New England Biolabs) along with a forward primer designed based on the flanking sequence next to P gene (PF1), and L gene specific degenerate oligonucleotide primer (LCR) as reverse primer. A virus specific amplicon of ~5.0 kb was amplified in SYDV cDNA containing sample, but not in healthy *N.benthamiana* cDNA containing control sample (Figure 5.16).

PCR condition to amplify the SYDV-specific 5.0 kb fragment (annealing temperature:

55°C):

98°C – 30 sec

98°C – 10 sec

55°C – 30 sec

X 35

72°C – 4 min

72°C – 10 min

5.11 Amplification of SYDV-G-L fragment:

SYDV-G-L fragment was amplified by RT-PCR (Superscript™ First strand cDNA synthesis system for RT-PCR, Invitrogen, Carlsbad, CA) using total RNA (5 ug) derived from SYDV infected *N. benthamiana* plants as template for first strand cDNA synthesis. The first strand cDNA synthesis reaction was catalyzed and primed by Superscript™ II reverse transcriptase enzyme (Invitrogen, Carlsbad CA) and SYDV-N gene specific primer (NRF1) respectively. Following first strand cDNA synthesis, PCR reaction was conducted using Phusion high fidelity DNA polymerase and SYDV-G and L gene specific degenerate primer combination (G2F/ LSR). PCR reaction results in amplification of SYDV specific 2.0 kb DNA fragment (Figure 5.17). Cross hybridization with the cDNA obtained from total RNA isolated from healthy *N. benthamiana* leaves was not observed using the same primer combination.

PCR condition for amplification of SYDV-G-L fragment (annealing temperature: 50°C):

98°C – 30 sec

98°C – 10 sec

50°C – 30 sec

72°C – 3 min

72°C – 10 min

X 35

Table 5.1: Complete chart of SYDV PCR product and cloned fragments.

#	PCR product	Size	Template cDNA	Primer	Annealing temp. (°C)	Cloning status
1	SYDV-N	465 bp	oligoDT primed	N2F/N1R	45	Cloned
2	SYDV-N 5'RACE	1.4 kb	oligoDT primed	UPM/NRR2	68	Cloned
3	SYDV-N 3'RACE	0.6 kb	oligoDT primed	NRF1/UPM	68	Cloned
4	N-P junction	0.6 kb	NRF1 primed	NPF1/NPR1	60	Cloned
5	N-P junction	0.5 kb	NRF1 primed	NPF2/NPR2	60	Cloned
6	SYDV-P 5'RACE	0.5 kb	oligoDT primed	UPM/P5R	68	Cloned
7	SYDV-P 3'RACE	0.7kb	oligoDT primed	P3R/UPM	68	Cloned
8	SYDV-P	0.9kb	NRF1 primed	PF/PR	55	Cloned
9	SYDV-N-G	2.0kb	NRF1 primed	NRF1/G2R	50	Cloned
10	SYDV-G	1.0kb	hexamer primed	G1F/G2R	45	Not cloned
11	SYDV-P-L	5.0kb	NRF1 primed	PF1/LCR	55	Not cloned
12	SYDV-L	2.0kb	NRF1 primed	LCF/LCR	55	Not cloned
13	SYDV-L	0.45kb	NRF1 primed	LCF/LCR	50	Not cloned
14	SYDV-G-L	2.0kb	NRF1 primed	G2F/LSR	50	Not cloned
15	SYDV-N-L	7.0kb	NRF1 primed	NRF2/LCR	55	Not cloned

List of cloned / not cloned SYDV PCR product with their size, template cDNA and primer used along with annealing temperature for the PCR reaction. Sequences of the primers are listed in chapter 2. Note SYDV-N-G 2.0 kb fragment was cloned in GeneJET (pJET1) vector; all others were cloned in pGEM-T vector (Promega).

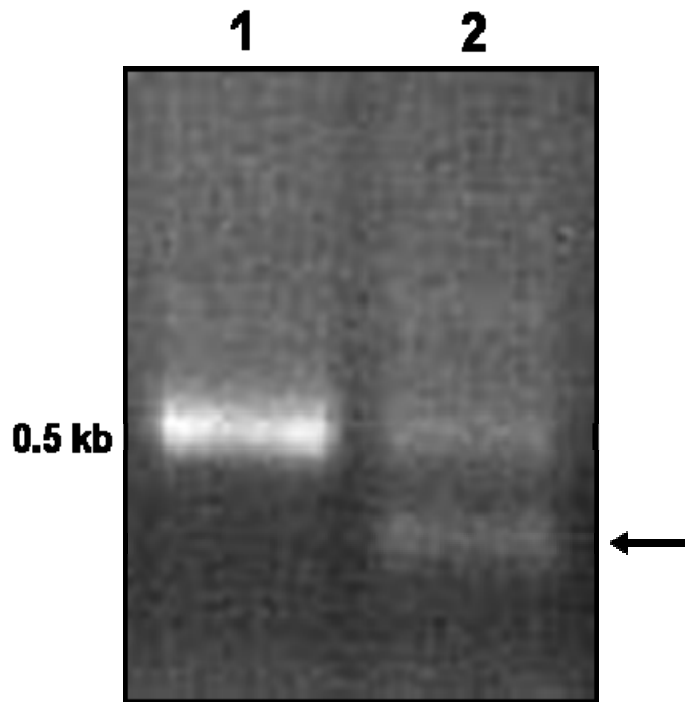


Figure 5.1: Amplification of SYDV-N gene. Amplification of SYDV-N by using N-specific degenerate forward and reverse primer (N2F/N1R). RT-PCR was conducted using total RNA derived from healthy as well as SYDV-infected *Nicotiana benthamiana* leaves as template for cDNA synthesis. Black arrow indicates the amplification of 0.45 kb SYDV-N specific amplicon.

