

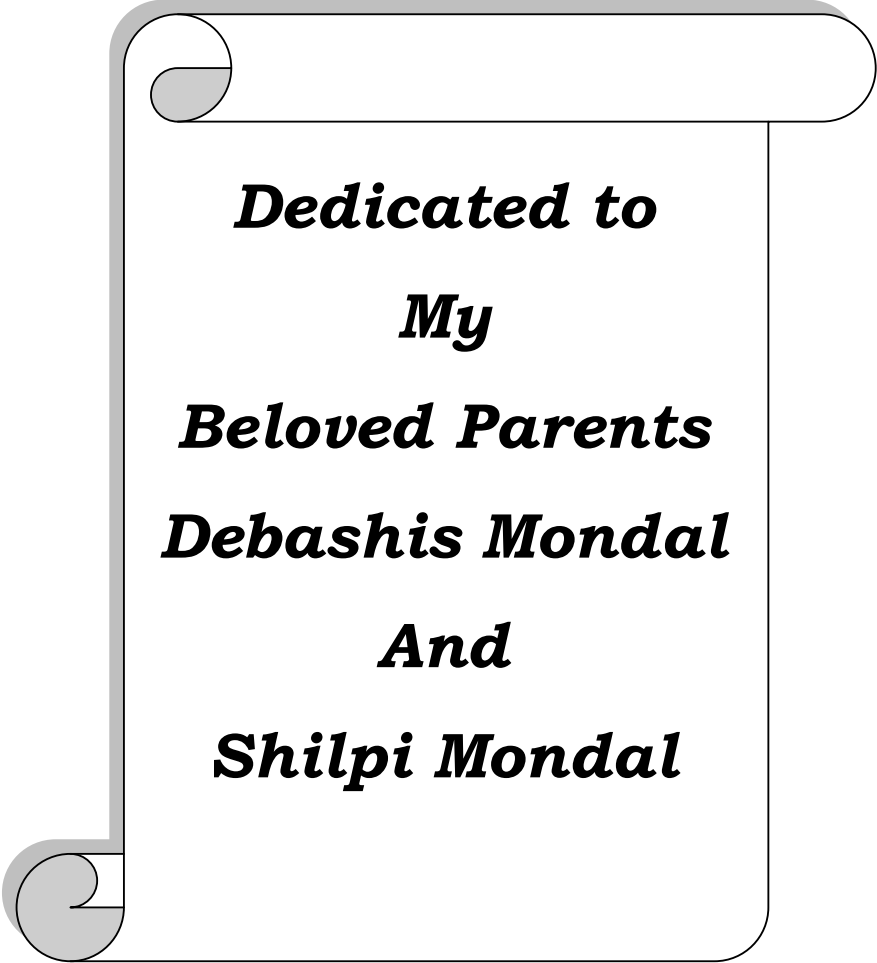
**Molecular Characterization of Geminiviruses
from Tomato (*Lycopersicon esculentum* Mill.)
Grown in Terai Region of West Bengal**

**A Thesis
Submitted to the
Uttar Banga Krishi Viswavidyalaya
In partial fulfilment of the requirements for the
Degree of Master of Science (Agriculture)
In
Biochemistry**

**By
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2017**



Dedicated to
My
Beloved Parents
Debashis Mondal
And
Shilpi Mondal

Uttar Banga Krishi Viswavidyalaya
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**APPROVAL OF EXAMINERS FOR THE AWARD OF THE DEGREE OF
MASTER OF SCIENCE (AGRICULTURE)
IN
BIOCHEMISTRY**

We, the undersigned, having been satisfied with the performance of Mr. Debayan Mondal, Regn. No. A-2015-7-M of 2015-16, in the *viva-voce* of final evaluation of the thesis entitled “Molecular Characterization of Geminiviruses from Tomato (*Lycopersicon esculentum* Mill.) in Terai Region of West Bengal”, conducted today, the 29th June, 2017 recommend that the thesis be accepted for the award of the degree of Master of Science (Agriculture) in Biochemistry.

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CERTIFICATE

This is to certify that the work recorded in the thesis entitled “Molecular Characterization of Geminiviruses from Tomato (*Lycopersicon esculentum* Mill.) in Terai Region of West Bengal” submitted by Mr. Debayan Mondal, Regn. No. A-2015-7-M of 2015-16 in partial fulfillment of the requirements for the degree of Master of Science (Agriculture) in Biochemistry of Uttar Banga Krishi Viswavidyalaya, is a faithful and bonafide research work carried out under my personal supervision and guidance. The results of the investigation reported in the thesis have not so far been submitted for any other Degree or Diploma. The assistance and help received during the course of investigation has been duly acknowledged.

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LIST OF ABBREVIATIONS

Abbreviation	Full form
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
NCBI	National Centre for Biotechnology Information
MEGA	Molecular Evolutionary Genetics Analysis
SDT	Sequence Demarcation Tool
ICTV	International Committee on Taxonomy of Viruses
ssDNA	single stranded DNA
OW	Old World
NW	New World
ROS	Reactive Oxygen Species
PCD	Programmed Cell Death
SA	Salicylic Acid
CAT	Catalase
POD	Peroxidase
PPO	Polyphenol Oxidase
PAL	Phenylalanine Ammonia Lyase
HR	Hypersensitive Response
TYLCV	Tomato Yellow Leaf Curl Virus
NLS	Nuclear Localization Signal
CP	Coat protein
UV-VIS	Ultraviolet-Visible
RPM	Rotation Per Minute
LB	Luria broth

Abstract

The present work entitled “Molecular Characterization of Geminiviruses from Tomato (*Lycopersicon esculentum* Mill.) in Terai Region of West Bengal” was carried out with the three objectives namely (i) identifying hypersensitive response in plants and estimation of defence related enzymes during begomoviral infection, (ii) study of evolutionary pattern of begomoviral genome and (iii) development of a PCR based detection system for begomoviruses from solanaceous vegetables.

Plants are often exposed to various environmental stresses which adversely affect the plants at physiological and cellular levels. Biotic stresses are results of attack from different potential microbial pathogens including viruses. *Geminiviruses*, particularly *begomoviruses* are major group of plant infecting viruses which causes severe damage to vegetable production. These viruses are white fly-transmitted and produces symptoms like yellowing, mottling and inward curling of the leaves. *Begomoviruses* typically have genomes consisting of two separately encapsidated genomic components, known as DNA-A and DNA-B. The DNA-A component contains six Open Reading Frames (ORFs) in both orientations that encode proteins which play major roles in symptom development, viral replication and infectivity, whereas the DNA-B component encodes two genes for viral movement and nuclear shuttling. The present study was focused on confirmation and identification of begomoviral infection in tomato and other solanaceous crops in *terai* region of West Bengal. The results of the present study confirm the presence of these viral particles from tomato and other solanaceous vegetable crops grown in this region by PCR mediated amplification of AV1 and AV2 genes from different symptomatic DNA samples extracted from plants. The study was conducted also to see the plant defence response against the virus infection.

Hypersensitive response was found from the infected sample which was evident by the presence of apoptotic cells in trypan blue staining of infected leaves. The stress responsive enzymes were also studied from these healthy and infected samples where enhanced activity of Polyphenol Oxidase, Catalase and Peroxidase were found during the infection. Moreover, to know the evolutionary pattern of *begomoviruses* in last few decades 31 nucleotide sequence of DNA-A component was downloaded from NCBI database. Phylogenetic analyses of these sequences through MEGA and SDT softwares have shown a geographical confinement of the viruses during the evolution. We also detected the occasional presence and absence of AC4 and AV2 genes in these viruses, but coat protein gene was found to be the least diversified gene during evolution in our study. Overall the study contributes towards the understanding of evolution, genome configuration of *begomovirus* and plant stress responses against the viral infection.

Introduction

Plants are often exposed to environmental stresses in both natural and agricultural conditions. To sustain in such condition they induce different stress responses at physiological and cellular levels. The stresses which are imposed on plants by the attack of different potential enemies including microbial pathogens like viruses, bacteria, insects, nematodes & fungi are known as biotic stress. Among all those microorganisms the viruses can be considered as the smartest of all as they have the potentiality to seize the plant defence system for their survival as well as progress of the infection without killing the host plant. The viruses impose serious threat to agricultural production as well as the productivity worldwide. According to International Committee for Taxonomy of Viruses (ICTV) there are 20 families with plant-infecting viruses sub grouped into 90 genera, which comprise ~800 plant virus species (Brown *et al.*, 2012). *Geminiviruses* belonging to the family *Geminiviridae* is considered to be the second largest plant virus family with broad spectrum host infectivity. *Geminiviruses* are characterized structurally by twinned (geminate) quasi-icosahedral capsids (Fig 1A) and genetically having one or two small circular ssDNA molecules (Harrison *et al.*, 1977). These viruses infect both monocot (monocotyledonous) as well as dicot (dicotyledonous) plants (Gutierrez, 2000). They replicate through an intermediate dsDNA molecule in the nuclei of infected host plant cells and depend upon the host DNA replication machinery (Jeske, 2007). *Geminiviridae* is comprised of seven genera; named as *Mastrevirus*, *Curtovirus*, *Topocuvirus*, *Begomovirus*, *Becurtovirus*, *Turncurtovirus* & *Eragrovirus* (ICTV 8th report). Among the entire genus, *Begomovirus* is the largest group of viruses having 196 member species infecting mostly vegetable crops and emerging in a very high speed. *Begomoviruses*, transmitted by ubiquitous White fly (*Bemisia tabacci*) (Fig 1B), has been proved to be economically most destructive (Brown *et al.*, 2012). These viruses

are sub-divided into two types based on the presence of different genetic components as monopartite or bipartite (Brown *et al.*, 2012; Seal *et al.*, 2006) (Fig 1C). On the basis of an evolutionary time scale i.e. prior to 1990 for which Koch's Postulates had been satisfied using cloned genomes were bipartite (Briddon *et al.*, 2010). *Begomoviruses* originating from the New World (NW) typically have genomes consisting of two separately encapsidated genomic components, known as DNA-A and DNA-B (Brown *et al.*, 2012). Till now, no monopartite new world *begomoviruses* has been found; the majority of OW *begomoviruses* are monopartite in nature, with some bipartite members (Zhang & Ling, 2011).

A short sequence of ~200 nt with high sequence identity known as "common region" is present in both DNA-A and DNA-B which is responsible for the initiation of rolling circle replication in the genomic components as well as the integrity of the divided genome (Elmer *et al.*, 1988). Apart from this small sequence there is no sequence similarity in these two components. The genomes of monopartite (and DNA-A components of bipartite) *begomoviruses* are typically ~2.7- 3.0 kb in length and have genes in both orientations from a non-coding intergenic region (IR), which contains the promoter elements and the *ori* of virion-strand DNA replication (Fauquet *et al.*, 2008). The virion strand *ori* consists of a predicted hairpin structure containing a conserved nonanucleotide (TAATATTAC) sequence in the loop and repeated upstream motifs known as "iterons". The DNA-A component contains six ORFs in both orientations that encode proteins which play major roles in symptom development, viral replication & infectivity.

The proteins encoded by DNA-A component with their corresponding responsible genes are as follows (Table 1):

Coat Protein (CP; V1): Required for encapsidation, insect transmission and movement in plants (Sharma and Ikegami, 2009).

Table 1: Functions of begomoviral genes

GENE NAME	FUNCTION	SIZE (bp)
AV1	Viral encapsidation (coat protein)	750
AV2	Movement, suppressor of RNA silencing.	250
AC1	Replication	1073
AC2	Interferes with TGS and PTGS pathways	407
AC3	Replication enhancer	404
AC4	Not clear (may be pathogenicity determinant)	293
BC1	Movement protein	878
BV1	Nuclear shuttle protein	845

