STUDIES ON PURIFICATION OF βC1 PROTEIN OF GEMINIVIRUS SATELLITE AND GEMINIVIRUS PATHOGENESIS

PROJECT SUBMITTED TO

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CERTIFICATE

This is to certify that Ms. Ananya Ghosh, student of Masters in Biotechnology, Department of Life Sciences and Biotechnology, Jadavpur University, India has worked under my supervision and guidance as a project trainee for two months in the Molecular Virology Laboratory in the School of Life Sciences, Jawaharlal Nehru University, New Delhi from 2nd July 2018 to 31st August 2018. The project entitled "Studies on Purification of βC1 protein of Geminivirus satellite and Geminivirus pathogenesis" and the data presented by her is original. It is certified that this work being original has been submitted so far, in part or full, for any other degree or diploma of any other institute or university.

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Last but not the least, I would like to praise and thank God, the almighty, who has granted countless blessings, knowledge and opportunity.

ABBREVIATIONS USED

APS	Ammonium Persulphate
ATP	Adenosine triphosphate
βME	β-mercaptoethanol
DNA	Deoxyribonucleic acid
Dpi	Days post inoculation
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic acid
GST	Glutathione S-transferase
IPTG	Isopropyl Thio-β-D-Galactoside
Kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani
µg	Microgram
µl	Microlitre
mM	Milimolar
ml	Millilitre
nm	Nanometer
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenyl Methyl Sulfonyl Fluoride
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-TetraMethylEthyleneDiamine

CONTENT

ABSTRACT

Begomoviruses constitute the largest genus of the family *Geminiviridae* which causes huge loss of the crops worldwide. Monopartite begomoviruses are usually associated with betasatellite which highly increases disease development process and therefore possess a great threat for the economically important crops. βC1, the only protein encoded by the betasatellite plays multitasking roles and aid helper virus to promote infection by increasing symptom severity, by suppressing the transcriptional and post transcriptional gene silencing etc. In this study PatnaβC1 protein encoded by *Tomato leaf curl Patna betasatellite* and its ATPase deficient mutant has been purified by affinity chromatography to examine the structural changes of the protein after the mutation. Furthermore to study the begomovirus mediated pathogenesis, *Nicotiana benthamiana* plants infected with *Tomato leaf curl New Delhi virus* DNA-A (ToLCNDV-A) and its mutants along with Radish leaf curl betasatellite (RaLCB). The plants infected with ToLCND-A along with RaLCB shows severe symptoms like veinal chlorosis, leaf curling, stunted growth etc. However plants infected with ToLCNDV-AC2 and ToLCNDV-AC4 along with RaLCB did not shows any symptoms. RaLCB specific PCR analysis did not support the result observed for ToLCNDV-AC4 – RaLCB inoculated plants, however the PCR analysis of ToLCNDV-AC2 – RaLCB inoculated plants suggests the influence of RaLCB for symptom development.

1. INTRODUCTION:

Virus are an infectious agent of small size and composition that can multiply only in living cells of animals, plants, or bacteria. The name is from a Latin word meaning “slimy liquid” or “poison”.

A virus particle has three main parts:

• Nucleic acid – This is the core of the virus with the DNA or RNA. This holds all the information for the virus and that makes it unique and helps it multiply.

• Protein coat (capsid) – This is covering over the nucleic acid that protects it.

• Lipid membrane (envelope) – This covers the capsid. Many viruses do not have this envelope and are called naked viruses.

Like all other viruses, plant viruses are obligate intracellular parasites that do not have the molecular machinery to replicate without a host. Tobacco Mosaic Virus (TMV) was the first virus to be described by Dutch microbiologist Martinus Beijerinck and Russian researcher Dmitrii Iwanowski who were investigating the cause of a mysterious disease of tobacco back to the late 19th century (Gergerich and Dolja.2006).

The main reason of studying plant viruses is the negative impact that viral diseases have on the crop production. It is responsible for huge losses in crop production and quality all over the world. Infected plants show a wide range of symptoms depending on the disease but often virus infection causes leaf yellowing (either of the whole leaf or in a pattern of stripes or blotches), leaf distortion (e.g. curling) and/or other growth distortions (e.g. stunting of the whole plant, abnormalities in flower or fruit formation).

1.1. GEMINIVIRUS

Geminiviruses are a large group of plant viruses responsible for causing crop losses worldwide especially in tropical and sub tropical regions. The family *Geminiviridae* have circular, single stranded genomes encapsidated in twinned icosahedral (geminate) virions (Kumar et al., 2016). The characteristic twinned or “Geminate” particles, which consists of

two joined, incomplete T = 1 icosahedra, are unique among viruses (Zhang et al., 2001). The genome size of Geminiviruses is about 2500 – 5200 bases. (Zerbini et al., 2017). Geminiviruses replicate their genomes in the nuclei of infected (usually phloem tissue) cells via the rolling-circle (RCR) mechanism which is initiated by virus-encoded replication initiation protein (Rep) that ranges in size from approximately 320 to 400 amino acid residues. (Ravantti et al., 2009). The 5-7 protein encoded by single-stranded DNA genomes of Geminiviruses reprogram the plant cell cycle and transcriptional regulation, inhibiting cell death pathways, hamper with cell signaling and protein turnover, and downregulate defence pathways (Hanley-Bowdoin et al., 2013). This family comprises of nine genera as *Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus,* and *Turncurtovirus* on the basis of differences in their organization of genome, their genome-wide pairwise sequence identities, their insect vector and host range (Carlos Medina et al., 2018). Members of the genus *Begomovirus* are transmitted by whiteflies, members those belong to the genera *Becurtovirus, Curtovirus, Grablovirus, Mastrevirus* and *Turncurtovirus* are transmitted by specific leafhoppers, one member of the genus Capulavirus is transmitted by an aphid and the single member of the genus Topocuvirus is transmitted by a treehopper (Zerbini et al., 2017). The *Geminiviridae* family infects a wide range of dicotyledonous and monocotyledonous plants and is responsible for important economic losses in tropical and subtropical regions worldwide (Silva et al., 2017).