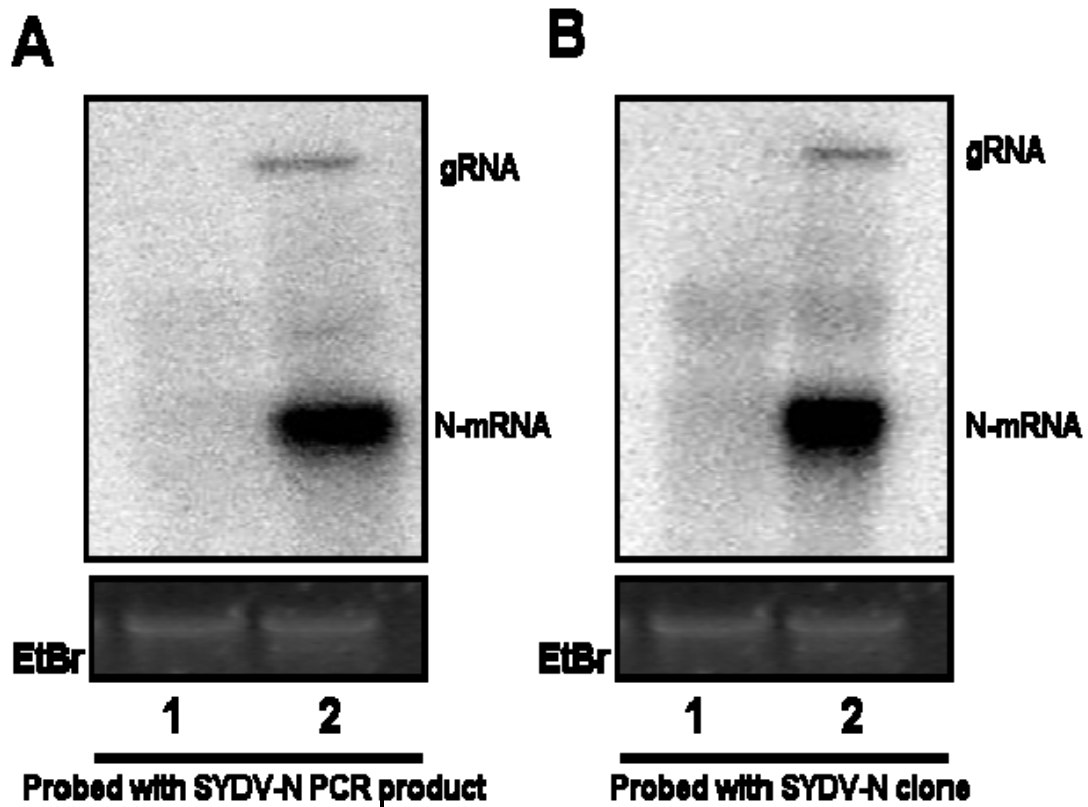


Figure 5.2: Northern hybridization showing the validation of SYDV-N clones. Equivalent amounts of total RNA from healthy *N. benthamiana* (lane 1), and SYDV-infected *N. benthamiana* (lane 2) leaves were separated on denaturing 1.2% formaldehyde-agarose gel, transferred to positively charged nylon membrane and probed with **A.** 0.5 kb SYDV-N PCR product as positive control. **B.** SYDV-N clone. Cross-hybridization with total RNA isolated from healthy *Nicotiana benthamiana* leaves (lane 1) was not observed. EtBr gel image of 25s rRNA was used as loading control.

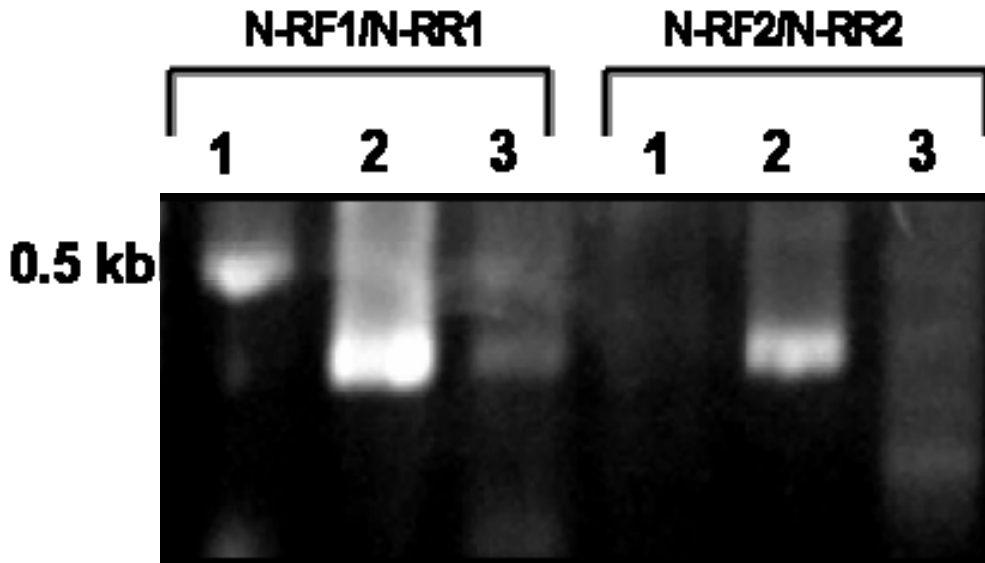


Figure 5.3: RT-PCR of SYDV-infected *Nicotiana benthamiana* cDNA using SYDV-N gene specific primer (N-GSP). RT-PCR reactions using cDNA synthesized from total RNA isolated from mock-inoculated (lane 1) and SYDV (lane 2) or CYDV-infected (lane 3) *Nicotiana benthamiana* leaves and primers designed from the initial 465 bp SYDV N mRNA sequence. The NRF1/NRR1 primers amplified a DNA fragment from cDNA synthesized from mock-inoculated *Nicotiana benthamiana* total RNA along with the expected fragment from SYDV-containing samples where as the primer pair NRF2/NRR2 was SYDV-specific only.