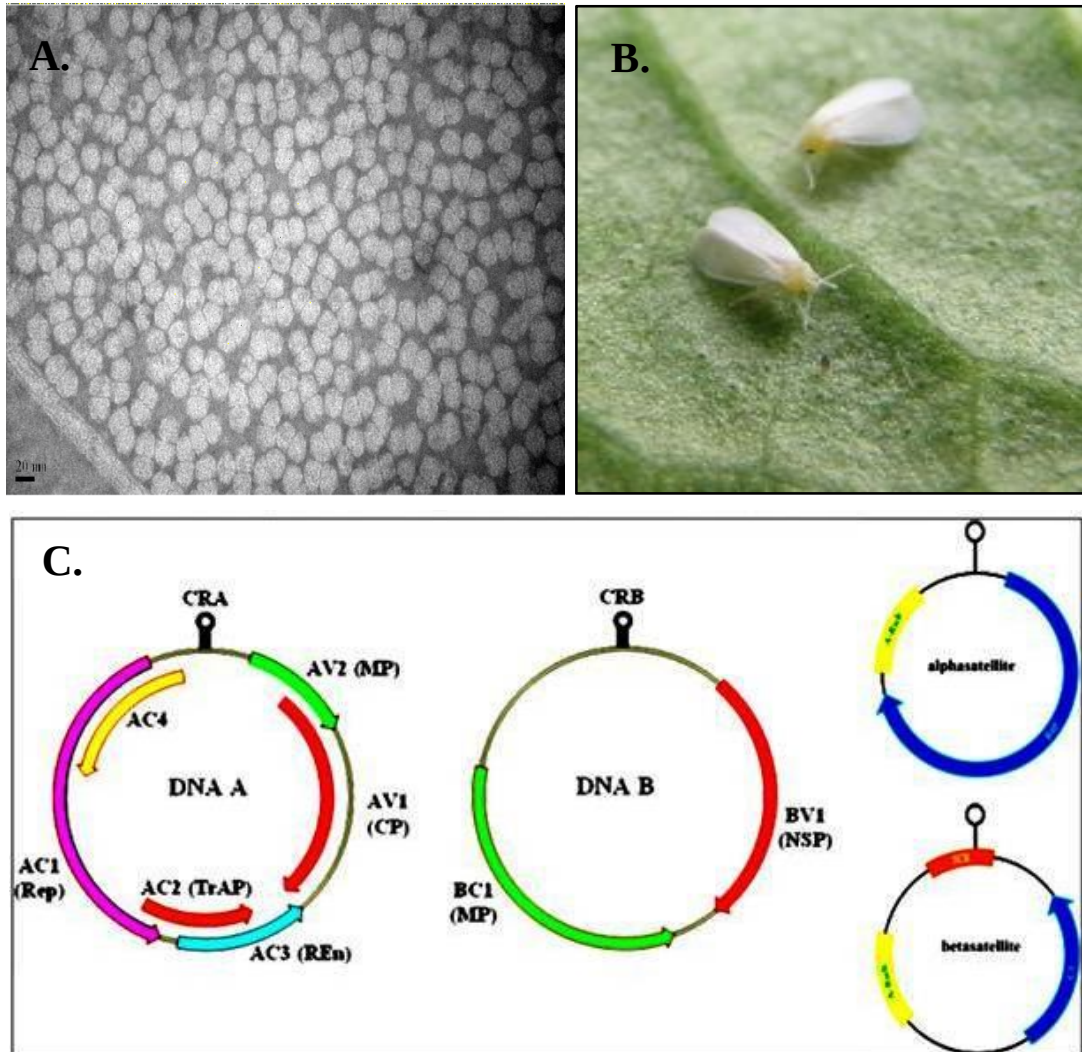


Fig 1: (A) Genomic components of *Begomoviruses*
(B) Electron microscopic view of the virus particle (source:
<https://Electron-micrograph-of-purified-Tomato-yellow-leaf-curl-virus-Bar-20-nm&psig=AFQjCNG57Rb-CffML-K9WrHPkmgmB6BRhw&ust=1498245065647815>
(C) Insect vector whitefly (*Bemisia tabaci*) helps in transmission of *Begomoviruses*

Pre-Coat Protein (Pre-CP; V2): Responsible for virus movement in plants and also known as pathogenicity determinant (Rojas *et al.*, 2005). It also acts as a suppressor of RNA silencing pathway in host (Yadava *et al.*, 2010).

Replication-Initiator Protein (Rep; C1): The one and only virus-encoded gene product required for viral DNA replication (Nash *et al.*, 2011).

Transcriptional activator protein (TrAP; C2): Acts as a suppressor of RNA silencing in bipartite (Yang *et al.*, 2007) and monopartite begomoviruses (Gopal *et al.*, 2007).

Replication enhancer protein (REn; C3): REn is involved in the establishment of an environment which is suitable for virus replication (Pedersen & Hanley-Bowdoin, 1994).

C4 protein (C4): The function of C4 is still unknown but for some viruses it has been shown as a pathogenicity determinant and a suppressor of PTGS by binding of siRNAs (Vanitharani *et al.*, 2004).

The DNA-B component contains two ORFs in opposite orientations encoding:

Nuclear shuttle protein (NSP; BV1): Responsible for transport of viral DNA from the nucleus into the cytoplasm (Sanderfoot *et al.*, 1996).

Movement protein (MP; BC1): Responsible for the movement of viral DNA across plasmodesmatal boundaries (Sanderfoot & Lazarowitz, 1996).

Majority of monopartite *begomoviruses* are associated with DNA satellites (betasatellites) and nanovirus-like DNA satellite molecules (alphasatellites) (Briddon & Stanley, 2006). Although alphasatellites have been found to be associated with bipartite NW *begomoviruses* (Paprotka *et al.*, 2010). The satellites are ssDNA molecules of approximately half the size of their helper *begomoviruses* (~1350nt) with no sequence homology with their

helper viruses, other than the presence of a potential stem-loop structure containing the nonanucleotide sequence TAATATTAC (Briddon *et al.*, 2003).

The *begomoviruses* have already undergone a lot of evolution and has differentiated into new strains which are geographically distributed in all over the world and can infect a wide range of host plants. West Bengal is well-known for its leadership in vegetable production and the cultivation is inevitably affected by *begomovirus* infection. But till now there is no characterization of these viruses and a detail study of these viral infections is very important to know about their identification strain differentiation, evolution and plant defence responses specific to this region.

To understand the molecular and evolutionary characteristics of the virus the present study was conducted with the following objectives.

1. Identifying hypersensitive response in plants & estimation of defence related enzymes during begomoviral infection.
2. Study of evolutionary pattern of Begomoviral Genome.
3. Development of a PCR based detection system for *begomoviruses* from solanaceous vegetables.

Review of Literature

Plants must continuously defend themselves against attack from intruder pathogens as their immobility precludes escape; each plant cell possesses both a preformed and an inducible defense capacity. In comparison to vertebrate immune system the striking contrast lies in the specialized cells devoted to defence for plants. Those cells are rapidly mobilized to the infection site, where they either kill the invading organism or limit its spread.

Viral infection is one of the major biotic stresses on plant which affects the metabolomes as well as the genome of the plant, particularly its expression in terms of transcriptome and proteome. Plant viruses enter cells only through wounds made mechanically or by vectors or by deposition into an ovule by an infected pollen grain (Diener *et al.*, 1993).

Begomoviruses are ssDNA genomic material containing viruses. After insertion of the genome into the host cell it replicates by following a rolling circle replication mechanism. Initially it produces a multimeric (-) strand, which serves as a template for the production of multimeric (+) strands that are then cleaved to produce unit length (+) strands. As soon as new viral nucleic acid is produced, some of it is translated, i.e., it induces the host cell to produce the protein molecules coded by its nucleic acid. (Gutierrez, 1999).

Biotic Stress and Biochemistry of Infection

Plant viruses do not contain any enzymes, toxins, or other pathogenic substances and yet cause a variety of symptoms on the host. The mere presence of viral nucleic acid or complete virus in a plant, even in large quantities, does not always cause disease symptoms. Viral diseases of plants are not due to the depletion of nutrients that have been diverted

toward synthesis of the virus itself, but rather due to other indirect effects on the metabolism of the host. These effects are brought about probably through the virus – induced synthesis of new proteins by the host, some of which are biologically active substances (enzymes etc.) and may interfere with the normal metabolism of the host. (Dhar and Singh, 1995). Viruses generally cause a decrease in photosynthesis through decreases in chlorophyll per leaf, chlorophyll efficiency, and leaf area per plant. Viruses usually cause a decrease in the amount of growth- regulating substances (hormones) in the plant, frequently by inducing an increase in growth – inhibiting substances. A decrease in soluble nitrogen during rapid virus synthesis is rather common in virus diseases of plants. The respiration of plants is generally increased immediately after infection with a virus (Gafni and Epel, 2002).

The activation of pathogen stress responses includes hormonal changes and induction of defence mechanism which can affect the total yield (Heil *et al.*, 2000). During the infection a major portion of produced energy is spent on defence. This is most evident in mutants that have constitutively activated defences and show higher resistance to pathogens (Alvarez, 2000). After recognition of the pathogen, plant defence mechanisms are elevated. This can initiate the production of signal compounds such as reactive oxygen species (ROS) and salicylic acid (SA), which may be harmful in high concentrations to the host itself. For instance, when a threshold for local ROS concentration is exceeded, the cells initiate programmed cell death (PCD) (Navarre and Wolpert, 1999).

Biotic Stress Responses: In Tomato during Viral Infection

Begomoviruses are DNA viruses of the family Geminiviridae having a single or double genomic segment which are comprised of overlapping five or six open reading frames in both orientations and these are translated into 10 KDa proteins (Bisaro, 2006; Vanitharani *et al.*, 2005).

Plant cells are protected against the oxidative damage caused by ROS through a complex antioxidant system, comprised of antioxidant enzymes like catalase (CAT), peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) which scavenge ROS within the tissues (Scandalios,1993).

The ROS produced by plants against invading pathogens have an important role in signal transduction and limiting pathogen progress (Peng and Kuc, 1992; Kinily *et al.*, 1993), but when produced in excess, they cause tissue necrosis (Eltner and Osswald, 1994; Baker and Orlandi, 1995).

Hypersensitivity: A Preventive Measure

The hypersensitive response, often referred to as HR, is a localized induced cell defence in the host plant at the site of infection by a pathogen. HR is the result of quick mobilization of a cascade of defence responses by the affected and surrounding cells and the subsequent release of toxic compounds that often kill both the invaded and surrounding cells and also the pathogen. The hypersensitive response is often thought to be responsible for limiting the growth of the pathogen and, in that way, is capable of providing resistance to the host plant against the pathogen.

In plant–virus interactions, the HR is an active defence response by plants, resulting from activation of defence-related pathways which lead to rapid cell death (Baker *et al.* 1997; Hammond-Kosack and Jones 1996; Heath, 2000). Hypersensitive response is followed by apoptosis, with the affected cells being metabolically active (Nozue *et al.* 1997).

During a viral infection a HR response is initiated by Avr/R protein interactions that lead to metabolic changes in signalling pathways and the accumulation of ROS, such as O²⁻ and hydrogen peroxide, both in the infected and noninfected tissues (Culver and

Padmanabhan, 2007; Carr *et al.*, 2010; Pallas and García, 2011; Mandadi and Scholthof, 2012).

During HR several caspase-like proteinases such as the vacuolar processing enzymes are activated. Vacuolar processing enzymes primarily act as effectors of cell death or necrosis during HR (Mur *et al.*, 2008). Although necrosis is observed concomitantly with HR-mediated resistance, necrosis can be uncoupled from the resistance response.

Begomoviral Evolution

Taxonomically the *Geminiviruses* are mainly divided into four genera, three of which (*Mastrevirus*, *Curtovirus*, and *Topocuvirus*) consist of viruses with monopartite genomes only. But the contrast lies in the case of the genus *Begomovirus* which are evolutionized to incorporate either monopartite or bipartite genomes (Stanley *et al.*, 2005). More than 133 *begomovirus* species have been characterized comprising of monopartite genomes and all originate from the Old World (OW). No monopartite *begomoviruses* natives to the New World (NW) have been identified, although TYLCV was constitutively introduced (Polston *et al.*, 1999). A major portion of monopartite *begomoviruses* have been found to be associated with ssDNA satellites known as betasatellites which are totally dependent on their helper viruses for replication, movement and encapsidation in plants and transmission between plants (Briddon and Stanley, 2006). Further study on the evolution of *begomoviruses* has revealed that the virus-betasatellite complex is also associated with a class of ssDNA components known as alphasatellites (Briddon and Stanley, 2006). Despite having a mechanism to maintain the integrity of their split genomes, component exchange, referred to as pseudo-recombination for *begomoviruses*, occurs frequently (Pita *et al.*, 2001).

There is a greater genetic variation of DNA-B components in comparison to the DNA-A components. Although the vast majority of NW and some OW *begomoviruses* show

near perfect co-evolution of the DNA-A and DNA-B components, this is not the case for the majority of OW viruses (Briddon *et al.*, 2010).

Geminiviruses evolve as rapidly as some RNA viruses but their coat protein encoding gene (AV1) is least diversified while the whole genome reflects a rapid mutational dynamics rather than frequent adaptive evolution (Duffy and Holmes, 2007).

Indian Tomato Leaf Curl Virus (ToLCV) strains are closely related in the context of geographical locations. The predicted NLS regions present in AV1 gene shares more similarity with experimentally known TYLCV coat protein NLS (Sivakumar *et al.*, 2012).

Bioinformatic Tools to Study Viral Genome Evolution

Bioinformatics is the interface between biology and computer science (Bios Instant Notes on Mol. Biology, 4th Edition). Biological Databases and bioinformatic tools are the two indispensable parts for any type of computational biology study. The maintenance of sequence of data and the other data types in databases are designed to allow their easy manipulation and inspection, often in the context of access on the Internet. Analytical methods, particularly for the comparison of DNA and protein sequences (sequence similarity searching, sequence alignment), used to identify unknown sequences and assign functions; identification of structural or functional motifs, protein domains (Pevsner, 2009). Analysis of phylogenetic relationships by multiple sequence alignment, most often of protein or DNA sequences; these methods help to identify evolutionary relationships between organisms at the sequence level, and aid the identification of functionally important regions of DNA, RNA and proteins. (Pop and Salzberg, 2008). Two DNA or two protein sequences will always show some similarity, if only by chance. The principle of searching for meaningful sequence similarity is to align two sequences, i.e. array them one above the other with instances of the same base or amino acid at the same position. Multiple sequence alignments are related to the

alignment of more than two sequences. It becomes advantageous by multiple alignments that evolutionary relationships between individual genes or sequences become clearer and it becomes possible to identify important residues or motifs from their conservation between genes or proteins from different organisms. The sequence similarity can be used to generate phylogenetic relationships and phylogenetic trees.