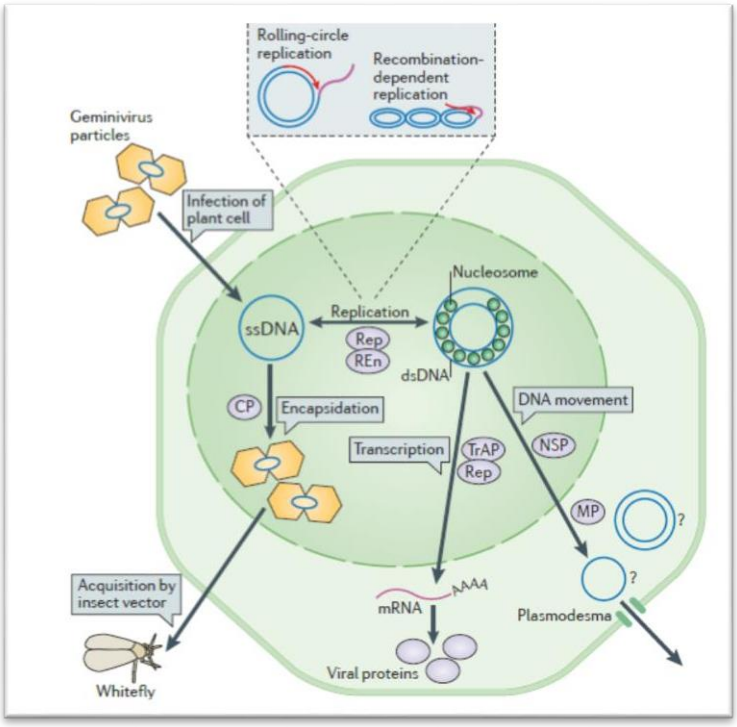


Figure 1: Single cell replication of Begomovirus genome (Hanley-Bowdoin et al., 2013)

1.2. BEGOMOVIRUS

The genus Begomovirus consists of more than 200 species or members and is the largest Geminivirus genus (Zhou, 2013). The vector is the white fly (*Bemisia tabaci*) that causes begomoviral infections in ornamentals, crops, and weeds and it is prevalent in the tropical and subtropical regions of the world (Marwal et al., 2013). Begomoviruses consists circular single strand DNA, they have either monopartite (DNA-A) or bipartite (DNA-A and DNA-B) genome. Most of the described begomoviruses are bipartite in nature, each being approximately 2.6-2.8 kb in size, which are responsible for different functions in the infection process and their life cycle (Hanley-Bowdoin L et al., 2000). The genomic DNA of monopartite begomoviruses and the DNA-A component of bipartite begomoviruses are homologous and have a similar genome organization that includes the V1/AV1, C1/AC1, C2/AC2, C3/AC3, and C4/AC4 genes, which encode the capsid protein (CP), replication-associated protein (Rep), transactivator protein (TrAP), replication enhancer protein (REn), and C4/AC4 proteins, respectively (Harrison et al.,1999 and Gutierrez, 1999). The genomic DNA of monopartite begomoviruses is larger than the DNA-A components of bipartite

begomoviruses and it has an additional virion-sense gene (V2), which encodes the precoat protein (Melgarejo et al., 2013). DNA-B component of bipartite begomoviruses encodes for two proteins, movement protein (MP) and nuclear shuttle protein (NSP) involved in cell to cell movement within the plant, host range and symptom modulation.(Hanley-Bowdoin et al., 2000). In the genome, the non coding region called intergenic region approximately 500 bp contains the origin of replication, where viral rep protein binds to initiate replication. Within the intergenic region, the region called conserved region present which is conserved in both DNA components of bipartite begomovirus of approximately 200-250 nucleotides (Zhou, 2013). Both DNA-A and DNA-B are required for infectivity. Tomato yellow leaf curl disease is one of the major viral diseases of tomato worldwide and bhindi yellow vein mosaic disease affects a lot in India and Within the last decade or so, a large number of begomoviruses have been investigated in India encompassing fibre crops, legumes, root crops and vegetables.(Borah and Dasgupta, 2012).