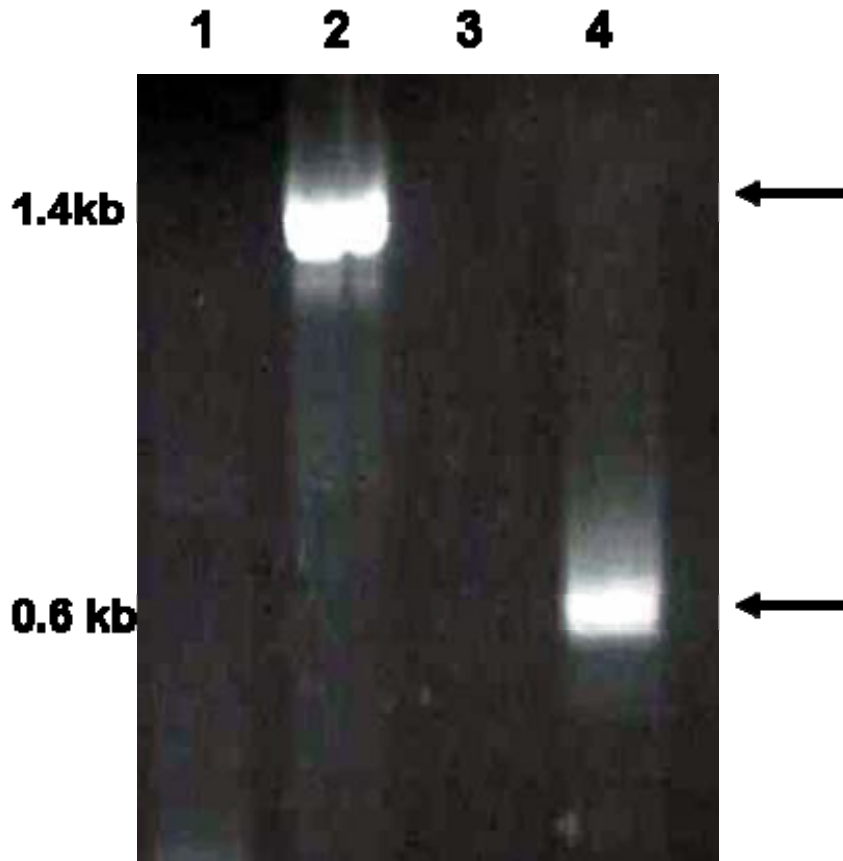


Figure 5.4: 5' and 3' RACE (Rapid Amplification of cDNA Ends) of SYDV-N mRNA. EtBr gel image of 5' and 3' RACE-PCR reaction showing the amplification of SYDV-N specific amplicon. Black arrow indicates 1.4 kb SYDV- N specific 5' RACE (lane 2) and 0.6 kb 3' RACE (lane 4) amplicon from SYDV-infected *Nicotiana benthamiana* cDNA. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1 and 3) was not observed.

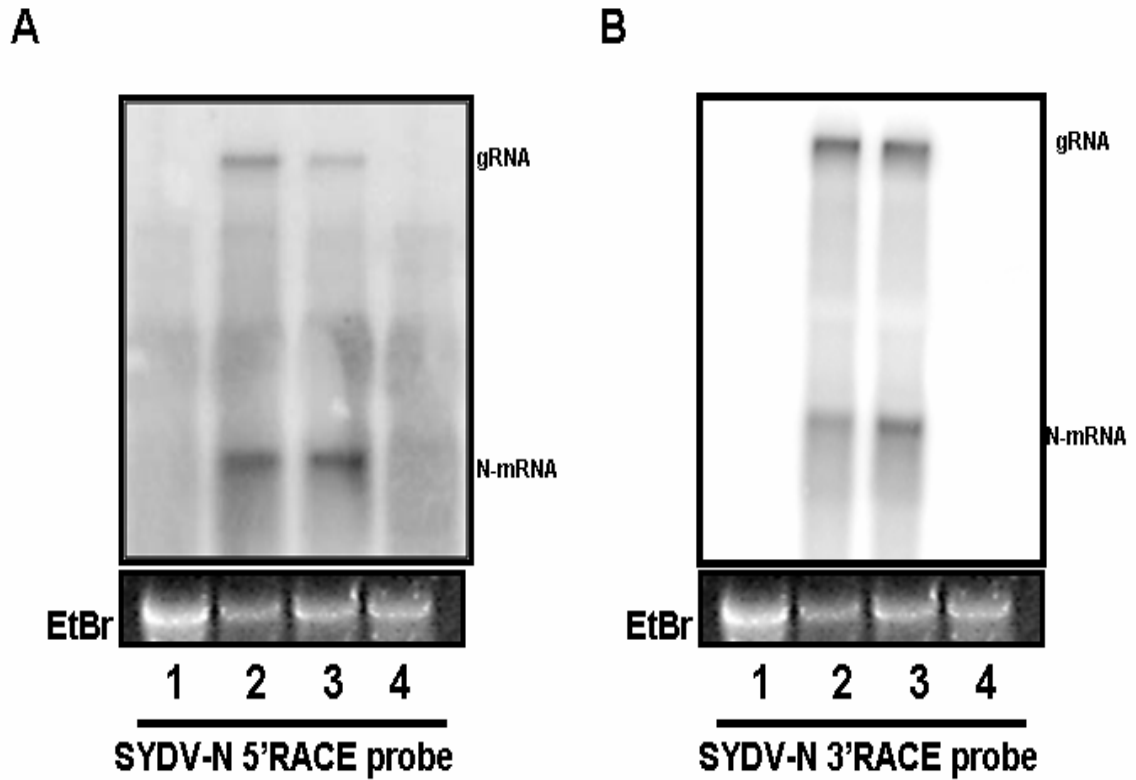


Figure 5.5: Validation of SYDV-N 5'- and 3' RACE amplicon by Northern Hybridization. Equivalent amounts of total RNA isolated from SYDV and CYDV infected *N. benthamiana* leaves (lane 2, 3 respectively) hybridized to genomic RNA (gRNA) and SYDV-N mRNA when probed with **A.** SYDV-N 5'RACE and **B.** SYDV-N 3' RACE PCR product respectively. Cross-hybridization with the RNA transcripts from total RNA isolated from healthy *N. benthamiana* (lane 1) and SYNIV infected *N. benthamiana* leaves (lane 4) was not observed. Ethidium bromide (EtBr) gel images of 25s ribosomal RNA were used as loading controls.