Genomic Study of *Begomoviruses*

Begomoviruses require only DNA-A for systemic infection (Dry *et al.*, 1999). The CP gene is the most highly conserved gene in the family *Geminiviridae* (Wyatt, 1996). CP is the sole structural protein of *geminiviruses* and has been shown to play a determinative role in the transmission of these viruses (Briddon *et al.*, 1990). This gene sequence efficiently predicts discrete strains, species, and taxonomic lineages of *begomoviruses*, which has been accepted by ICTV as a desirable marker for virus identity when a full length genomic sequence is not available (Mayo, 1998).

Material and Methods

To study the stress responses during the viral infection in the samples the following experiments were done.

a. Trypan Blue Staining:

Leaves of chilli (*Capsicum annuum* L.), potato (*Solanum tuberosum*) & tomato (*Lycopersicon esculentum* Mill.) from healthy as well as infected plants were collected from UBKV campus and farmer's fields of nearby village. For staining of the leaves staining stock solution is made by mixing 10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml water and 0.02 g of trypan blue. The working solution is prepared by diluting the stock solution with ethanol (96%; 1:2 v/v). The destaining solution is made by dissolving 1 kg of chloral hydrate in 400 ml water. Chloral hydrate takes long time to dissolve. After the chemical gets dissolved it is covered with an appropriate lid and stored.

The staining procedure is carried out by transferring the leaf tissues into a conical flask filled with appropriate quantity of diluted trypan blue working solution and a lid is placed on the flasks. Then the flasks are placed in a heated water bath and the staining solution is boiled for one minute. After boiling, the flasks along with the dipped leaf tissues are left overnight in a shaker with a uniform speed. On the next day destaining is done by dipping the leaves in chloral hydrate solution and shaking the flask vigorously until the complete destaining of the healthy leaves.

b. Enzyme Assay:

Assays were done for the enzymes Catalase (EC 1.11.1.6), Peroxidase (EC 1.11.1.7), Polyphenol Oxidase (EC 1.14.18.1) and Phenylalanine Ammonia Lyase (EC 4.3.1.5). For all

the enzyme assay the healthy and infected leaf tissues were taken from chilli because of the unavailability of other host tissues (potato, tomato) during experiment. Chilli is also infected by begomoviruses as this is also a solanaceous vegetable host plant.

c. Assay of Catalase:

The assay of the enzyme catalase was done by the method of Sadasivam and Manickam (1996) with some modifications. Briefly, 0.5 mg of fresh plant leaf was extracted with 2 ml. of phosphate buffer (M/15; pH=7.0) in a pre-cooled mortar and pestle at 4°C and centrifuged at 10,000 RPM for 30 minutes in a centrifuge (Sigma 2-16 KL) and the supernatant was used as enzyme source.

In a cuvette 2.3 ml of phosphate buffer(M/15), 0.2 ml of enzyme extract and 0.5 ml of H₂O₂ (0.2 M) were taken and the change in absorbance was recorded at 240 nm at 30 second interval for 5 minutes in a UV-VIS spectrophotometer (Shimadzu UV-1800). The enzyme activity was expressed as “ $\Delta A_{240} \text{min}^{-1} \text{g}^{-1}$ fresh weight”, where ΔA_{240} represents the change in absorbance at 240 nm.

d. Assay of Peroxidase:

The extraction of the enzyme peroxidase was carried out by the method of Sadasivam and Manickam (1996) with some modifications. Briefly, 0.5 mg of fresh plant leaves taken in a pre-cooled mortar and pestle, 2 ml of phosphate buffer (0.1 M, pH= 7.0) was added and crushed uniformly. The homogenate was centrifuged at 10,000 RPM at 4°C for 30 minutes in a centrifuge and the supernatant was used as the enzyme source. It was stored in ice till the assay was carried out.

The assay of peroxidase was assayed by the method of Neog *et al.* (2004). The assay mixture, containing 2.5 ml of phosphate buffer (pH = 6.5, 0.1 M), 0.2 ml of enzyme extract

and 0.1 ml of *o*-dianisidine (1 mg ml^{-1} in methanol) was incubated at 28°C in a water bath for 2 minutes. The reaction was started by adding 0.2 ml of H_2O_2 (0.2 M). The change in absorbance was recorded at 430 nm at 30 second interval for 5 minutes in a UV-VIS spectrophotometer. The enzyme activity was expressed as “ $\Delta A_{430} \text{ min}^{-1} \text{ g}^{-1}$ fresh weight”, where ΔA_{430} represents the change in absorbance at 430 nm.

e. Assay of Polyphenol Oxidase:

The assay of the enzyme polyphenol oxidase was done by the method of Mayer et al. (1965). Briefly, 0.5 mg of finely cut and well mixed fresh leaf was crushed in pre-chilled mortar-pestle with the addition of 2 ml phosphate buffer (0.1M, pH = 6.6). The homogenate was centrifuged in a centrifuge at 10,000 RPM for 30 minutes and the supernatant was used as enzyme source. The extract was kept on ice till the assay was carried out.

The polyphenol oxidase was assayed using pyrogallol as substrate. The assay mixture was prepared by taking 0.2 ml freshly prepared enzyme extract, 2.4 ml of phosphate buffer (pH=6.0, 0.1 M) and 0.4 ml pyrogallol (0.05 M) and the change in absorbance was recorded at 495 nm at 30 second intervals for 5 minutes in a UV-VIS spectrophotometer. The enzyme activity was expressed as “ $\Delta A_{495} \text{ min}^{-1} \text{ g}^{-1}$ fresh weight”, where ΔA_{495} represents the change in absorbance at 495 nm.

f. Assay of Phenylalanine Ammonia Lyase:

The assay of the enzyme phenylalanine ammonia lyase was done by the method Sadasivam and Manickam (1996) with some modifications. Briefly, 0.5 mg of fresh plant leaf was crushed with 2 ml of borate buffer (0.2 M, pH=8.7) taking in a pre-chilled mortar and pestle at 4°C . The homogenate was centrifuged at 12,000 RPM for 20 min and the supernatant was used as enzyme source.

In a test tube 0.5 ml borate buffer, 0.2 ml enzyme solution and 1.3 ml water was taken and the reaction was initiated by the addition of 1 ml L-Phenylalanine solution. Then the whole mixture is incubated for 30 minutes at 32⁰C. The reaction is stopped by the addition of 0.5 ml 1M trichloroacetic acid. The change in absorbance was measured at 290 nm at 10 min. interval for 30 minutes. Separately a standard graph was prepared for trans-cinnamic acid at a concentration of 1mg/1 ml.

For the PAL assay the protein content of the fresh leaf tissues were estimated by the method given by Lowry *et al.* (1951). For that a standard curve of protein was made by using Bovine Serum Albumin as the reference. The absorbance was taken at a wavelength of 660 nm. The enzyme activity was expressed as “micromole *trans*-cinnamic acid formed per mg protein per min”.

g. Statistical Analysis:

The data obtained from the laboratory experiments were subjected to statistical analysis in t-test for independent samples. The computation as well as the analysis was done in Microsoft Excel-2007.

h. Viral evolution study:

The complete genome sequences of *Begomoviruses* along with individual sequence of the genes were retrieved from the National Centre for Biotechnology Information (NCBI) Database. The 31 begomoviruses which were used in our study varied in affecting different crop plants. Some of the viruses are also devoid of one or two genes in their genomic component. The 31 begomovirus members are given in the Table 2. The sequences were aligned by running Clustal W, integrated within the software program BioEdit (version 7.1.11).

Table 2: Thirty one begomoviruses used in our study.

Name of virus	Reporting year	Country where reported	Reference
Ageratum yellow vein virus (AYVV)	1995	Singapore	Tan, H.N.P
Tomato yellow leaf curl virus (TYLCV)	2009	China	Liu, P.et.al.
Tomato severe leaf curl virus (ToSLCV)	2015	USA	Rosario, K.et. al.
Papaya leaf curl virus (PLCV)	2004	China	Zhou, X and Ma, X.
Pepper golden mosaic virus (PepGMV)	1996	Mexico	Torres-Pacheco, I.et.al.
Indian cassava mosaic virus (ICMV)	2015	India	Patil, B.L.et.al.
Cotton leaf curl virus (CLCuV)	2002	Sudan	Idris, A.M,
Okra leaf curl virus (OLCV)	2009	India	Singh, A.K.et.al.
Bhendi yellow vein mosaic virus (BYVMV)	2009	India	Taware, S.D.
Pepper yellow leaf curl virus (PYLCV)	2015	Thailand	Chiemsombat, P.
Sweet potato leaf curl virus (SPLCV)	2008	Japan	Onuki, M.
Tobacco leaf curl virus (TLCV)	2011	India	Singh, M.K.et.al.
Squash leaf curl virus (SLCV)	2003	India	Muniyappa, V.et.al.
Tobacco mottle leaf curl virus (TbMoLCV)	2008	Cuba	Dominguez, M.
Mesta yellow vein mosaic virus (MeYVMV)	2015	India	Rishishwar, R.et.al.
Alternanthera yellow vein virus (AIYVV)	2006	China	Huang, J.et. al.
Cotton leaf crumple virus (CLCrV)	2005	Mexico	Brown. J.K.
Hollyhock yellow vein mosaic virus (HoYVMV)	2015	India	Venkataravanappa et.al.
Tomato golden mosaic virus (TGMV)	1984	USA	Hamilton, W.D.
Ageratum enation virus (AEV)	2012	India	Raj, S.K. et. al.
Mungbean yellow mosaic virus (MYMV)	1998	Japan	Ikegami, M.
Melon chlorotic mosaic virus (MeCMV)	2013	Venezuela	Romay, G.et.al.
Watermelon chlorotic stunt virus (WmCSV)	2014	Jordan	Lapidot. M. et.al.
Macropitilium yellow spot virus (MaYSV)	2014	Brazil	Sobrinho, R.R. et.al.
Soybean chlorotic spot virus (SoCSV)	2014	Brazil	Sobrinho. R .R. et.al.
Corchorus golden mosaic virus (CoGMV)	2014	Bangladesh	Hasan, M.M.
Gossypium darwinii symptomless virus (GDarSLV)	2007	Pakistan	Rehman, M.,
Ramie mosaic virus (RamMV)	2009	China	Zhou, X.P.
Rhynchosia golden mosaic virus (RhYMV)	2000	Honduras	Potter, J.L. et.al.
Horsegram yellow mosaic virus (HYMV)	2012	India	Bhatt, B.S.et. al.
Honeysuckle yellow vein mosaic virus (HYVMV)	2004	Japan	Takanami, Y.
Ageratum enation virus (AEV)	2012	India	Raj, S.K.et. al.

Source: NCBI Database

i. Phylogenetic Analysis:

After alignment in Clustal W the sequences were incorporated for evolutionary analysis in Molecular Evolutionary Genetical Analysis tool (MEGA v 7.0.18). An evolutionary model was determined using a maximum-likelihood model and the maximum likelihood tree was constructed using the Tamura- Nei model with the highest log likelihood (-47187.7813). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioN-J algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

j. Pair-wise sequence diversity analysis:

Sequence diversity of the sequences acquired from NCBI Database was analyzed by using Sequence Demarcation Tool (SDT v 1.2). The genes residing in the genome sequences were indexed as per their similarity patterns. Heat map generated using Sequence Demarcation Tool version 1.2 (SDT v1.2). Sequence diversity in Begomovirus genes from 31 different viruses was analyzed.

The same analysis was done for Indian Tomato Leaf Curl Virus strains of different geographical locations in India retrieved from NCBI Database (Table 3).

k. Nuclear Localisation Signal Sequence Identification:

Additionally the coat protein gene (AV1) of Tomato Leaf Curl New Delhi Virus (ToLCNDV) was utilized to map the Nuclear Localization Signals (NLS) in the entire region of the gene as well as in all ToLCV Indian strains. For this purpose the online mapping software cNLS Mapper tool was used by fixing the cut off score at 5.00.

Table 3: Seventeen Indian ToLCV strains used in our study.

TLCV STRAIN	Characteristics		Reporting Year	Reported from	Reference
New Delhi isolate eggplant	Old World	Bipartite	2009	New Delhi, Maharastra	Pratap, D.et.al.
Strain MPT (Coimbatore, TN)	Old World	Monopartite	2013	Coimbatore(TN)	Deivamani, M.et.al.
Isolate Lucknow	Old World	Monopartite	2012	Lucknow(UP)	Raj,S.K.et.al.
Gujarat virus (Vadodara)	Old World	Monopartite	2003	Vadodara district(Gujarat)	Shih,S.L.et.al.
Patna virus	Old World	Monopartite	2009	Patna,Bihar	Kumari,P. et.al.
Isolate Raebareli	Old World	Monopartite	2012	Raebareli,UP	Aslam,M.
Bangalore II	Old World	Monopartite	unpublished	Bangalore	Chatchawank anphanich,O. et.al.
Bangalore virus (India: Kerala IV, 2005)	Old World	Monopartite	2006	Kerala	Pandey,P.et.al .
Bangalore virus (Kolar)	Old World	Monopartite	2001	Kolar,Bangalore	Kirthi,N.
Karnataka virus	Old World	Monopartite	1995	Bangalore	Maxwell,D.P.
Bangalore virus	Old World	Monopartite	2006	Kerala	Pandey,P.et.al
Patna virus	Old World	Monopartite	unpublished	Joydebpur,Bangla desh	Ansari,A.et.al .
Ranchi virus (isolate GUW 2)	Old World	Monopartite	2011	Guwahati	Saha,B.et.al.
Palampur virus (isolate Punjab)	Old World	Bipartite	2013	Punjab	Kanakala,S.et .al.
Gujarat virus (isolate Ramgarh)	Old World	Monopartite	2009	Gujarat	Kumari, P.et.al.
Joydebpur virus (Kalyani)	Old World	Monopartite	2006	Kalyani,West Bengal	Tiwary N.et.al.
Kerala virus	Old World	Monopartite	2008	Kerala	Mukhopadhy ay,S.
New Delhi virus (Jessore)	Old World	Bipartite	2005	Jessore,Banglades h	Maruthi,M.N. et.al.
New Delhi Virus	Old World	Bipartite	2008	New Delhi	Padidam,M. et al.