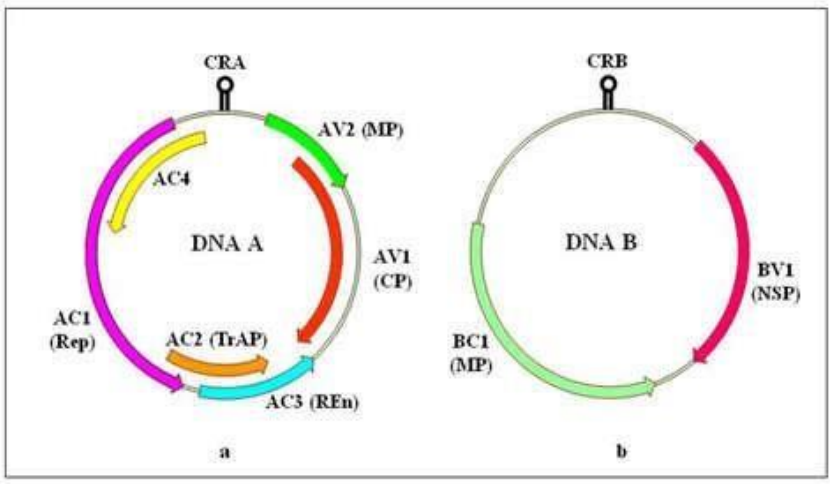


Figure 2: Genomic organization of begomovirus showing various ORFs (genes) in virion sense and complementary sense: DNA-A (a) and DNA-B (b) (Snehi et al., 2017)

1.3. SATELLITE

Satellites are defined as viruses which have a life cycle dependent on a helper virus, but lack extensive nucleotide sequence homology to the helper virus and are dispensable for helper virus proliferation (Belén Frígols et al., 2015). Generally numerous satellite RNAs are usually associated with RNA plant viruses (Simon et al., 2004). However, the first DNA

satellite 682nt in size was identified in association with *Tomato leaf curl virus*, a monopartite begomovirus, lacking an open reading frame (ORF) or an apparent promoter but contains binding motifs for the TLCV replication-associated protein, and was not necessary for replication of helper virus but depends on helper virus for its own replication and encapsidation (Dongmei et al., 2007). Monopartite begomoviruses are usually associated with satellite DNAs, known as alphasatellites and betasatellites (Karyna Rosario et al., 2016). The alphasatellites are not strictly considered as satellites, since they are capable of autonomous-replication in plant cells, but are dependent on their helper begomoviruses for movement within plants and insect transmission between plants (Gloria Lozano et al., 2016). The betasatellites are circular single stranded DNAs which are approximately 1,350 nucleotides in size and depends on helper begomovirus for its replication and help in inducing typical disease symptoms (Zhou, 2013). Another class of DNA satellite has been found in association with begomovirus that are approximately one quarter size of a begomovirus genome and are called deltasatellite (Gloria Lozano et al., 2016)

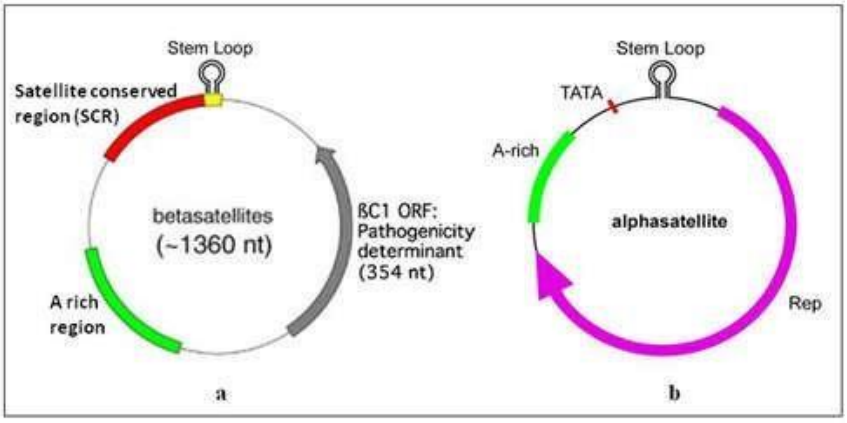
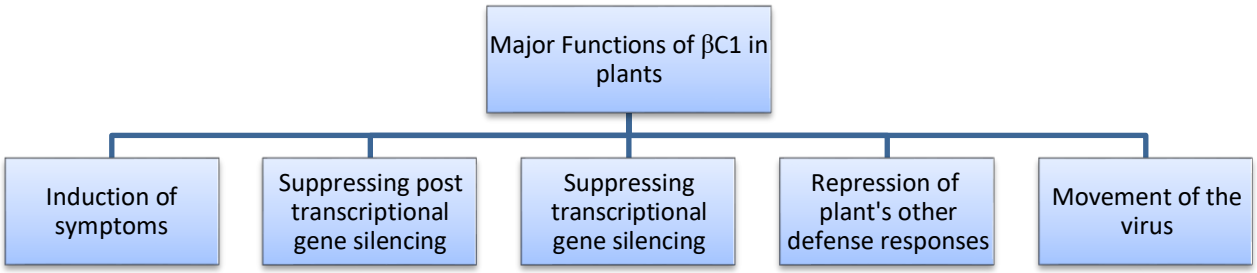


Figure 3: Genomic structure of betaasatellite DNA component (a) and alphasatellite DNA component (b) (Snehi et al., 2017)

1.4. BETASATELLITE

Betasatellites have a highly conserved structure gene encoded in the complementary-sense known as βC1 which translate into protein of around 13.5 kDa. The satellite region also contains a hairpin-loop structure TAA/GTATTAC similar to the helper begomovirus (Sahu et al., 2014). In addition to the ORF, genome of the betasatellites have SCR i.e. satellite conserved region

which is nearly 120 nucleotides long, highly conserved region among all betasatellites and A rich region typically 160-280 nucleotide long with 57%-65% Adenine content. The A rich region plays a role in maintaining the size of the betasatellites and also may have role in regulation of the promoter activity of βC1 ORF (Zhou , 2013). Betasatellites can be trans replicated by the different non cognate helper virus which increases the occurrence of different begomovirus betasatellite complexes resulting into the expansion of the specific host requirement to non specific hosts. Betasatellites have evolved in such a way that it can play multiple roles, assisting virus to establish a successful infection.