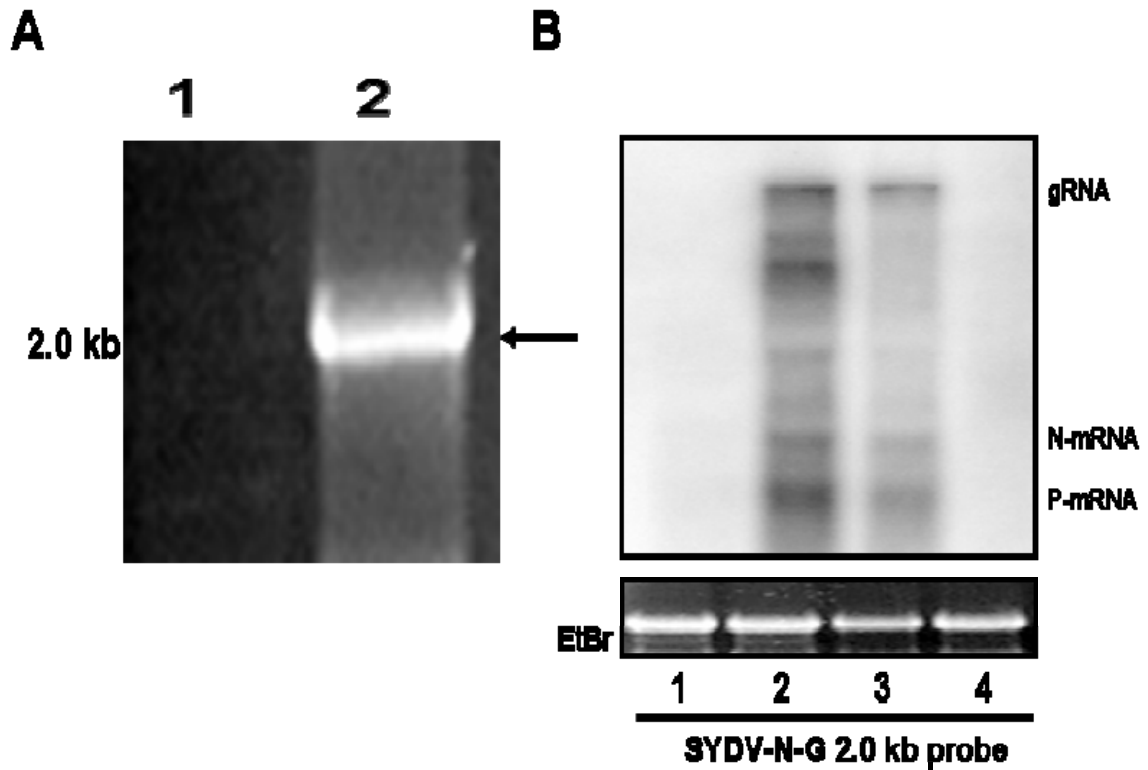


Figure 5.6: Amplification and validation of SYDV-N-G 2.0 kb amplicon. **A.** EtBr gel image of RT-PCR reaction showing amplification of 2.0 kb SYDV-specific DNA fragment (black arrow) from SYDV-infected *Nicotiana benthamiana* cDNA (lane 2) using N specific (NRF1) forward and G (G2R) reverse primer. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1) was not observed. **B.** **Validation of SYDV-N-G 2.0 kb amplicon by Northern Hybridization.** Equivalent amounts of total RNA isolated from SYDV and CYDV infected *N. benthamiana* leaves (lane 2, 3 respectively) hybridized to genomic RNA (gRNA) and SYDV-N, SYDV-P mRNA. Cross-hybridization with the RNA transcripts from total RNA isolated from healthy *N. benthamiana* (lane 1) and SYNV infected *N. benthamiana* leaves (lane 4) was

not observed. Ethidium bromide (EtBr) gel images of 25s ribosomal RNA were used as loading controls.

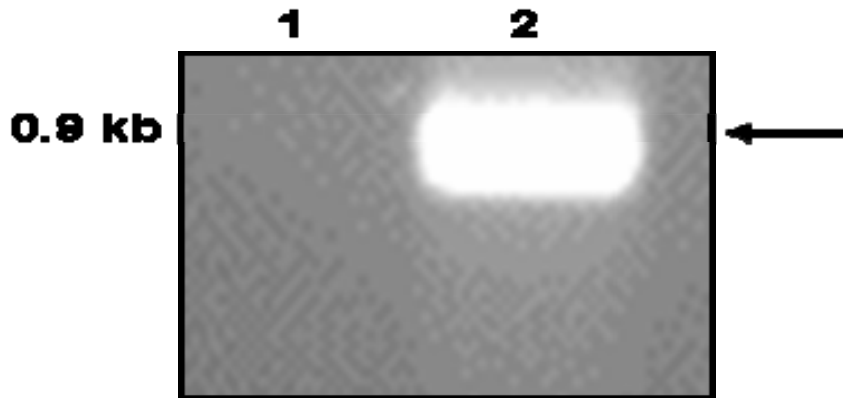


Figure 5.7: Amplification of SYDV-P gene. Amplification of SYDV-P by using P gene specific (P-GSP) forward and reverse primer. RT-PCR was conducted using total RNA derived from healthy as well as SYDV-infected *Nicotiana benthamiana* leaves as template for cDNA synthesis. Black arrow indicates the amplification of 0.9 kb SYDV-P specific amplicon (lane 2). Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1) was not observed.

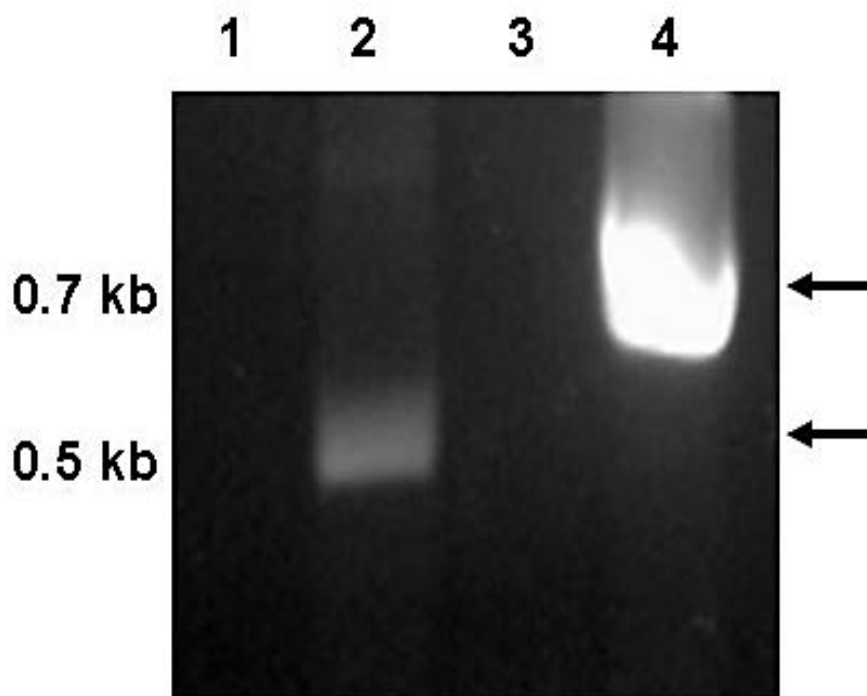


Figure 5.8: 5' and 3' RACE (Rapid Amplification of cDNA Ends) of SYDV-P mRNA. EtBr gel image of 5' and 3' RACE-PCR reaction showing the amplification of SYDV-P specific amplicon. Black arrow indicates 0.5 kb SYDV- P specific 5' RACE (lane 2) and 0.7 kb 3' RACE (lane 4) amplicon from SYDV-infected *Nicotiana benthamiana* cDNA. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1 and 3) was not observed.