Source: NCBI Database

l. Identification of Conserved Sequence :

The identified NLS regions were further analyzed in WebLogo (version 2.8.2) tool for analyzing the conserved sequences in the coat protein gene of Indian ToLCV strains. WebLogo generate sequence logos i.e. a graphical representation of an amino acid or nucleic acid multiple sequence alignment developed by Tom Schneider and Mike Stephens. Each logo consists of stacks of symbols, one stack for each position in the sequence. The overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino or nucleic acid at that position. In general, a sequence logo provides a richer and more precise description of, for example, a binding site, than would a consensus sequence.

m. Genomic DNA Isolation from Plant Leaves:

The leaves from infected plants were taken from chilli, potato & tomato from our University campus and from nearby farmer's field. The leaves were selected on the basis of visualizing the typical *begomoviral* symptoms. The tissues were collected from field in a container of liquid nitrogen. After taking it to laboratory the leaves were crushed in a pre-chilled mortar-pestle to a fine powder and the CTAB method for isolation of genomic DNA from plant tissues was carried out. The extraction buffer is made by adding 1.4 M NaCl, 2.0% CTAB, 100 mM Tris-HCl, pH=8.0, 20 mM EDTA. 1 ml of pre-warmed extraction buffer along with ample amount of 0.2% β -mercaptoethanol is added to that fine powder. Then the mixture is incubated at 60°C for 30 minute with occasional mixing with gentle swirling. An equal volume of chloroform- isoamyl alcohol mixture is added to it & gentle mixing is done followed by centrifugation at 10,000 RPM for 10 minutes in eppendorf tubes. The upper aqueous phase is collected and the above step is repeated till no white interface is visualized. In that aqueous phase 0.6 volume of isopropanol is added and mixed gently and the whole thing is left at -20°C for overnight. On the very next day the DNA is pelleted by swirling it in

ultracentrifugation at a speed of 10,000 RPM for 1 minute. The DNA pellet is washed with 70% ethanol and then it is air dried & dissolved in appropriate quantity of TE buffer. TE buffer is made with 10 mM Tris-HCl, pH=8.0 & 1 mM EDTA.

n. Primer Designing:

The primers for PCR protocol were designed by taking the already known and sequenced genes of begomoviruses as reference (Table 4). The sequences were aligned using BioEdit version 8 and the 20 to 30 bases were taken for forward and reverse primers.

The infection clone of Tomato Leaf Curl Joydevpur Virus (ToLCJV) was used to standardize the primers. Infection clones are made by using the vector pBin19. The ToLCJV genomic components DNA-A into this plasmid vector. This construct was kindly provided by Dr. Shelly Praveen, Head, Department of Biochemistry, IARI, New Delhi.

o. Competent Cell Preparation:

The bacterial cells were transformed by following the protocol given by Joe Graber. At first the competent cells were prepared by following the steps below:

E.coli was grown in a 3 ml LB media overnight at 37°C. 500 µl of O/N culture was placed into 100 ml of LB broth. The flask was immediately immersed in ice and swirled. Spinning out of cells at 5000g was done for 5 minutes. Then the cells were resuspended in 24 ml of ice cold MgCl₂ 0.1 M and were centrifuged at 5000 RPM. The supernatant was discarded and the cells are suspended in 24 ml of ice cold sterile CaCl₂ 0.1 M by gentle pipetting. Again spinning was done at 4000 RPM & the supernatant was discarded. The cells were suspended in 4.3 ml of 0.1 M sterile CaCl₂.

Table 4: Designed primers used in our study.

Name of primer	Sequence
AVI Forward	ATGGCGAAGCGACCGGC
AV1 Reverse	TTAATTTGTTACGGAATCATAG
AV2 Forward	ATGTGGGATCCGTTAGTAAAC
AV2 Reverse	CTAGGAACATCTGGGCTTC
AC1 Forward	ATGGCTCCTCCAAATAAATTC
AC1 Reverse	TCAACGCGACGACGCCTG
AC2 Forward	ATGCGACCTTCATCACC
AC2 Reverse	CTAAAGACCCTTAAGAAATG
AC3 Forward	ATGGATTCACGCACAGG
AC3 Reverse	TTAATAAATATTGAATTTTATTATG
AC4 Forward	ATGGGGAACCTCATCTAC
AC4 Reverse	TTAAAATATTGAGGGCCGC

p. Transformation of the Cells:

To transform the competent cells the CaCl_2 treated cells 200 μl of each suspension of competent cells were incorporated with 2 μl plasmid DNA. Mixing is done gently by swirling and the tubes were stored on ice for 30 minutes. Then the tubes were placed in a preheated 42°C circulating water bath. The tubes were kept for 90 seconds. Rapidly the tubes were transferred to an ice bath and allowed to chill for 1-2 minutes. The cultures were then incubated for 1.5 hours in an incubator shaker and then centrifuged at 7000 RPM for 3 minutes and the supernatant is discarded. The appropriate volumes (200 μl) of transformed competent cells were transferred into a LA medium plate with proper antibiotic. The plates were incubated at 37°C for 12- 16 hours.

q. Plasmid Isolation:

The protocol for isolation of plasmid was followed from the laboratory manual of Molecular Cloning by Sambrook and Russell. The following materials were used for plasmid isolation-

1. Alkaline Lysis solution I = 50 mM glucose + 25 mM Tris-HCl (pH= 8.0) + 10 mM EDTA (pH = 8.0).
2. Alkaline Lysis solution II = 0.2 N NaOH+1% SDS + water.
3. Alkaline Lysis solution III = 5 M potassium acetate + Glacial acetic acid + H_2O
4. Antibiotic for plasmid selection
5. Ethanol
6. Phenol: Chloroform
7. TE buffer (pH = 8.0) containing 20 $\mu\text{g}/\text{ml}$ RNase A
8. Luria Broth (LB) Media

Two ml of rich medium is inoculated containing appropriate antibiotic with a single colony of transformed bacteria. The culture is incubated overnight at 37°C with vigorous shaking. Then 1.5 ml of the culture is poured in eppendorf tubes and centrifuged at 10,000 RPM for 2 minutes at 4°C. The bacterial pellet is collected by discarding the supernatant. The bacterial pellet is resuspended in 100µl of ice-cold alkaline lysis solution I by vigorous vortexing. Then freshly prepared 200 µl alkaline lysis solution II is added without any kind of vortexing. After that 150µl of ice- cold lysis solution III is added and mixed thoroughly by repeated gentle inversion. Centrifugation is done at 10,000 RPM for 10-15 minutes at 4°C. The resultant supernatant is recovered followed by addition of equal volume of chloroform to precipitate the plasmid DNA. Mixing is done thoroughly by gentle inversion. Centrifugation is done at 10,000 RPM for 10 minutes at 4°C. The aqueous upper layer is transferred to a fresh tube. Rinsing is done with ice-cold isopropanol and kept in ice for 10-15 minutes for coagulation. Again the tubes are spinned for 10 minute at 10,000 RPM and then a final wash is given with 70% ethanol. Then the pellet was dried and dissolved in warm TE buffer.

r. PCR reaction:

After standardization the primers were used to amplify the isolated genomic DNA. For PCR protocol the following reagents are used:-

1) Genomic DNA / Viral DNA	:1µl
2) Buffer	: 1.8 µl
3) Forward primer	: 1 µl
4) Reverse primer	: 1 µl
5) MgCl ₂	: 1 µl
6) dNTP mix.	: 1 µl
7) Water	: 14 µl
8) Taq polymerase	: 0.2 µl
Total	: 20 µl

The PCR amplification was carried out in a thermal cycler (Veriti, Applied Biosystems).

During this experiment the PCR protocol followed is as follows:

- Denaturation : 94°C for 1 minute.
- Annealing : 55°C for 30 seconds (35 cycles).
- Extension : 72°C for 1 minute.
- Final extension : 72°C for 10 minutes.
- Infinite Storage : 4°C.

Amplified DNAs were electrophoresed in a 1% agarose gel (BioRad). The agarose gel is made by adding 0.36 g agarose in 30 ml of 0.5X TAE buffer. Photograph of the gel was taken in Alpha imager.

Results and Discussion

1st Objective: Identifying hypersensitive response in plants & estimation of defence related enzymes during begomoviral infection

The reactive oxygen molecules have been found to be accumulated in various crops under biotic stress (Culver and Padmanabhan, 2007). In case of viral infections plant faces similar situations and produces stress responsive enzymes (Jones and Dangl, 2006). In the present study the level of stress response was measured by two independent experiments using healthy and infected symptomatic chilli (Fig 2A), potato (Fig 2B) & tomato (Fig 2C) plants. In the first experiment the infected as well as healthy plants were stained with trypan blue to quantify the cell death in tomato, chilli, and potato. Cell death is a measure of stress responses during the viral infection. In all the three solanaceous crops infected leaves were found to retain higher level of stain even after complete destaining of the healthy leaves (Fig 3A, B & C). The severely symptomatic areas of leaves were deeply stained in comparison to other areas of leaf lamina. Healthy leaves of all the three crops were found absolutely devoid of any stain. When seen under microscope the cells were found to be transparent with light blue demarcation (Fig 4A, B & C). On the other hand infected cells were visualized as dark blue spots. In both the cases leaf veins were clear of any stains. Both the staining and microscopic study clearly suggest presence of more dead cells in case of infected leaves and indicate that hypersensitive response has occurred during the begomoviral infection in all three host plants.

Induction of hypersensitive response has been evident in many plant viral infections including *begomovirus* (Baker *et al.*, 1997). The purpose of the apoptosis after viral infection is to restrict the virus by destructing the tissue or cell in the particular area where the

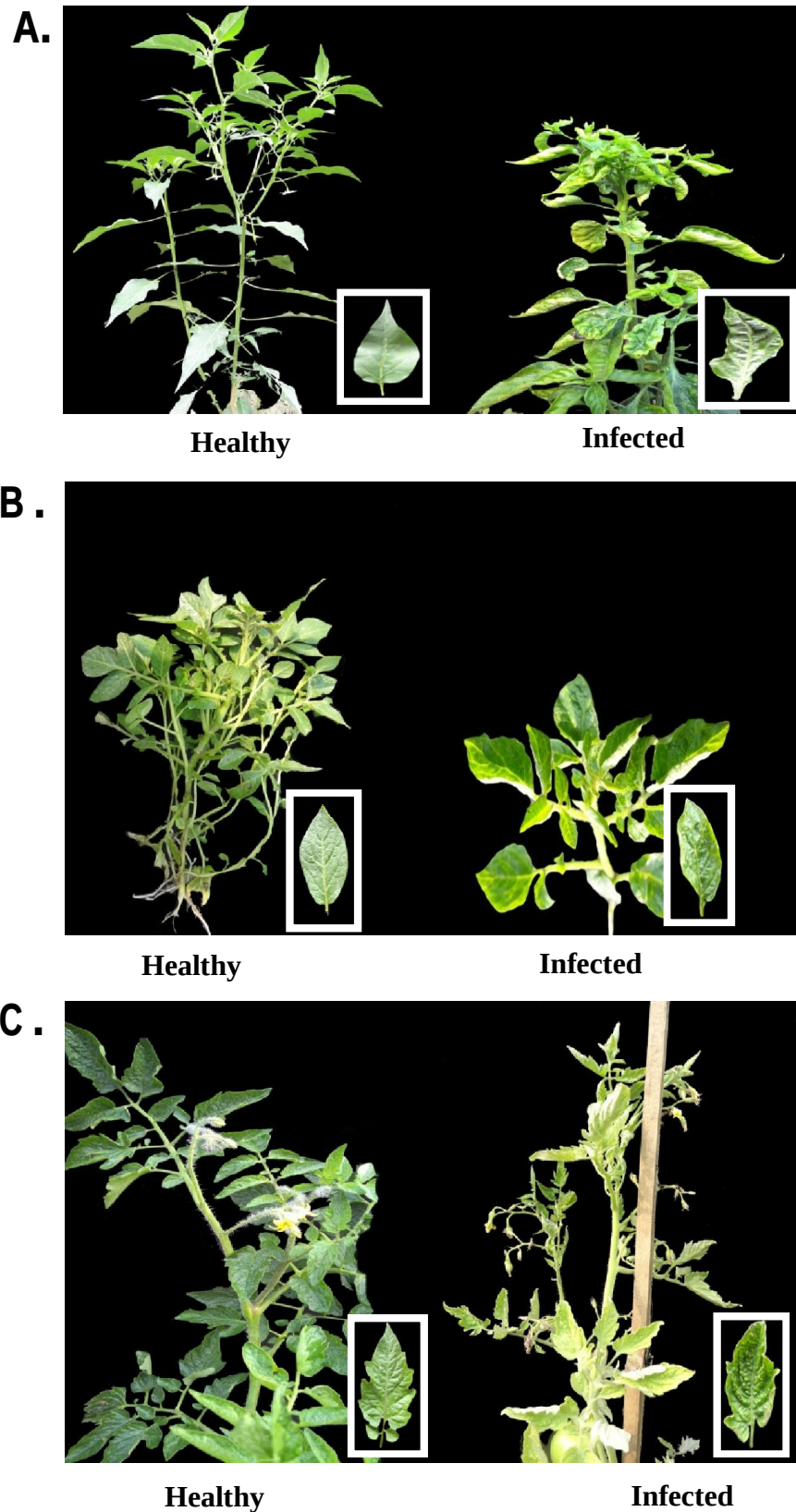


Fig 2: Plant samples (Healthy & *Begomovirus* Infected) of Chilli (A.), Potato(B.) & Tomato(C.) collected from university campus and nearby villages. In all the three crop plants symptoms of viral infection like curling and yellowing of leaves, stunted growth etc. are visible.