- **Induction of symptoms:**

  It is found in studies that βC1 forms large multimeric complexes which are important for the induction of symptoms (Cui et al., 2005; Saunders et al., 2004; Dry et al., 1997). When βC1 is expressed in the plant the leaves showing vein clearance and the genes are downregulated which are involved in chlorophyll biosynthesis, chloroplast development and translocation of plastid (Bhattacharyya et al., 2015).

- **Suppressing post transcriptional gene silencing:**

  Nuclear localization and central portion of βC1 protein is necessary for the suppression of silencing (Yang et al., 2011). Calmodulin-related protein found to be essential for suppression of PTGS by βC1. RNA-DEPENDENT RNA POLYMERASE 6 is repressed by βC1 controlling the upregulation of this calmodulin related protein (Li et al., 2005).

- **Suppressing transcriptional gene silencing:**

  DNA modification like methylation regulates gene expression and invading DNA viruses, The dsDNA intermediates formed during replication of Geminiviruses become associated with histones forming minichromosomes which are the main target of

methylation causing suppression by plant system as a defense strategy (Raja et al., 2010). βC1 is crucial for the suppression of transcriptional gene silencing of viral gene.When βC1 was expressed with *Tomato yellow leaf curl China virus* (TYLCCNV) it showed low levels of methylation of both host genome and helper virus which further leads to finding that βC1 interacts with S-adenosyl homocysteine hydrolase inhibiting its activity. It is an enzyme required in methyl cycle for the Transcriptional gene silencing (Yang et al., 2011).

- **Repression of plant's other defense responses:**

  Plant defense response against biotic stress includes two signaling cascade pathway i.e. Jasmonic acid pathway and Salicylic acid. Repression of Jasmonic acid response pathway occurs due to the presence of βC1 protein however, it doesn't affect salicylic acid pathway (Zhang et al., 2012). Also, when the βC1 protein of Chilli leaf curl betasatellite was infiltrated in *Nicotiana benthamiana* under 35S promoter through differential display analysis, eight genes were isolated and cloned and sequence analysis of differentially amplified products showed that these genes were involved in ATP synthesis, and used in electron transport chain for photosynthesis and respiration processes suggesting that βC1 regulates genes differentially which are mostly related to chloroplast and mitochondrial functions , these genes play role in cell protection, defense processes, plant growth and development, detoxification process and replication mechanisms (Andleeb et al., 2010).

- **Movement of virus:**

  In case of bipartite begomoviruses, DNA-B encodes movement protein which allows viruses to pass through plasmodesmata and nuclear shuttle protein for viral particles getting out of the nucleus, responsible for systemic spread of the virus in host (Noueiry et al.,1994). However in case of monopartite begomoviruses, βC1 complements the function of DNA-B to produce infection (Patil et al., 2010). The βC1 protein gets accumulated inside the nucleus and cell's periphery, thus involved in intracellular transport of viral particles from nucleus to periphery (Saeed et al., 2007).

2. OBJECTIVES:

Since betasatellites play a major role in pathogenesis of begomovirus in host plants, we have attempted to elucidate the biochemical role various betasatellite-encoded βC1 protein. In the lab, it has been observed that the βC1 protein of *Tomato leaf curl Patna betasatellite* displays ATPase activity in vitro. Also by studying bioinformatic analysis, mutants of this protein were created which lacks ATPase activity. These mutation are at 49^{th} , 69^{th} and 91^{st} residue in the protein. Also betsatellites have important role in the pathogenesis of begomoviruses. Another attempt was taken to study the association of *Tomato leaf curl New Delhi virus* and its mutants with *Radish leaf curl betasatellite* to know which protein of the virus is regulating somehow by betasatellite to increase the pathogencity.

To investigate the structural changes in the protein after mutation, the following objectives were set :-

1. To purify βC1 of *Tomato leaf curl Patna betasatellite* and PatnaβC1Δ69 mutant by affinity chromatography.
2. Role of with *Radish leaf curl betasatellite* in regulating *Tomato leaf curl New Delhi virus.*

3. MATERIALS AND METHODS:

3.1. Sources of viral DNA:

Tomato leaf curl Patna betasatellite (Ac.no.EU862324) βC1 ORF and its mutant ORF cloned in pGEX-6p2 expression vector were available in our laboratory. (Molecular Virology Laboratory, School of Life Sciences, Jawaharlal Nehru University, and New Delhi.). Similarly, infectious tandem repeats of *Tomato leaf curl New Delhi virus* (NA); accession no, U15015 and its mutants (NAΔAC2, NAΔAC4, NAΔAV2, NAΔAC2AV2, NAΔAC2AC4 and also *Radish leaf curl betasatellite* (rlβ) accession No. EF175734 in pCAMBIA2300 vector for the agroinfection were available in the laboratory.

3.2. Source plant:

Nicotiana benthamiana plants were grown in glass house in maintained conditions (25^{o}C , 70% humidity and 16hr of light plus 8hr of dark).

3.3. Overexpression analysis of GST-βC1 protein:

Arctic express harboring pGEX6p2-βC1 construct was grown on LB media (10ml) containing Ampicillin(100µg/ml) at 30^{o}C, 200rpm till the growth reaches to 0.5 OD at 600nm and then the culture. After the growth, 1ml culture was pellet down and stored directly for loading and remaining culture was induced with 1mM IPTG and grown for 24h at 12ºC followed by again storing of 1ml culture for loading. The culture was pelletized and sonicated. After the sonication, the sample was centrifuged. The supernatant and pellet was used for analysis of protein on SDS-page.