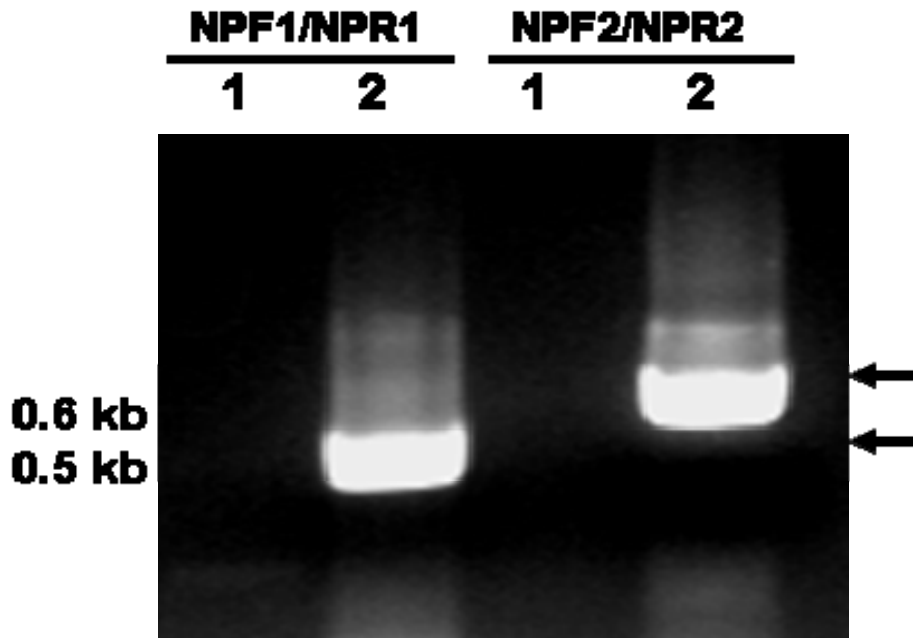


Figure 5.9: Amplification of the region between SYDV-N and P gene. EtBr gel image of RT-PCR reaction showing amplification of 0.6 and 0.5 kb SYDV-specific DNA fragment (black arrow) from SYDV-infected *Nicotiana benthamiana* cDNA (lane 2) using the N- and P- gene specific primer (N-GSP, P-GSP) combination NPF1/NPR1 and NPF2/NPR2 respectively. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1) was not observed.

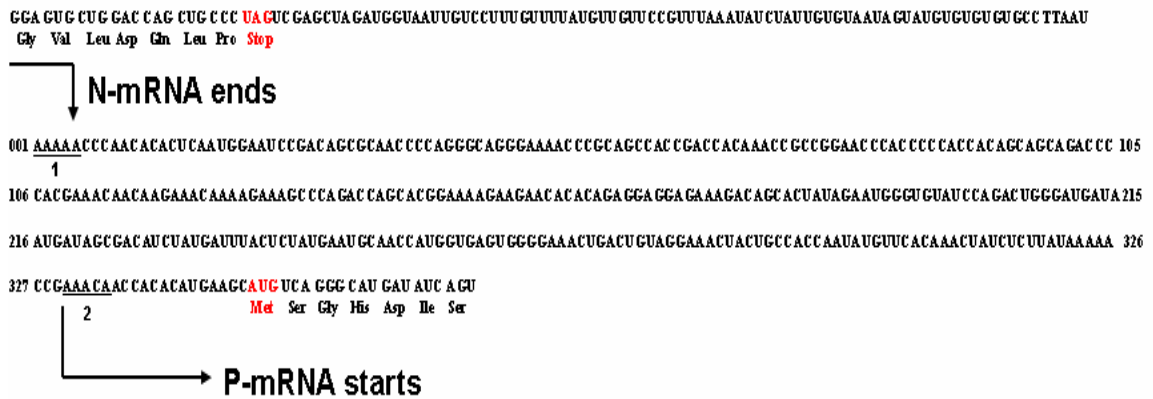


Figure 5:10: Nucleotide sequence of the region between SYDV-N and P gene. The nucleotide sequence is presented as messenger RNA sequence. The N and P mRNA sequence is not taken into consideration for numbering. The underlined sequence 1 and 2 represents the polyadenylation signal of N-mRNA and conserved transcription initiation signal of P-mRNA, respectively. The stop codon (UAG) of N-mRNA and start codon (AUG) of P-mRNA are shown in red.

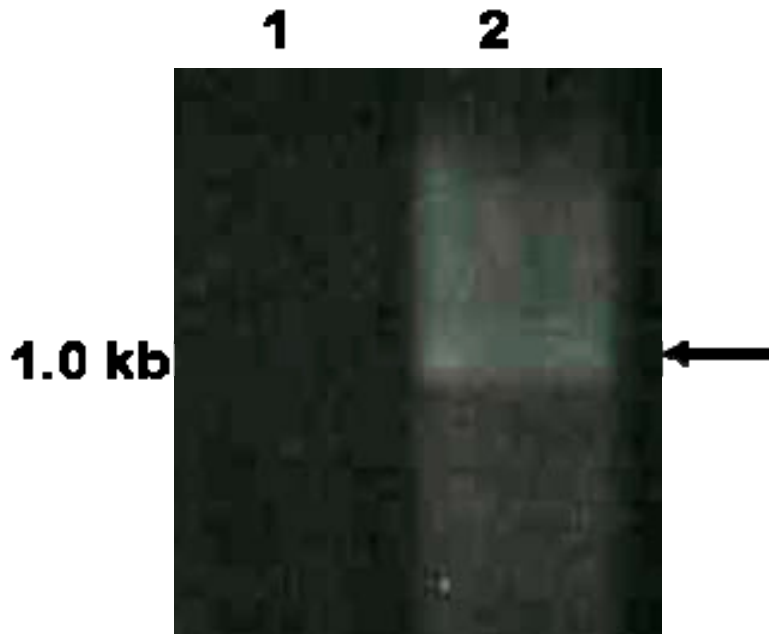


Figure 5.11: Amplification of SYDV-G gene. Amplification of SYDV-G by using G-specific degenerate forward and reverse primer (G1F/G2R). RT-PCR was conducted using total RNA derived from healthy as well as SYDV-infected *Nicotiana benthamiana* leaves as template for cDNA synthesis. Black arrow indicates the amplification of 1.0 kb SYDV-G specific amplicon. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1) was not observed.