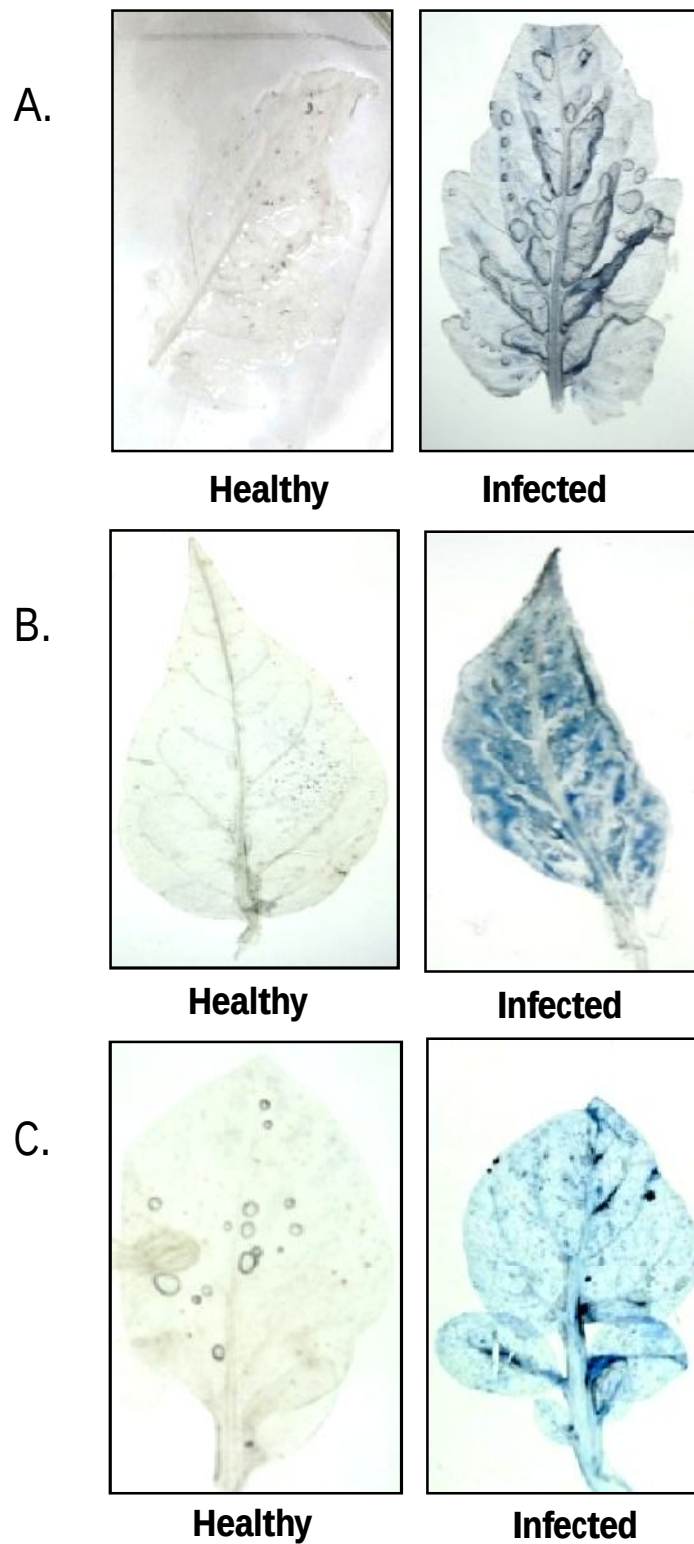


Fig 3: Trypan blue staining of healthy and infected leaf samples of A. Tomato, B. Chilli C. Potato showing hypersensitive response during infection.

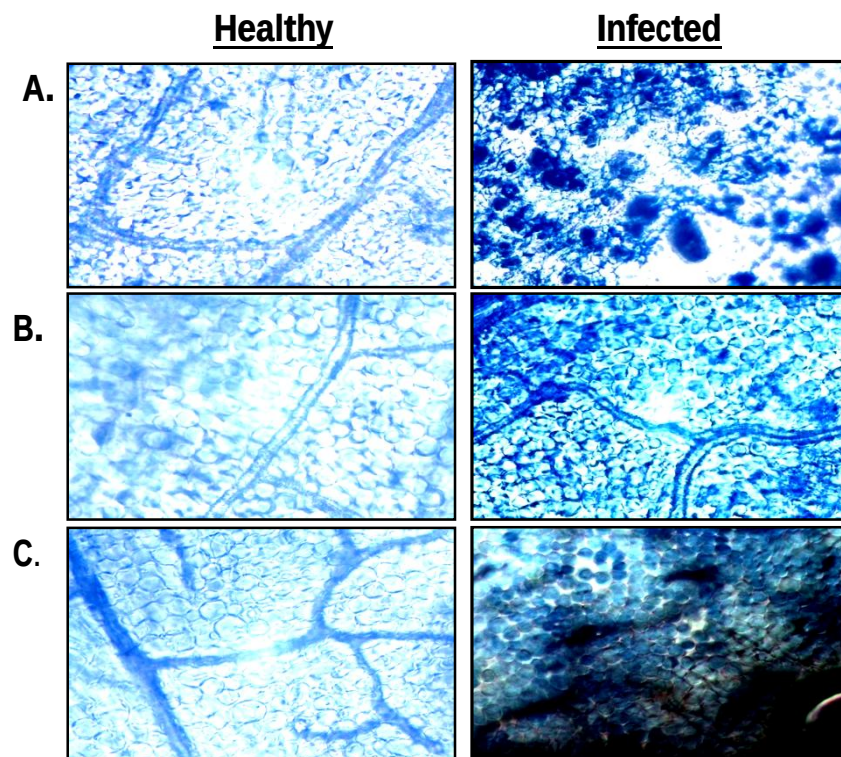
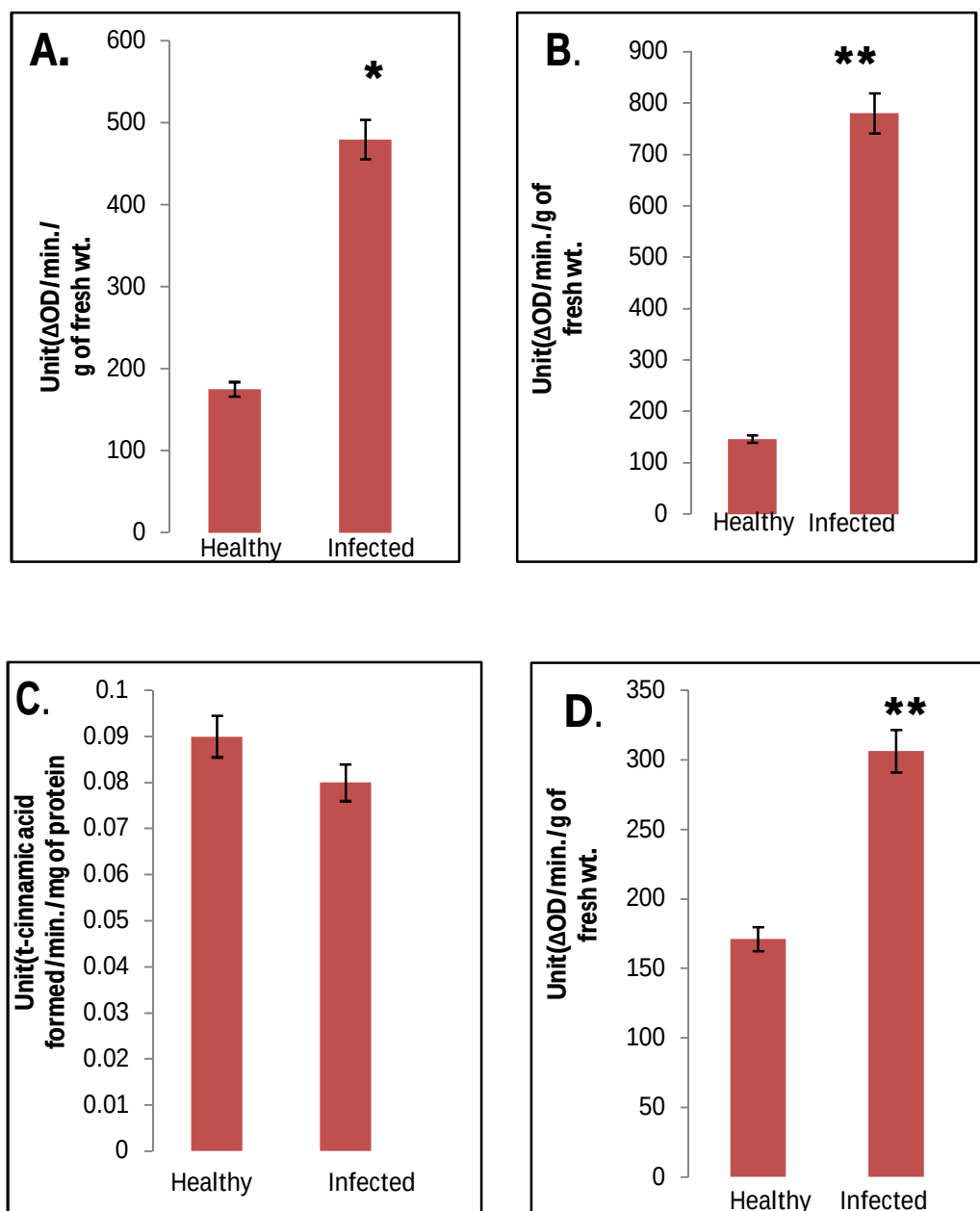


Fig 4: Microscopic Identification of cell death in the infected Leaf samples of A. Tomato, B. Chilli C. Potato during infection.

preliminary attack has happened (Balague *et al.*, 2003). The hypersensitivity involves induction of different stress responsive enzymes like CAT, PPO, lipid peroxidase, SOD, PAL, lipoxygenase etc. (Scandalios, 1993). In our study the levels of some of these enzymes were estimated by performing specific assays for the enzymes both from infected and healthy plant samples (Fig 5). The results state that CAT, PPO and peroxidase enzymes have a statistically significant aggravated level in infected plant sample compared to the healthy leaves. In case of CAT the value for healthy sample was 175.33 (Δ OD/min/mg of fresh tissue) whereas in infected sample the value was much higher 480 (Δ OD/min/mg of fresh tissue) (Fig 5A). Similarly in case of peroxidase the healthy sample showed less enzymatic activity i.e. 171.2 (Δ OD/min/mg of fresh tissue) in comparison to infected ones i.e. 306.5 (Δ OD/min/mg of fresh tissue) (Fig 5D). So the enzymes like CAT & peroxidase showed approximately 2 fold increase in the infected samples. PPO enzyme showed a much higher enzymatic activity in infected ones i.e. 780 (Δ OD/min/mg of fresh tissue) compared to healthy ones i.e. 146.66 (Δ OD/min/mg of fresh tissue) (Fig 5B). This indicates approximately seven fold increase in enzymatic activity. So, these enzymes play a vital role during the infection. PAL enzyme showed a decreased level in infected plants, the enzymatic activity for PAL was 0.61 (Δ OD/min/mg of fresh tissue) for healthy samples and for infected samples it was 0.56 (Δ OD/min/mg of fresh tissue) (Fig 5C), but the results were not statistically significant. Both in infected plants as well as transgenics made from different begomoviral gene have shown an increase in these enzymes (Prasad, 2016). There are many reports in literature about the induction of PAL enzyme activity during any biotic stress which helps in lignifications of the cell wall in order to protect the physical barrier of the cell to restrict the further invasion. In our case we found a reduced level of enzyme activity probably due to completion of the lignifications activity or PAL activity at the early stage of infection which is evident from other studies as well. (Dixon and Paiva, 1995).



<1% LOS=; >1% LOS=***
(LOS = Limit of significance)

Fig 5: Assays of stress responsive enzymes, A. Catalase, B. Polyphenol Oxidase, C. Phenylalanine Ammonia Lyase and D. Peroxidase, performed with infected and healthy leaf samples

2nd Objective: Study of evolutionary pattern of begomoviral genome

Based on the sequence alignments a phylogenetic tree was generated from DNA-A sequences of 31 *begomoviruses* (Table 2) reported in last twenty years in part of the world. The result shows the grouping of the viruses according to their geographical distribution into three major clades (Fig 6). The first group of viruses are reported from the south Asian countries like China, Thailand, Singapore and India where as the viruses falling in the second clade are invariably from India and are reported to infecting different host plants. The third group of viruses were found to be isolated from Central and South American countries. The grouping of the viruses originated from same geographical origin has been evident from other studies (Briddon *et al.*, 2010). It is presumed that due to presence of barriers like big ocean and big water bodies the viruses that prevails in south Asian countries does not move to countries of America and Latin America. Some viruses in the phylogenetic tree do not fall in any of the major clades and remains distance from the entire three cluster. Among them WmCSV and CLCuV are of African origin and thus remain together. The other four viruses SPLCV, MYMV, HYMV and CoGMV are of South East Asian origin but they differ largely on their preferred host for example CoGMV and SPLCV infects host chorchorus and sweet potato respectively where as legume infecting MYMV and HYMV fall in one cluster (Qazi, 2007). Comparative analysis of evolutionary aspect of DNA-A and B components of Bipartite viruses has revealed that DNA-B component is more prone to genetic variations than DNA-A (Briddon *et al.*, 2010). The possible reason could be the presence of major genes for the establishment of infection and survival of the virus in DNA-A which emphasize on the fact that DNA-A is an indispensable part of begomoviral genome (Navot *et al.*, 1991).