3.4. GST-βC1 fusion protein purification by affinity chromatography:

Arctic express harboring pGEX6p2-βC1 construct was grown on LB media containing Ampicillin (100µg/ml) at 30^{o}C, 200rpm till the growth reaches to 0.5 OD at 600nm and then the culture was induced with 1mM IPTG. After induction, cells were grown at 12^{0}C for 24 hours at 200rpm. The cells were harvested at 5000rpm for 5 minutes. The pellet was resuspended in the 50ml of Lysis Buffer (25mM Tris,100mM NaCl, 5% glycerol, 5mM $MgCl_2$, 0.05% Tween 20, 0.05% TritonX100, 1mM PMSF, 5mM βME, 1mM EDTA) and 100µl (stock 40mg/ml) of

lysozyme added and kept in 4^0C for 2-3hr. After that sonicated by 15 cycle with 20seconds ON and 40Second OFF. The supernatant was filtered was allowed to bind to Glutathione beads for three times. After this, the column was washed with 100ml of wash buffer (25mM Tris, 500mM NaCl, 5% glycerol, 5mM $MgCl_2$, 0.05% Tween 20, 0.05% TritonX100, 1mM PMSF, 5mM βME, 1mM EDTA). Then 10ml of equilibrate buffer(25mM Tris,100mM NaCl, 5% glycerol, 5mM $MgCl_2$, 0.05% Tween 20, 0.05% TritonX100, 1mM PMSF, 5mM βME, 1mM EDTA) Finally, GST fusion protein was eluted with freshly prepared elution buffer (25mM Tris,100mM NaCl, 5% glycerol, 5mM $MgCl_2$, 0.05% Tween 20, 0.05% TritonX100, 1mM PMSF, 5mM βME, 1mM EDTA, 50mM Reduced Glutathione).

3.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of GST-βC1:

Proteins are separated by electrophoresis where matrix for separation is polyacrylamide. The polymerization of polyacrylamide creates a cross-linked network of tiny pores which allows small protein molecules to pass through rapidly and slowing the migration of larger proteins. This results in the separation of proteins based on their molecular size. Sodium Dodecyl Sulfate, an anionic detergent, denatures the proteins, unfolding each polypeptide chain into a linear polypeptide and applies an evenly distributed negative charge to each protein in accordance with its mass so that protein thus can be separated on the basis of their size only.

The chemical ingredients used for resolving and stacking gel preparation.

COMPONENTS	**STACKING GEL (12%)**	**RESOLVING GEL (12%)**
30% Acrylamide-bisacrylamide mix	1.34ml	4 ml
Distilled Water	5.4ml	3.3ml
1M Tris	1ml (pH 6.6)	2.5ml (pH 8.6)
10% APS	80µl	100µl
10% SDS	80µl	100µl
TEMED	10µl	10µl

The protein samples were prepared by mixing with that volume of 6X loading dye buffer (200mM Tris-Cl, pH 6.8, 400mM DTT, 10% SDS, 0.4% bromophenol blue, 40% glycerol) such that its final concentration is 1X. The mixture was heated at 100ºC for 15 minutes to denature the protein. Samples were loaded in slots and run initially at 100 volt/cm till dye front moved up to the spacer gel, and later at 120 volt till the dye reached at the bottom of the gel. The gel was removed from plates and transferred to staining solution (0.25% w/v Coomassie brilliant blue R - 250, 45% methanol, 10% glacial acetic acid). The gel was incubated for 1hour at room temperature with gentle shaking. The stained gel was transferred from the staining solution to the excess volume of destaining solution (25% methanol and 10% glacial acetic acid) and kept at room temperature on shaker till it gets properly destained.

3.5. *Agrobacterium* mediated plant inoculation:

Agrobacterium cells harbouring pCAMBIA2300-NA, NAΔAC2, NAΔAC4, NAΔAV2, NAΔAC2AV2, NAΔAC2AC4 and Rlβ constructs were grown at 28°C and the cells were centrifuged down at 5000rpm for 5 minutes at room temperature. The pellet was dissolved in sterile distilled water. Five leaf stage *Nicotiana benthamiana* growing in the glass house conditions were wounded by pricking and the culture was put on the wound. The plants were given water daily for the maintenance.

3.6. Genomic DNA isolation of infected plants:

Grind the leaf sample with liquid nitrogen

↓

Add 750 µl of extraction buffer (5M NaCl, 0.5M EDTA P^H 8.0, 1M Tris P^H 8.0, βME, SDW)

↓

Add 100 µl of 10% SDS and keep at 65^0C for 20-30 min

↓

Cool it to room temperature and add 250 µl of solution (potassium acetate 0.5M)

↓

Keep on ice for 10 minutes and spin at 13,000rpm at 4^0C for 10 minutes

↓

Remove the supernatant and add equal volumn of CI (Chloroform: Isoamylalcohol=24:1)

↓

Spin at room temperature for 10 minutes, remove the supernatant and add 0.8 volumn of isopropanol

↓

Incubate in 20^0C for 30 minutes and spin at 13,000rpm, 4^0C for 20-25 minutes

↓

Discard the supernatant carefully, add 500 µl of 70% ethanol, spin at 13,000rpm, 4^0C for 10 minutes

↓

Dry at room temperature and add 100-150 µl of SDW

3.7. Polymerase Chain reaction:

Polymerase Chain Reaction (PCR) is method developed by Kary Mullis in the 1980s. It is used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. PCR is based on utilizing the ability of DNA polymerase to synthesize new strands of DNA complementary to templates provided.