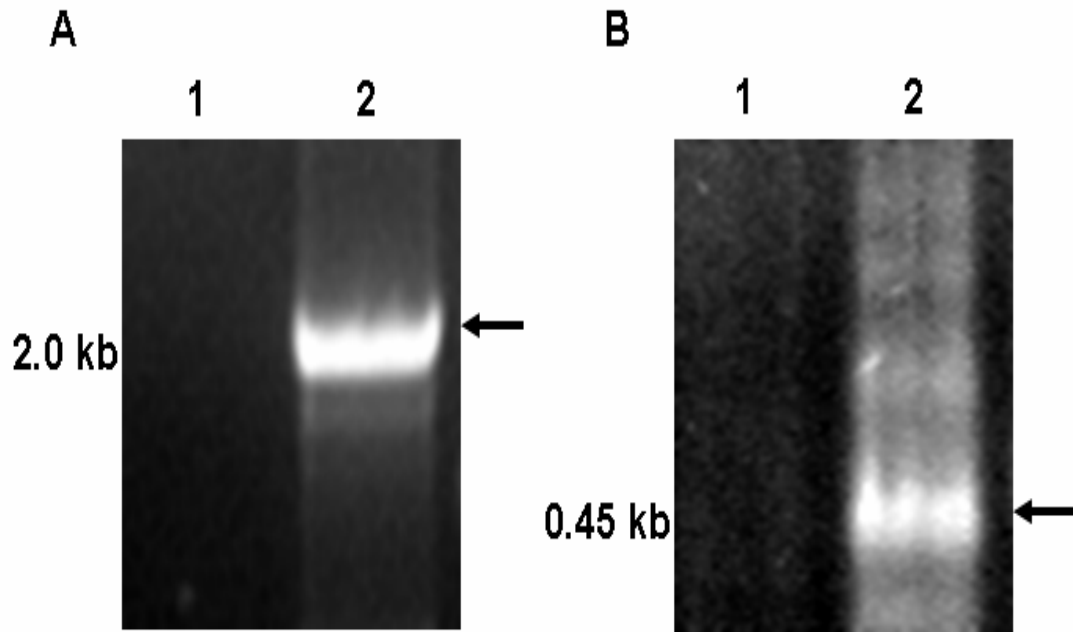


Figure 5.12: Amplification of SYDV-L gene. Amplification of SYDV-L gene by using L-specific degenerate forward and reverse primer (LCF/LCR) designed based on conserved block III region of rhabdoviral L gene. RT-PCR was conducted using total RNA derived from healthy as well as SYDV-infected *Nicotiana benthamiana* leaves as template for cDNA synthesis. Black arrow indicates the amplification of authentic SYDV specific **A.** 2.0 kb and **B.** predicted 0.45 kb SYDV-L amplicon. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1) was not observed.

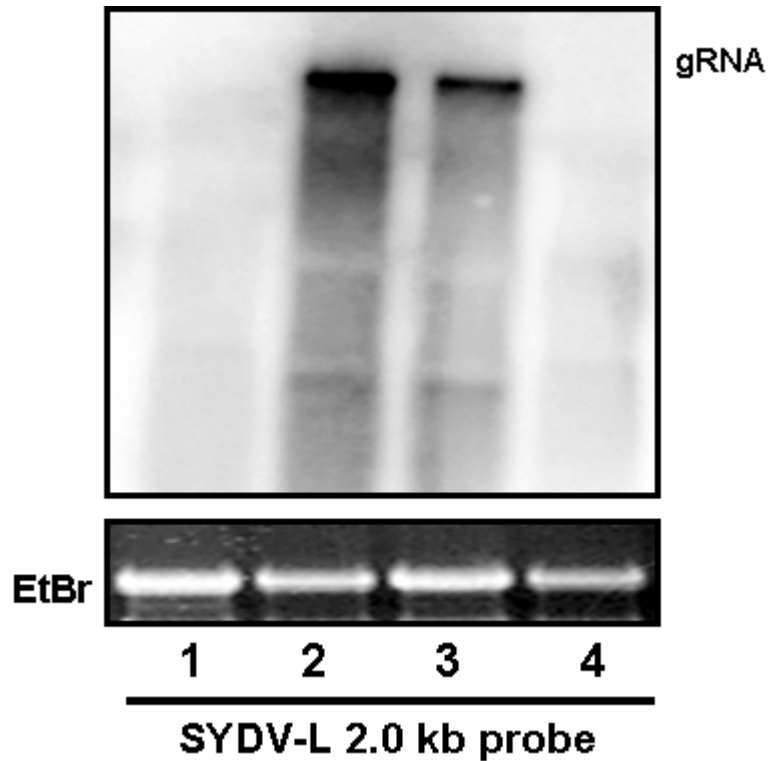


Figure 5.13: Validation of SYDV-L 2.0 kb amplicon by northern hybridization.

Equivalent amounts of total RNA isolated from SYDV and CYDV infected *N. benthamiana* leaves (lane 2, 3 respectively) hybridized to genomic RNA (gRNA) and SYDV-L mRNA. Cross-hybridization with the RNA transcripts from total RNA isolated from healthy *N. benthamiana* (lane 1) and SYNIV infected *N. benthamiana* leaves (lane 4) was not observed. Ethidium bromide (EtBr) gel images of 25s ribosomal RNA were used as loading controls.

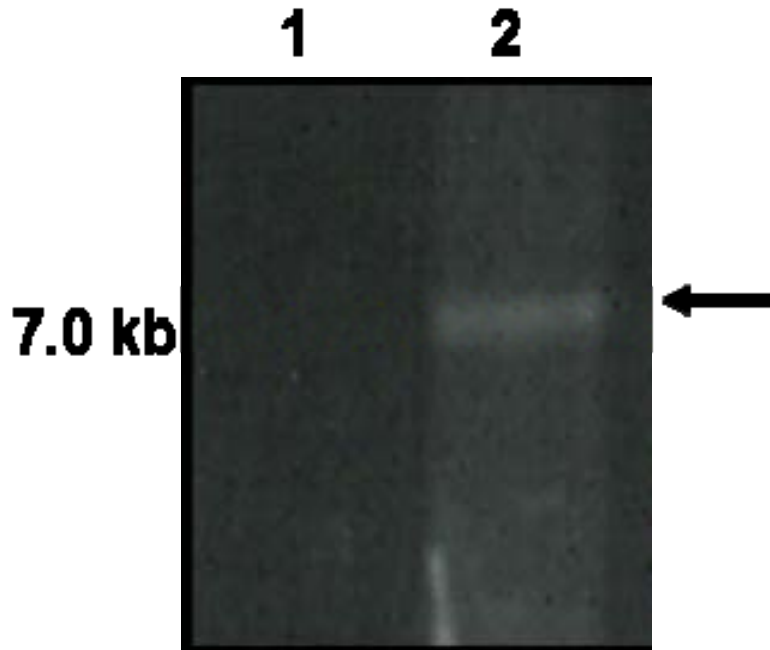


Figure 5.14: Amplification of SYDV-N-L fragment. EtBr gel image of RT-PCR reaction showing amplification of 7.0 kb SYDV-specific DNA fragment (black arrow) from SYDV-infected *Nicotiana benthamiana* cDNA (lane 2) using SYDV-N specific (NRF2) forward and L degenerate (LCR) reverse primer. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1) was not observed.