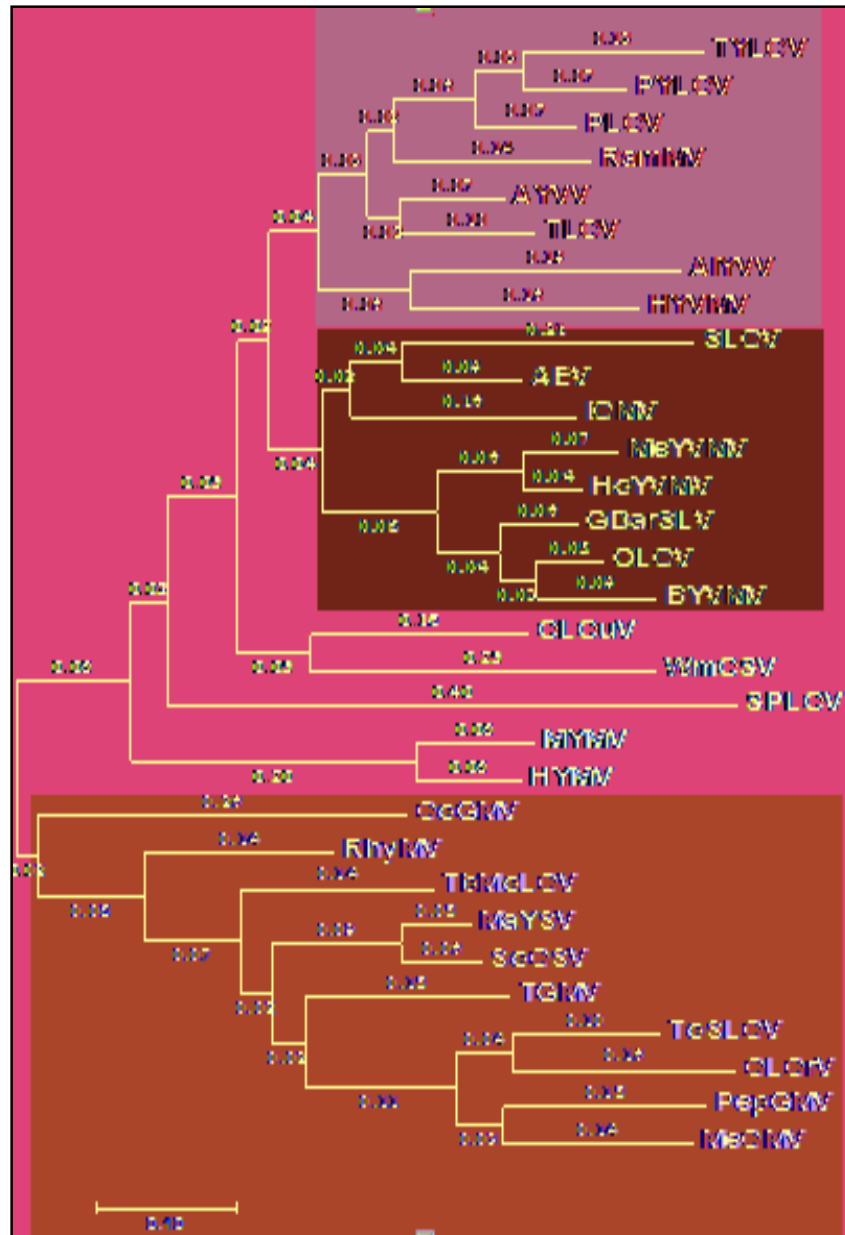


Fig 6: Phylogenetic analysis of DNA-A genomic component of 31 Begomovirus sequences reported in past two decades. Clades based on geographical distribution are indicated by different colors.

Because of the presence of this important gene DNA-A has less flexibility in sequence diversity than other DNA molecules that are found in *geminiviruses*. Even then mutations are found to be occurring in DNA-A at a high rate. The mean genomic substitution rate for DNA-A component of TYLCV has been estimated to be 2.28×10^{-24} nucleotide substitution/site/year (Duffy and Holmes, 2008) which is as high as RNA viruses. The level of the genetic diversity in the genic regions of DNA-A and its impact on the evolution of the individual genes has been analysed in our work.

Phylogenetic analysis of the begomoviral genes present in DNA-A:

DNA-A encodes total six proteins; the virion strand encodes coat protein (av1) required for encapsidation and movement in plants (Hanley-Bowdoin *et al.*, 1999) and pre-coat protein (AV2), a pathogenicity determinant believed to be involved in virus movement in plants (Rojas *et al.*, 2005). The complementary sense strand encodes Replication associated protein (AC1) required for viral replication (Nash *et al.*, 2011), Transcriptional activator protein (AC2) serves as the suppressor of RNA silencing mechanism (Yang *et al.*, 2007), Replication enhancer protein (AC3) establishes an environment conducive for virus replication (Pedersen & Hanley-Bowdoin, 1994) and C4 protein (AC4) may be the pathogenicity determinant and a suppressor of PTGS by binding of siRNAs (Pandey *et al.*, 2009). DNA-B component contains two ORFs in opposite orientations encoding nuclear shuttle protein (BV1) responsible for transport of viral DNA from the nucleus into the cytoplasm (Malik *et al.*, 2005) and movement protein (BC1) coordinates the movement of viral DNA across plasmodesmatal boundaries (Sanderfoot and Lazarowitz, 1996).

The *begomoviruses* also differ in the number of ORF in the DNA-A component. Among those 31 *begomoviruses* AV2 gene were present in AYVV, TYLCV, ToSLCV, PLCV, ICMV, CLCuV, OLCV, BYVMV, PYLCV, SPLCV, MeYVMV, AIYVV,

HoYVMV, AEV, MYMV, WmCSV, GDarSLV, RamMV, HYMV, HYVMV, AEV and AC4 were present in AYVV, TYLCV, ToSLCV, PLCV, PepGMV, ICMV, CLCuV, OLCV, BYVMV, PYLCV, SPLCV, TLCV, SLCV, TbMoLCV, MeYVMV, AIYVV, HoYVMV, TGMV, AEV, MYMV, MeCMV, MaYSV, CoGMV, GDarSLV, RamMV, RhYMV, HYMV, HYVMV, AEV. The phylogenetic analysis of AV2 & AC4 genes for the available viral sequences showed no similarity with other genes or among themselves. AC4 is normally functional in monopartite viruses. The entire ORF of C4 gene is located within the AC1 coding region in a different reading frame (Crake *et.al.*, 1998) and has role in viral disease symptom development (Stanley and Natham, 1992). It is reported that tomato infecting old world *begomoviruses* possess overlapping ORFs i.e. CP and AV2 while viruses infecting in new world have a single encoding ORF only (Padidam *et.al.*, 1996). The proposed role for AV2 protein is in symptom development and viral movement. Together it can be stated that presence and absence of these two genes affects the severity of infection and symptom development but these genes are not an absolute requirement for viral survival and replication. And thus may be lost from the genome of the virus during evolution. When these 4 genes were analysed to generate phylogenetic tree were analyzed to generate phylogenetic tree it was found that the evolution of individual genes doesn't follow the same pattern of the DNA- A evolution. In case of AV1 gene two major clades were found to be similarly distributed with the DNA-A phylogenetic tree with few exceptions suggesting AV1 gene has been evolutionarily parallel to DNA-A (Fig 7A), but phylogenetic tree of AC1 gene merely showed any similarity with other genes of the virus. For e.g. TYLCV and ToLCV falls in the same clade in case of AC2 (Fig 7C) and AC3 (Fig 7D) genes but which is not the case in AC1 (Fig 7B). The phylogenetic tree of AC1 shows that TYLCV and ToLCV are farly distant species for these genes. In all the cases some viruses have maintained to remain in close proximity for all the genes as well as DNA-A. Like HoYVMV, MeYVMV suggesting

these two virus to be very closely related throughout the evolution. Because AV2 and AC4 are unstable components of viral genome that's why we have focused on comparisons on rate of evolution among 4 genes, AV1, AC1, AC2, AC3 in our study. Substitution rates for genic region of coat protein in a recombination pre data set has been shown to be lower than the intergenic regions which was found to evolve even more rapidly in a higher substitution rate (Duffy and Holmes, 2008). However comparison of the rate of evolution between the genic regions has not been studied till date. It is believed that along with base substitution recombination among different DNA-A component are major source of variation in the genomic component of *Geminiviruses*. Our work first time demonstrates a comparative analysis of the rate of evolution for coding regions of the virus.

To address the differences between nucleotide sequences in the genes of these 31 *Begomoviruses* sequence diversity analysis was performed by Sequence Demarcation Tool version 1.2 (Fig 8A,B,C & D). All the other 4 genes were found to be present in all the *begomoviruses* among which AV1 showed the highest level of similarity between the different viruses as evident from the heat map. AC2 and AC3 showed a similarity index of 58-80% with a reduced peak of 65-70%. AC1 gene showed the lowest pair wise similarity index in the heat map for the 30 viruses suggesting that the AC1 or the Rep gene has been changing its sequences during the course of evolution with a higher rate than the other genes. AV1 gene has been remained least diversified according to the generated heat maps from different gene sequences in these viruses with a sharp peak ranging from 55-85% of pair wise identity percentage. AV2 showed a pair wise identity percentage of 41- 85% with no prominent peak and for AC4 gene the pair wise similarity ranged from 52- 92% with no sharp prominent peak with quite dispersed in their similarity index respectively.

To verify the stability of nucleotide sequences of the different genes of *begomoviruses* we studied ToLCV occurring only in India (Table 3) which has been reported in past decades

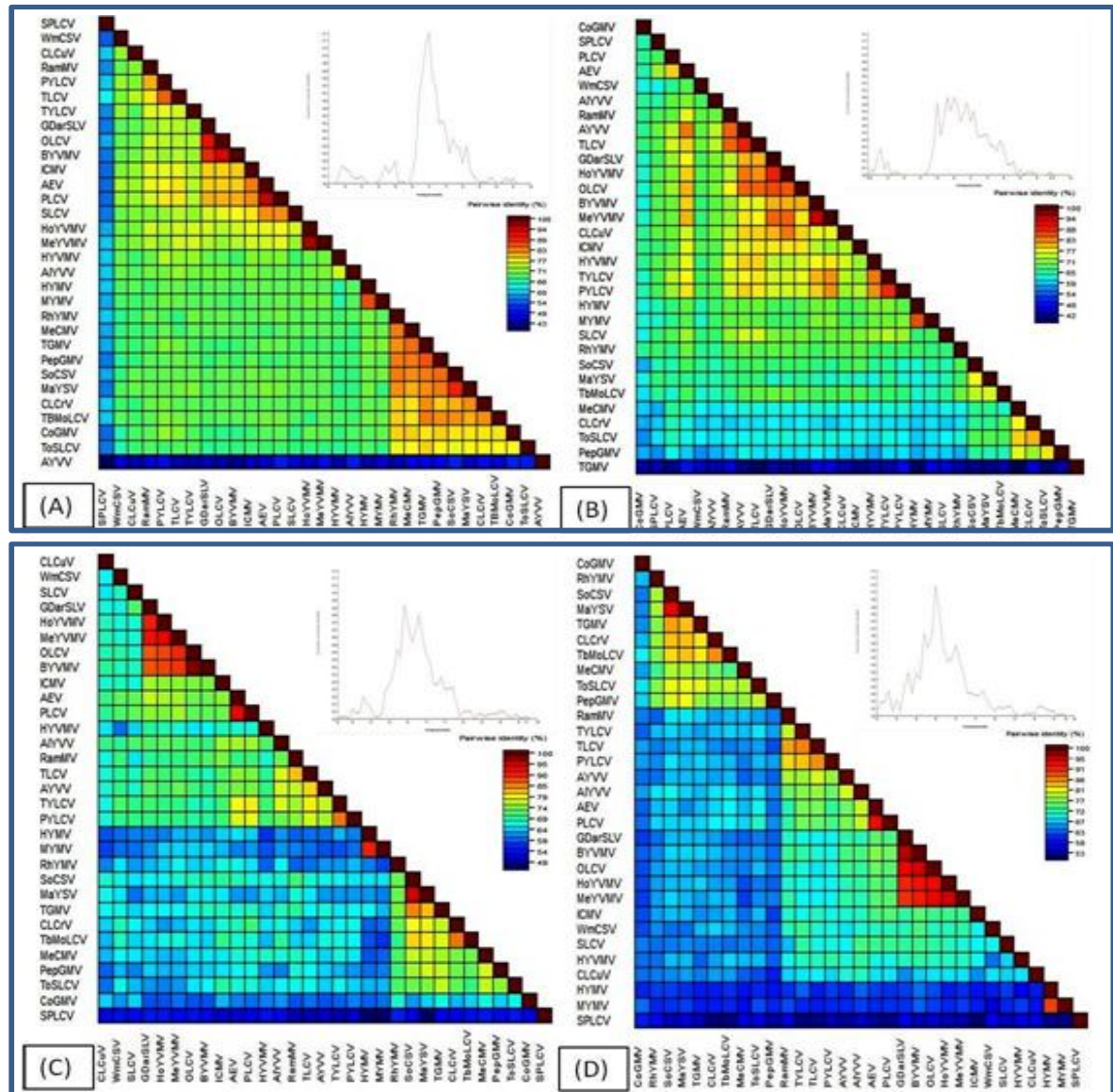


Fig 8: Sequence Demarcation Tool version 1.2 (SDTv1.2) generated heat maps of different genes present in DNA A genomic component of *Begeomoviruses*. (A) *av1*, (B) *ac1*, (C) *ac3*, (D) *ac4*. Presence of Blue color indicates high level of dissimilarity among the sequences. Presence of red and green color shows higher similarity index among the sequences (*av2* & *ac4* genes are not found in all viruses).

in different geographical locations in India. When the similarity index was analyzed for AV1 gene it was found that these viruses range from 71-95% pair wise identity percentage with peaks at 76% and 80% whereas AC1 showed the highest peak at 75%, AC2 and AC3 genes showed higher variability ranging from 66% which emphasize the same hypothesis that in only one virus also the trend of diversification of genes during evolution remains same and coat protein encoding gene AV1 is the most stably inherited across *begomoviruses* as well as different strains of ToLCV in India (Fig 9A, B, C & D).