PCR Reaction Components:

INGREDIENTS	REACTION VOLUME
10X Reaction buffer	2.5µl
2.5mM dNTP	1µl
100µM Forward Primer	0.2µl
100µM Reverse Primer	0.2µl
Phusion	0.2 µl
100ngTemplate	1µl
Sterile distilled Water	19.9 µl
Total	25µl

PCR program used for amplification :

Step	Temperature(^{o} C)	Time	No. of Cycles
Initial Denaturation	94	4 min	1
Denaturation	94	1 min	
Annealing	61	45sec	28
Extension	72	45 sec	
Final Extension	72	5 min	1

3.8. Agarose Gel Electrophoresis:

It separates DNA fragments by size in a solid support medium (an agarose gel). DNA samples are pipetted into the sample wells. Application of an electric current at the top (anodal, negative) end causes the negatively-charged DNA to migrate towards the bottom (cathodal, positive) end. The rate of migration is proportional to size: smaller fragments move more quickly, and wind up at the bottom of the gel. DNA is visualized by including in the gel an intercalating dye, ethidium bromide. DNA fragments take up the dye as they migrate through the gel. Illumination with ultraviolet light causes the intercalated dye to fluoresce.1% gel is made using 1X TAE buffer. After melting the agarose, cool it down to nearly bearable temperature and Ethidium Bromide. After mixing pour into the Horizontal electrophoresis apparatus and let it cool down. The amplified product was loaded into the wells and run the assembly.

4. RESULTS:

4.1. Overexpression analysis:

The overexpression analysis of two pGEX-6p2-PatnaβC1 clones was carried out by inducing small scale of bacterial culture by 1mM IPTG and grown for 24h at 12^{O} C. The cells were centrifuged at 5000rpm for 5minutes at 4^{O} C. The pellet was dissolved in lysis buffer and sonicated. The sonicated sample was centrifuged at 10,000 rpm for 20 minutes and supernantant was stored for loading. The pellet was also dissolved in lysis buffer and loaded on the sds-page for the analysis.

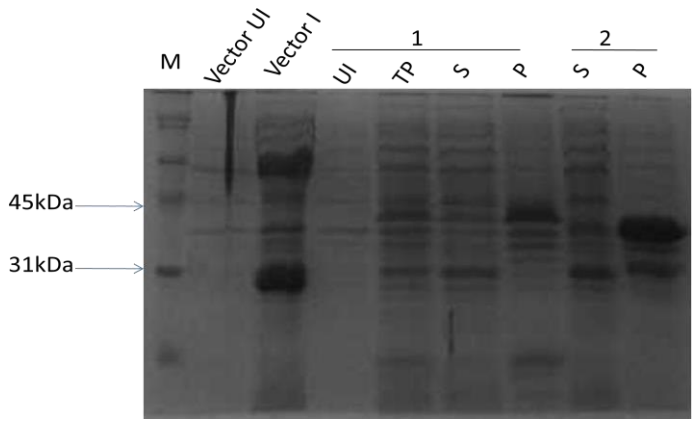


Figure 4: Overexpression analysis of GST-βC1 protein by IPTG. M represents the marker. Vector UI- vector uninduced; Vector I- vector induced; TP-total protein; S-supernatant; P-pellet. Clone no. 2 was taken for the purification.

4.2. Purification of GST-PatnaβC1 and GST-PatnaβC1Δ69 mutant:

The Arctic express DE3 strain harboring GST-PatnaβC1 and GST-PatnaβC1Δ69 expression construct was induced to express the fusion protein. The GST-PatnaβC1 and GST-PatnaβC1Δ69 protein of approx. 40KDa was purified by affinity chromatography using Glutathione sepharose bead.

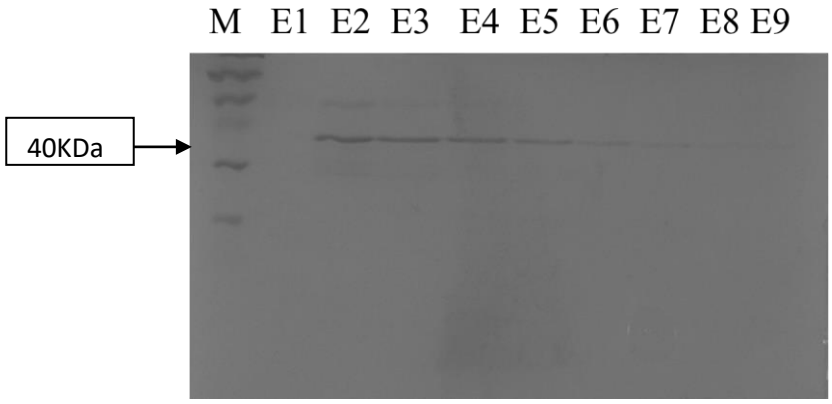


Figure 5: Purification of GST tagged PatnaβC1 clone by Affinity chromatography using Glutathione sepharose beads.