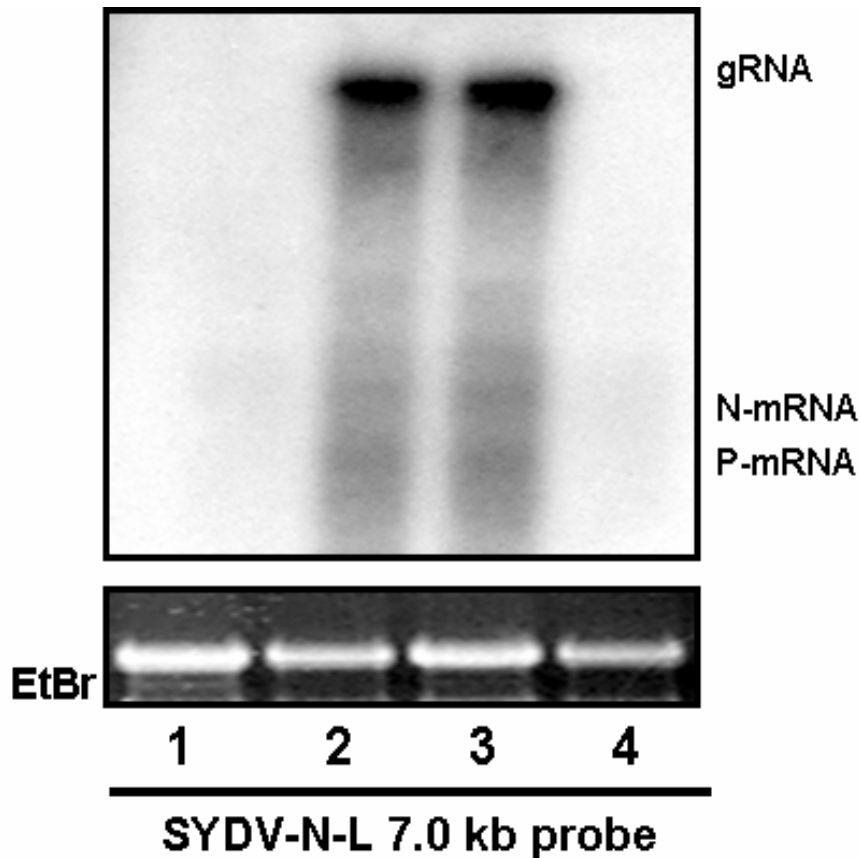


Figure 5.15: Validation of SYDV-N-L 7.0 kb amplicon by northern hybridization. Equivalent amounts of total RNA isolated from SYDV and CYDV infected *N. benthamiana* leaves (lane 2, 3 respectively) hybridized to genomic RNA (gRNA) and SYDV-N and P-mRNA. Cross-hybridization with the RNA transcripts from total RNA isolated from healthy *N. benthamiana* (lane 1) and SYNV infected *N. benthamiana* leaves (lane 4) was not observed. Ethidium bromide (EtBr) gel images of 25s ribosomal RNA were used as loading controls.

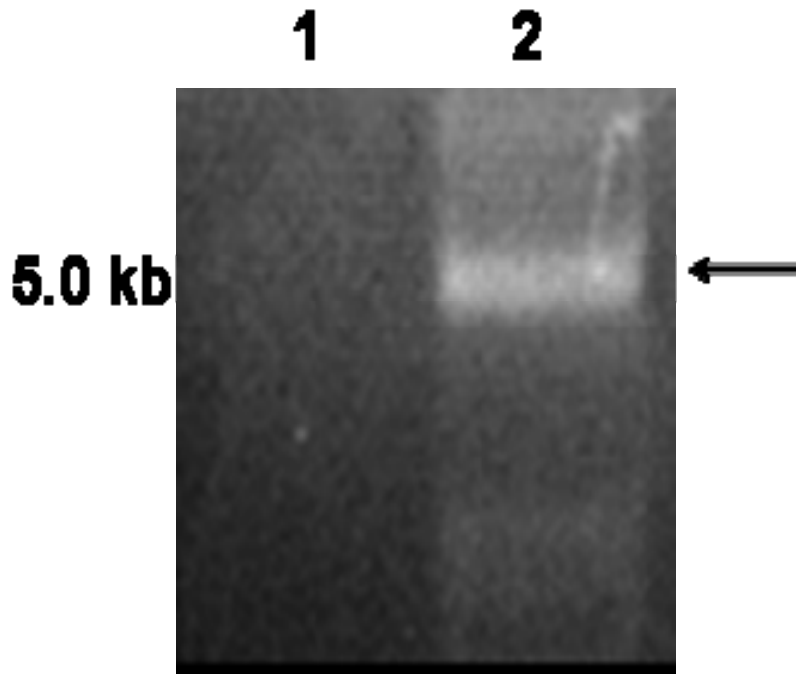


Figure 5.16: Amplification of SYDV specific 5.0 kb fragment. EtBr gel image of RT-PCR reaction showing amplification of 5.0 kb SYDV-specific DNA fragment (black arrow) from SYDV-infected *Nicotiana benthamiana* cDNA (lane 2) using forward primer (PF1) next to P gene and L degenerate (LCR) reverse primer. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1) was not observed.