To justify our resultant hypothesis we had taken the help of BioEdit tool (Fig 10) and the online tool cNLS Mapper (Kosugi *et al.*, 2009) which precisely identifies the NLSs present in the protein encoded sequences of certain viral genomes (Fig 11A). We had taken the ToLCNDV coat protein sequence as reference which gave prediction of 4 NLS sequences with a cut off score above 5 in the entire region. Using WebLogo software we analyzed the conservedness of these NLS sequences present in all the Indian isolates of tomato leaf curl virus. The NLS sequences were found to be highly conserved among the viruses as evident from Fig 11B.

Begomoviruses are most devastating plant viruses encroaching in different geographical location and new host very quickly. Many attempts have been made to generate resistant plants against these viruses by pathogen derived resistant (PDR) approach targeting different genic sequences of the virus. Most of these transgenics are based on generation on small interfering RNA or by antisense RNA against AC1 gene, AV2 gene or AC4 gene. Few attempts have been made against AV1 genes to establish resistance against cotton leaf curl virus (Amuda, J., 2011). Largely it can be stated that except few of transgenics (Faria, 2013) all the other attempts of incurring siRNA based transgenic resistance has failed in field trials. The prime reason is rapid evolution rate of the virus as well as presence of other *begomoviruses* in the field conditions. So identification of evolutionarily stable sequences in

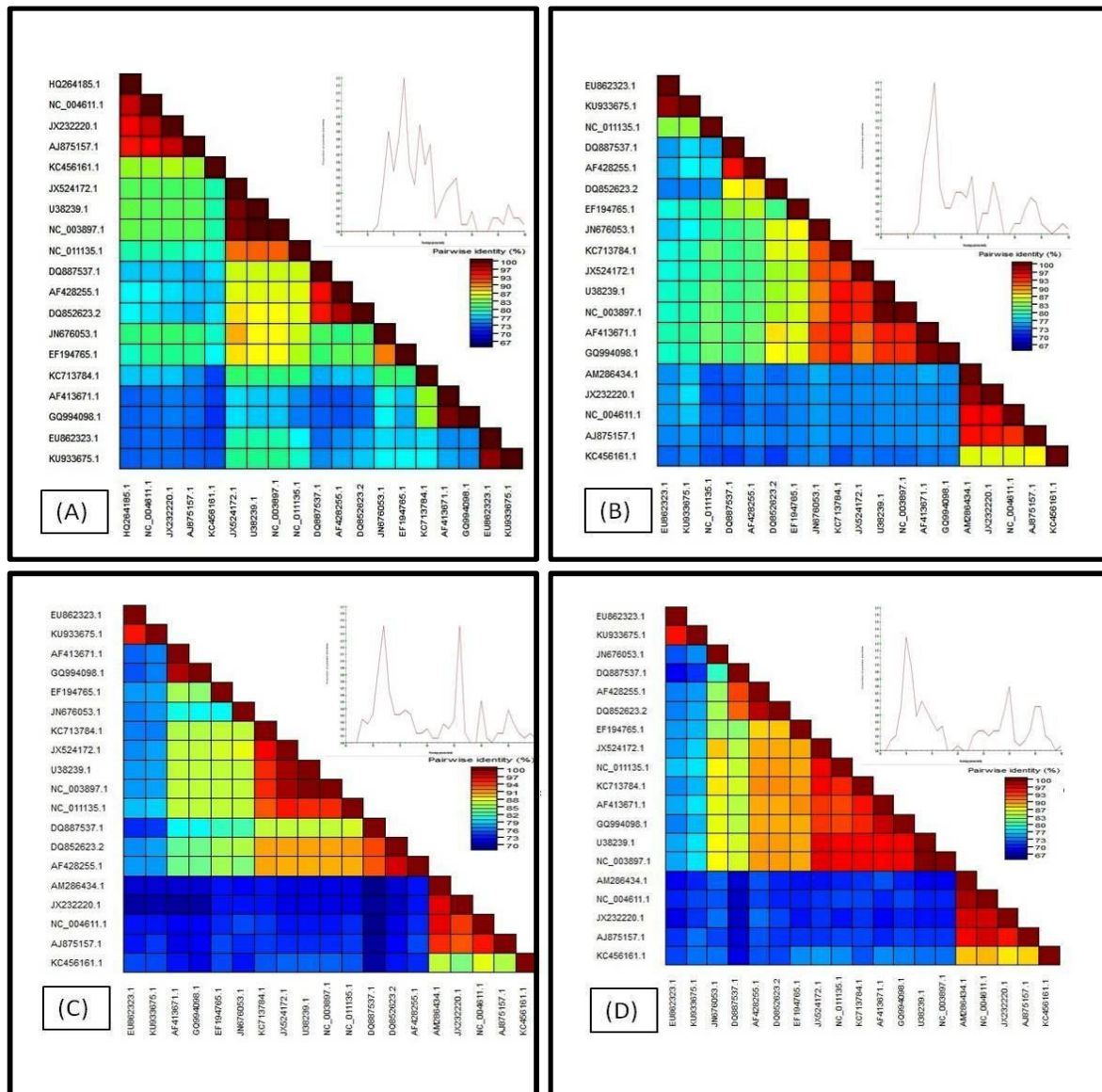


Fig 9: Sequence Demarcation Tool version 1.2 (SDT v1.2) generated heat maps. (A) *av1*, (B) *ac1*, (C) *ac2*, (D) *ac3* genes in Indian ToLCV strains. Presence of Blue colour indicates high level of dissimilarity among the sequences. Presence of red and green colour shows higher similarity index among the sequences.

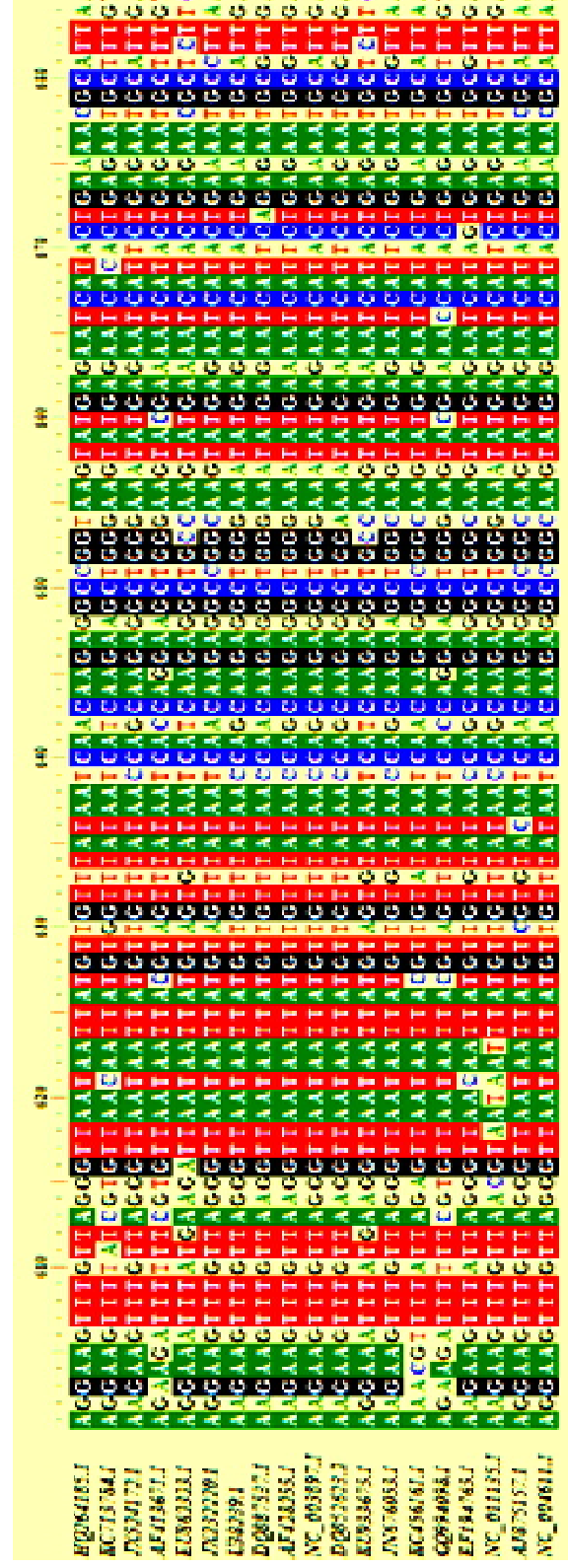


Fig 10: BioEdit generated graphic view of some most conserved regions towards 3' end of coat protein gene of all ToLCV Indian strains.

A.

Predicted bipartite NLS		
Pos.	Sequence	Score
1	MAKRPADIIISTPASKVRRR	8.7
47	RPMNRKPRMYRMYRSPDVPRGCEGPCKVQSFE	5.3
104	RVGKRFCVKS VYVLGKI WMDENIKTKNHTNS	5.6
173	RDRYQVLRKWHATVTGGTYASKEQALVRKFVRV	5

B.

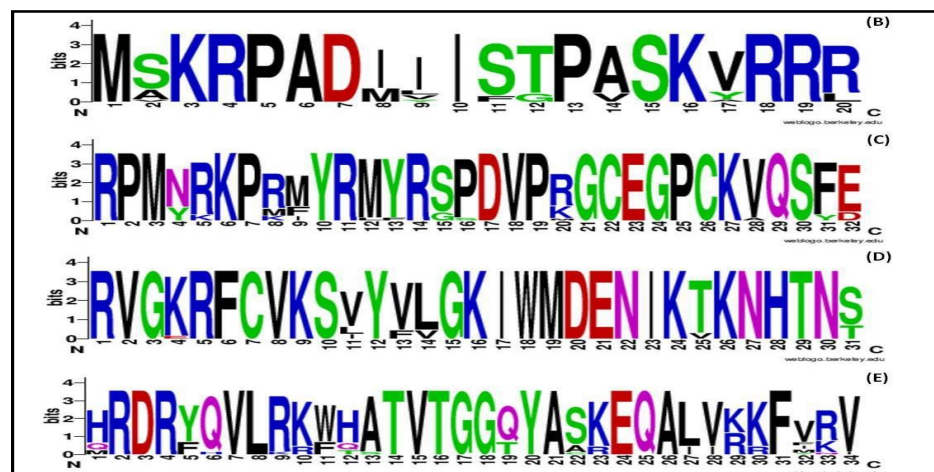


Fig 11: A. Predicted Nuclear localization signal (NLS) sequences in ToLCNDV above the cut off score 5.0 in cNLS Mapper tool.
 B. Presence of the conserved NLS sequences in Indian ToLCV strains represented by WebLogo software.

the different viruses of this group is of high importance in order to strategise RNAi based transgenics for broader impact in field condition. Our work provides important information about the stability of the genic regions in terms of their nucleotide sequences during evolution. We have able to elucidate the phylogenetic relationships of different *begomoviruses* reported during last two decades. We observed that the pattern of evolution of different genes in DNA-A component of *begomoviruses* differs among themselves as well as DNA. Notably AV1 coat protein gene has shown a near parallel evolution with DNA-A sequences. Also the similarity index of different genes in case of different *begomoviruses* as well as tomato leaf curl virus in India has shown highest pair wise identity among the viruses. In comparison AC1, AC3 and AC2 has shown diversified pattern of nucleotide sequences as evident from similarity index analysis suggesting these genes are more prone to mutations in comparison to AV1 gene.