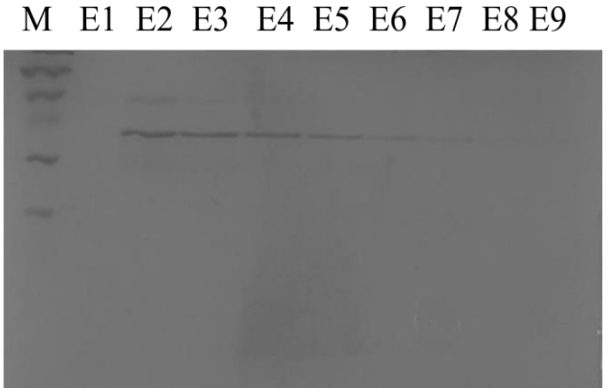


Figure 6: Purification of GST tagged PatnaβC1Δ69 clone by Affinity chromatography using Glutathione sepharose beads.

4.3 Agroinoculation:

Agrobacterium strain EHA105 carrying infectious partial tandem repeat of ToLCNDV-A, ToLCNDV-AC2, ToLCNDV-AC4, ToLCVND-AV2, ToLCNDV-AC2AC4, ToLCNDV-AC2AV2, RaLCB were inoculated in the *Nicotiana benthamiana* plants and symptom severity has been observed. Plants inoculated with *Agrobacterium* harbouring pcambia 2300 did not show any symptom. Plants inoculated with ToLCNDV DNA-A showed very mild symptoms. However, the plants infected with ToLCNDV DNA-A and RaLCB showed leaf curling, vein clearing and stunted growth at 14dpi and the severity increased later. The mutants ToLCNDV-AC2 and ToLCNDV-AC4 did not showed any symptom.ToLCNDV-AV2 with RaLCB showed

slightly less symptom compared to wild type virus. In ToLCNDV-AV2, there was mild vein clearing.

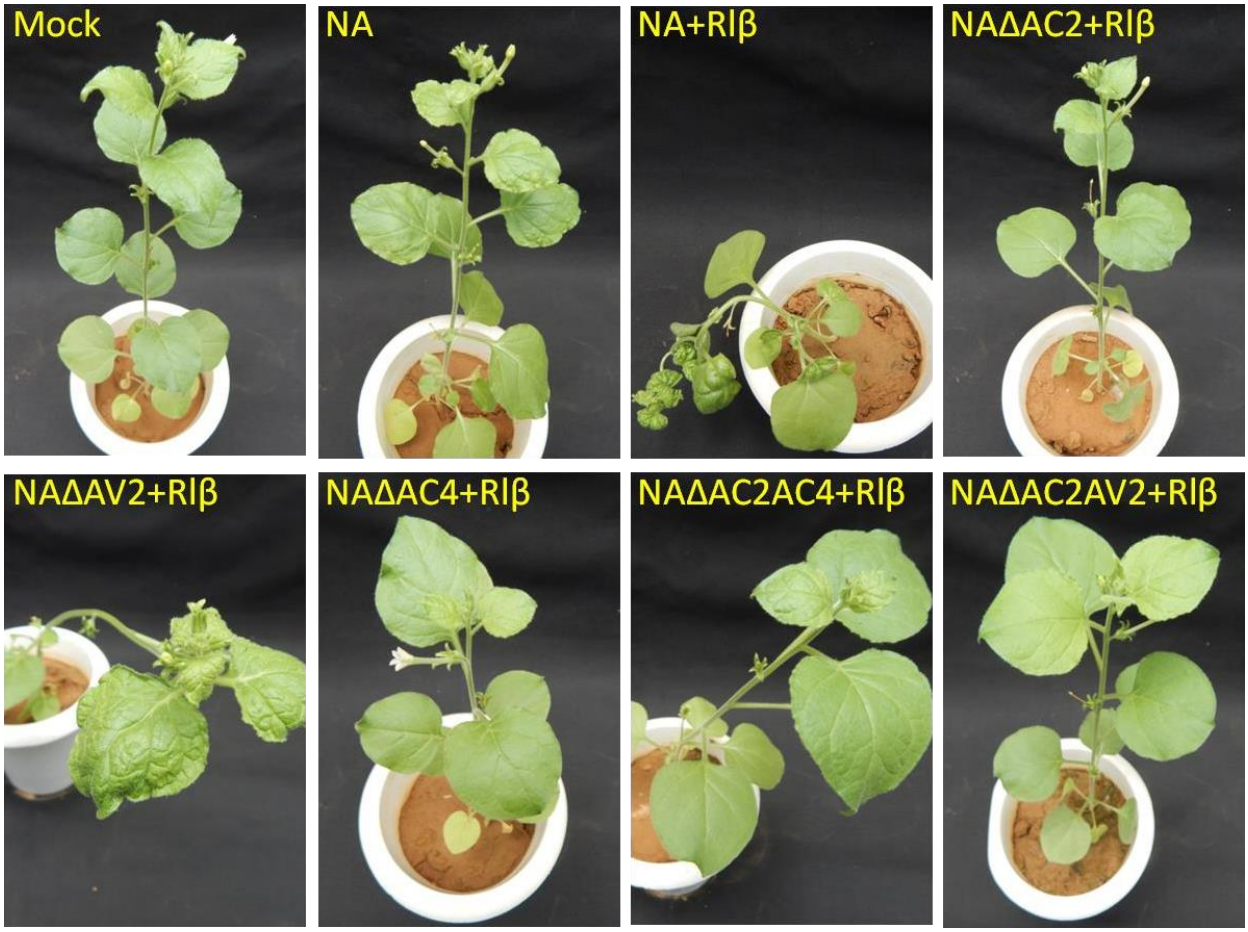


Figure 7: *Agrobacterium* strain EHA105 carrying infectious partial tandem repeat of ToLCNDV-A, ToLCNDV-AC2, ToLCNDV-AC4, ToLCVND-AV2, ToLCNDV-AC2AC4, ToLCNDV-AC2AV2 along with RaLCB were inoculated in the *Nicotiana benthamiana* plants. The photographs were taken at 28dpi.