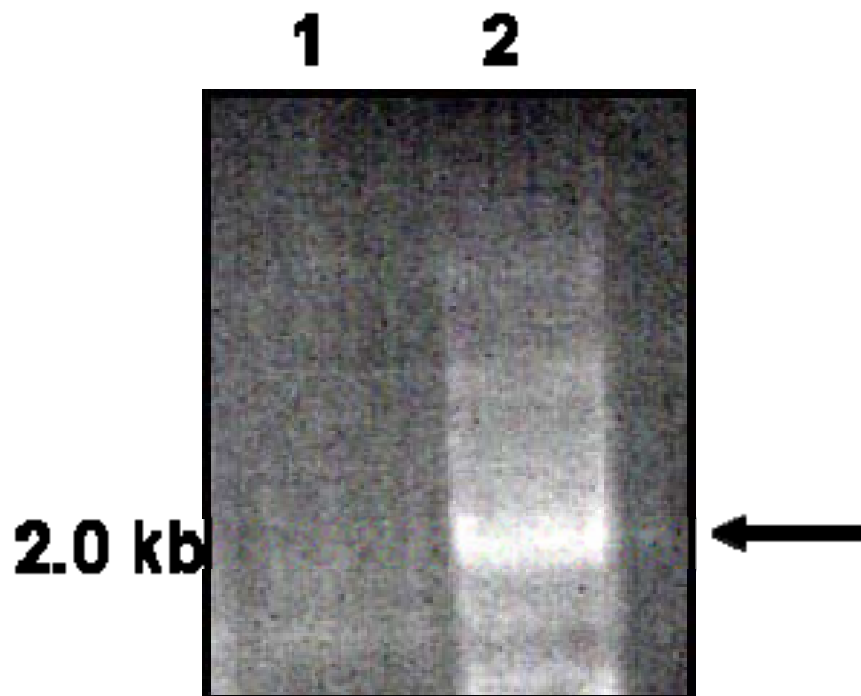


Figure 5.17: Amplification of SYDV-G-L fragment. EtBr gel image of RT-PCR reaction showing amplification of 2.0 kb SYDV-specific DNA fragment (black arrow) from SYDV-infected *Nicotiana benthamiana* cDNA (lane 2) using SYDV-G degenerate (G2F) forward and L degenerate (LSR) reverse primer. Cross hybridization with cDNA synthesized from healthy *Nicotiana benthamiana* (lane 1) was not observed.

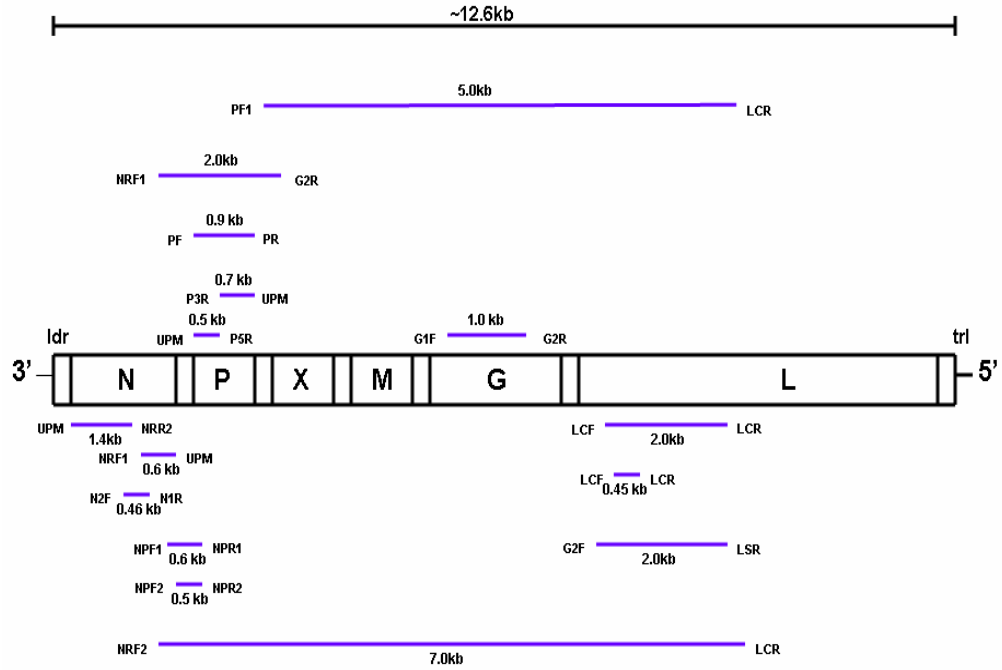


Figure 5.18: PCR amplification map of SYDV genome. Each amplified PCR product is represented by a single line along with their respective size. Flanking is the combination of forward and reverse primer used to amplify each specific PCR fragment (*Please see the primer section for the sequence of each primer used*).

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VITA

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August, 2001 - present
University of Kentucky, Lexington, KY (USA); GPA: 3.654/4
2. MS, Zoology
Specialization in Neuroendocrinology
Graduated August' 1999
University of Calcutta, India, First Class, 67.4%
3. BS, Zoology (Honors), Chemistry, Botany
Graduated July' 1997
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4. Diploma in Network Centered Software Engineering (GNIIT Program)
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E. PROFESSIONAL POSITION HELD

1. Faculty in Biology (Southwestern Oklahoma State University, Weatherford OK): 08/2007 – present
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F. AWARDS & PROFESSIONAL AFFILIATIONS:

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2. Awarded Teaching Assistantship at the Department of Biology, University of Kentucky, Lexington, KY
3. Awarded Best Teacher Award from the Department of Biology, University of Kentucky (Spring' 2005)

4. Awarded National Scholarship on the basis of the results of BS and MS Exam. By Ministry of Education & Human Resource Development, Govt. of India
5. Awarded Merit Certificates of All India Science Aptitude and Talent Search Test
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G. PUBLICATION:

1. **Ghosh D.**, Brooks R.E., Lesnaw J.A., Goodin M.M. (2007). Cloning and subcellular localization of the phosphoprotein and nucleocapsid proteins of Potato yellow dwarf virus, type species of the genus Nucleorhabdovirus (Submitted to Virus Research)
2. **Goodin**, M., Yelton, S Ghosh, D., Mathews, S., Lesnaw, J.: Live-cell imaging of Rhabdovirus-Induced Morphological Changes in Plant Nuclear Membranes. Molecular Plant-Microbe Interactions. (March, 2005)
3. **Andrew O. Jackson**, Ralf G. Dietzgen, Michael M. Goodin, Jennifer N. Bragg, and Deng. 2005 : BIOLOGY OF PLANT RHABDOVIRUSES. Annual Review of Phytopathology Vol. 43: 623-660. (*Please see Acknowledgement*)
4. Rohr, J.R., A.A. Elskus, B.S. Shepherd, P.H. Crowley, T.M. McCarthy, J.H. Niedzwiecki, T. Sager, A. Sih, **B.D. Palmer**. 2003. Lethal and sublethal effects of atrazine, carbaryl, endosulfan, and octylphenol on the Streamside Salamander, *Ambystoma barbouri*. Environmental Toxicology and Chemistry 22(10):2385–2392. (*Please see Acknowledgement*)
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H. Professional Meetings:

Live cell imaging of plant rhabdovirus movement (Co-author of the poster)

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