3rd Objective: Development of a PCR based detection system for *begomoviruses* from solanaceous vegetables

North Bengal region is famous for *solanaceous* crop production mainly potato, tomato and Chillies. All these crops are prone to begomoviral infection. Survey of the vegetable growing agricultural fields in and around Coochbehar during winter 2015 we found the symptoms of begomoviral infection like curling and yellowing of leaves, stunted growth, crinkled leaves in potato, tomato and chilli which clearly indicates the occurrence of these viruses in this particular area. To identify the particular viral strain affecting this area it is important to molecularly characterize the virus. In order to do so we designed a set of primers from the available nucleotide database (NCBI) the sequences of the genes of different *begomoviruses* were downloaded from NCBI and by the use of BioEdit software the conserved genic regions were identified both from 5' and 3' end of the ORF (Table 4). Synthesized oligonucleotides were out sourced (Sigma Primer, 50 nanomoles) with proper dilution these primers were used for PCR amplification. To standardize the primers we have used a clone of DNA-A component of Tomato leaf curl Joydebpur virus under pBIN 19 binary vector (Fig 12). Plasmid was isolated from *E. coli* bacterial culture by standard method (Fig 13A). The plasmid was used as template in the PCR reaction to standardize the PCR programme for different primers. When the PCR mixtures were analyzed in 1% gel electrophoresis different bands of amplified gene products was seen to be appeared. In lane 3 band length was 1100 bp approx., in lane 4 band length was 300 bp approx., in lane 5 band length was around 2500 bp, in lane 6 band was around 1500 bp, in lane 7 the band length was around 1000 bp & in lane 8 it was around 250 bp (Fig 13B).

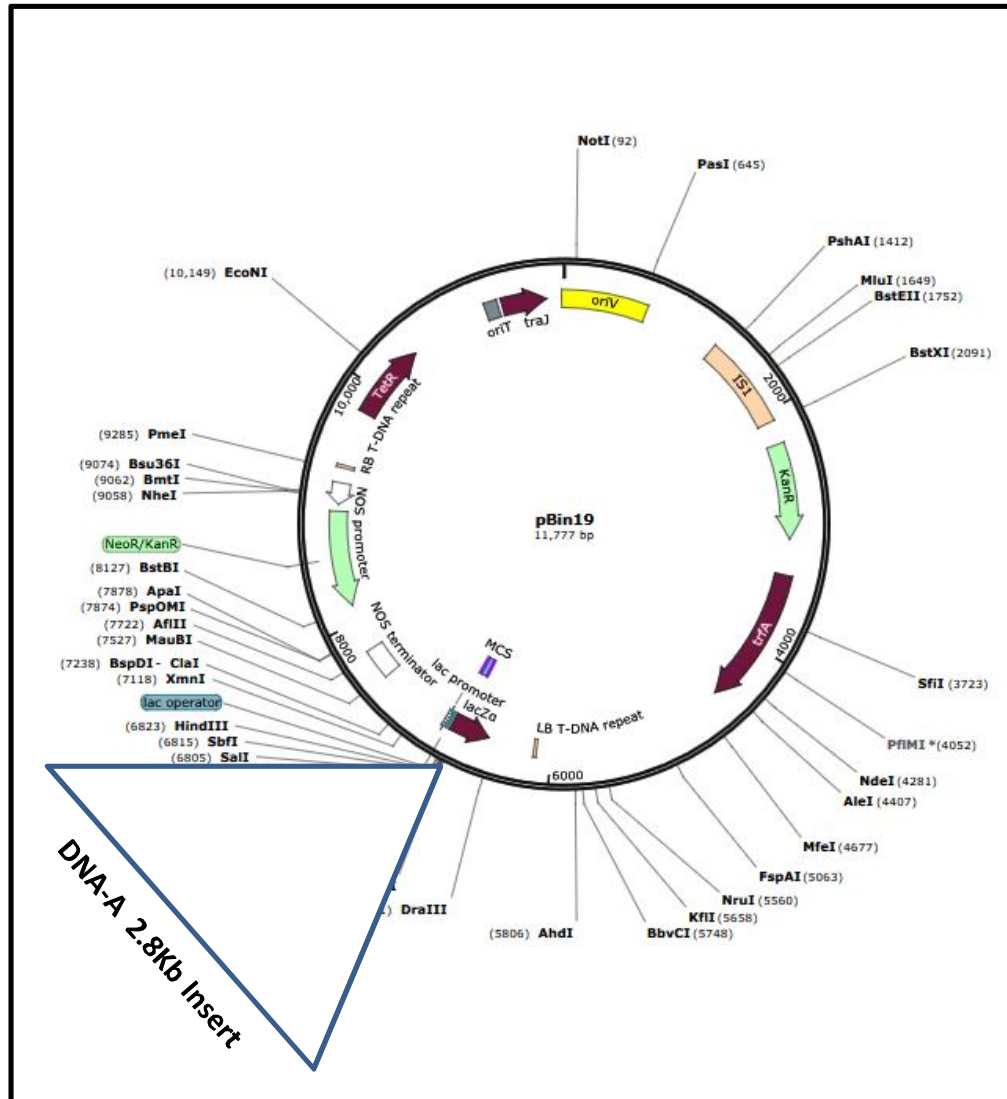


Fig 12: The infectious clone of TolCV Joydebpur virus containing DNA A genomic component in pBin 19 vector (The construct was kindly provided by Dr. Shelly Praveen, Head, Department of Biochemistry, IARI).

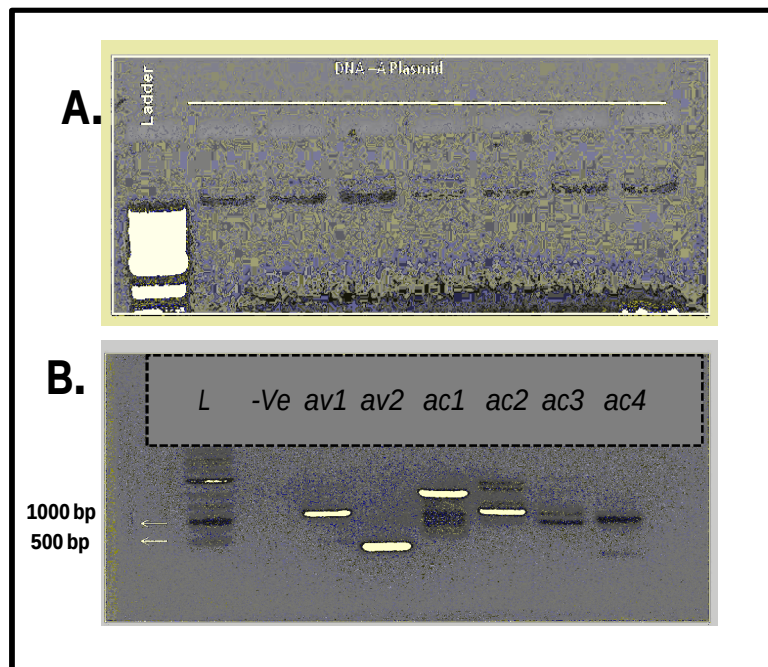


Fig 13: Standardization of the primers with ToLCJV DNA-A construct for amplification of the genes amplification.

- A. Isolated plasmid of pBIN 19-DNA-A.**
- B. PCR Amplification of Genes**

To screen the plant samples for the presence of the virus the above said primer set for AV1 and AV2 were used. Infected plant samples were collected from fields of UBKV and surrounding villages based on the appearance of characteristic begomoviral symptoms on the leaves. Plant DNA extraction was done with CTAB method from tomato and chilli young infected leaves. The extracted DNA was checked by agarose gel electrophoresis (Fig 14A & B). Six samples of these plant DNA was used as templates in the PCR reaction for AV1 and AV2 genes along with positive controls. Out of the six samples three samples showed the amplification of AV1 gene (Fig 14C) whereas AV2 amplification was found in 4 samples (Fig 14D). Our results confirm the presence of begomoviruses in the vegetable fields of UBKV and nearby villages. These primers can be also used for detection of *begomoviruses* in seeds as well as vegetative planting materials.

PCR mediated detection methods for *begomoviruses* is of prime importance. As many *solanaceous* vegetables are reproduced from grafting, cutting or vegetative propagation material. For this reason vegetable cultivation is always under risk of seed borne diseases. Thus it is very important to detect the presence of viral particles at early stage of the crop even before the appearance of the symptoms. Several reports are available regarding the use PCR mediated detection method of *begomoviruses* (Ghosh, 2009; Maruthi, 2007; Sivalingam, 2007; Khan, 2006). Amplification of coat protein gene (AV1) has been used in various experiments to confirm the presence of *begomoviruses* as well as the detection of particular viral strain profiling. According to ICTV the differences in the coat protein nucleotide sequence may differentiate two begomoviral strains (Mayo and Pringle, 1998). Our study is a preliminary attempt to identify and characterize the *begomoviruses* present in terai region of west Bengal but further cloning and sequencing of the viral gene is of prime importance for knowing the virus at molecular level.

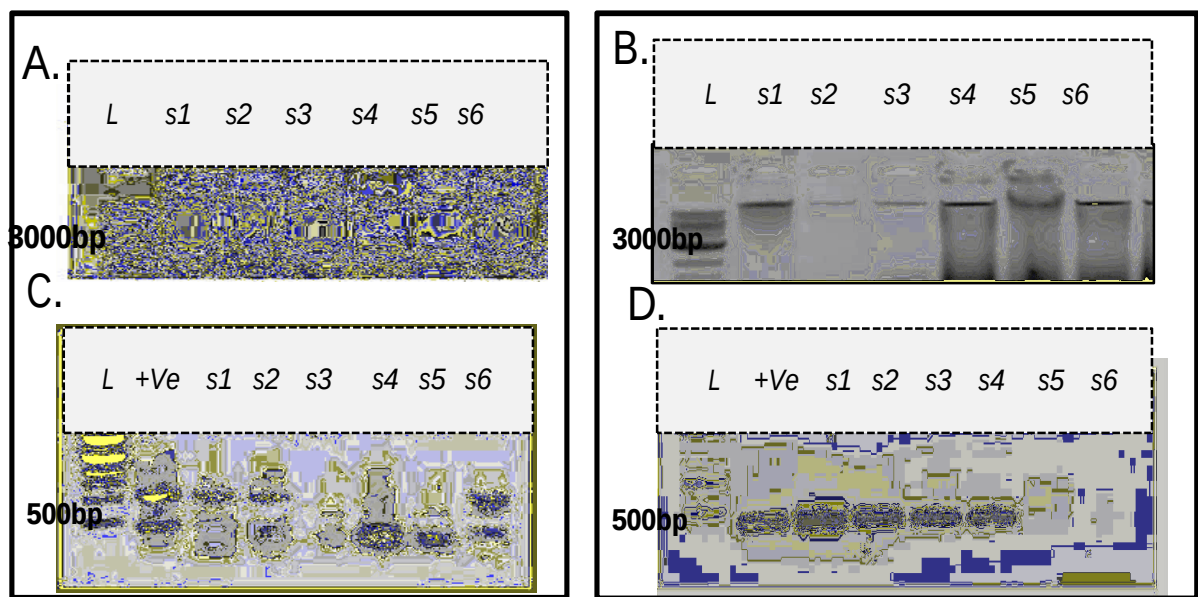


Fig 14: Genomic DNA of tomato & chilli samples collected from UBKV fields and nearby villages (A. & B.). PCR amplification of *Begomoviruses* with *av1* (C.) and *av2* (D.) gene specific primers.

Summary and Conclusion

The terai region of west Bengal is a niche of biodiversity along with different forest as well as medicinal plants this region is also renowned for cultivation of vegetables and fruits. Recently it has been observed in different areas of terai region that the vegetable cultivation is largely affected by different plant infecting viruses predominantly geminiviruses. Geminiviruses are single stranded DNA viruses which are evolving very rapidly and severely affecting a broad range of host plants including solanaceous vegetable like tomato. Among Geminiviruses begomoviruses are the largest group which has reportedly caused maximum damage to the vegetable crops. The present study was focused on confirmation and identification of begomoviral infection in tomato and other solanaceous crops. The results confirms the presence of these viral particles from the vegetable crops grown in the fields in and around UBKV by PCR mediated amplification of AV1 and AV2 genes from different symptomatic plant extracted DNA samples. After the confirmation of the infection the study was continued to see the plant defense response against the virus infection. Hypersensitive response was found from the infected sample which was shown in trypan blue staining of apoptotic cells of infected leaves. The stress responsive enzymes were also studied from these healthy and infected samples where Polyphenol Oxidase, Catalase and Peroxidase were found to be enhanced during the infection. Moreover to know the evolutionary pattern of begomoviruses in last few decades 31 nucleotide sequence of DNA-A component was downloaded from NCBI database. Phylogenetic analyses of these sequences through MEGA and SDTv softwares have shown a geographical confinement of the viruses during the evolution. We also detected occasional presence and absence of AC4 and AV2 gene in these

viruses but coat protein gene has been found to be the least diversified gene during evolution in our study. The possible reason can be the presence of conserved NLS sequences in coat protein AV1 gene which is evident from our study.

Future Scope of the Study

By the present study the presence of *begomoviruses* in the tomato and other *solanaceous* crops grown in the terai region of West Bengal is confirmed. But it is very important to identify particular strain of *begomovirus* present in this area. In order to do so cloning of the viral DNA fragment and sequencing is an absolute necessity. Further the plant defense responses have to be characterized in molecular detail. The response of specific plant defense pathways against this virus has to be addressed in detail. The role of viral proteins on these defence pathways of the plant needs to be addressed. Complete genome sequencing of these viruses will be very fruitful to identify and categorize the virus. It will also help to know the evolutionary pattern of the viruses. Screening of different genotypes of tomato and other *solanaceous* crops is important to identify resistant cultivars against these viruses that can be recommended for cultivation in this area.

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