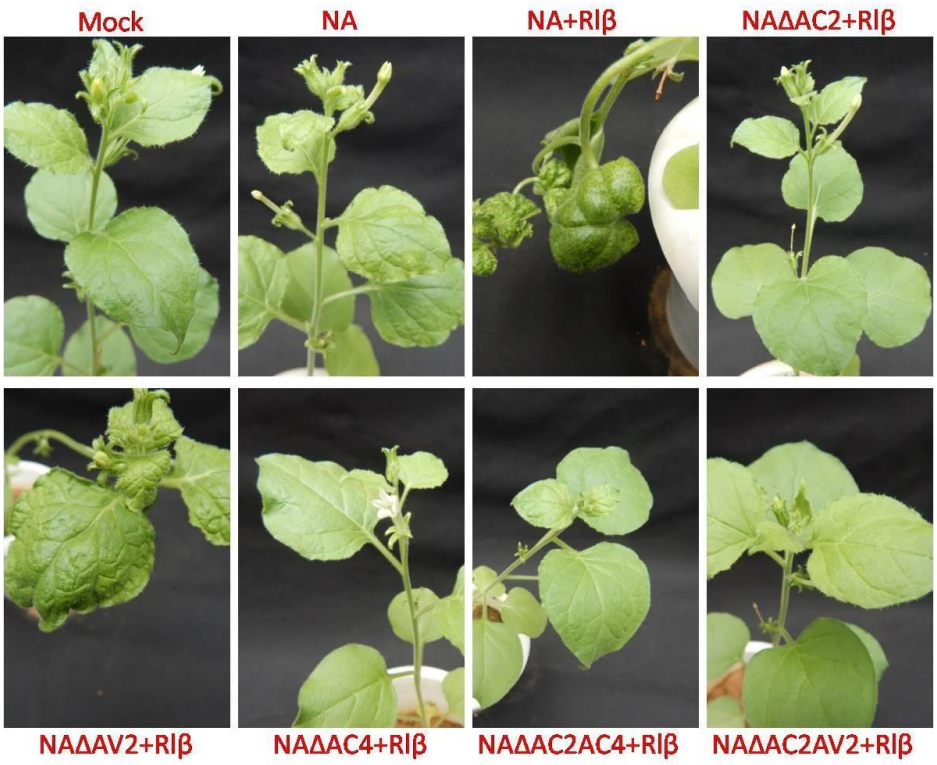


Figure 8: Enlarged view of the infected plants.

4.4. Amplification of rlβC1 ORF by Polymerase Chain reaction

PCR detection of betasatellite was carried out in agroinoculated samples collected at 21dpi. The positive was taken rlβC1 cloned in pJET1.2 vector. Samples infected with *Tomato leaf curl New Delhi virus* along with *Radish leaf curl betasatellite* showed presence of betasatellite by amplification. Also AC2 and AV2 mutant of Tomato leaf curl New Delhi virus showed PCR amplification.

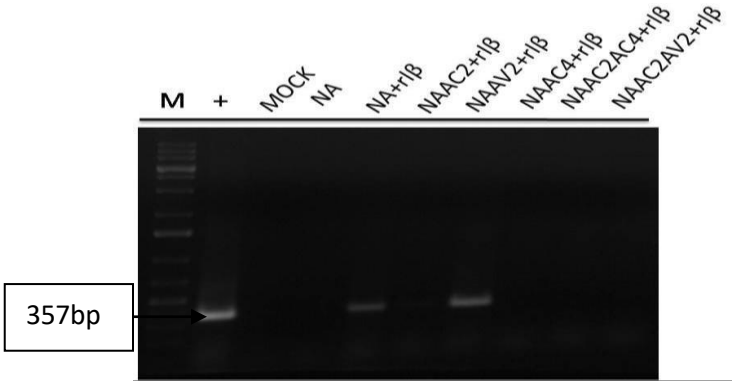


Figure 9: PCR amplification of rlβC1 ORF using rlβC1 specific primers.

5. INTERPRETATION AND CONCLUSIONS:

a) In SDS-PAGE it is seen that the size of GST-PatnaβC1 and GST-PatnaβC1Δ69 proteins is around 40kDa and in both cases the elute 4&5 showing no contamination protein band so we can proceed further experiments for the proteins with that elutes considering it as pure. As it is already known that PatnaβC1Δ69 lacks the ATPase activity further experiments will done to check whether this loss of activity due to some structural changes or not.

b) It is observed that the plants inoculated with wild type ToLCNDV DNA-A and RaLCB showed severe symptoms, ToLCNDV-AV2 with RaLCB showed slightly less symptom compared to wild type and ToLCNDV-AC2 and ToLCNDV-AC4 with RaLCB did not show any symptom. From this result we can interpret that AC2 and AC4 may be interacting with the βC1 protein and thereby showing severity in the symptoms, whereas AV2 might not be interacting directly with the RaLCB for the symptom severity.

Later the genomic DNA was isolated from 28dpi samples and RaLCB specific PCR was employed. It was observed that the sample ToLCNDV-AC4 with RaLCB did not show any amplification for RaLCB. From the result we got in plants it cannot be concluded whether AC4 and RaLCB interacting or not as the PCR result is an indication of absence of RaLCB in the sample. As ToLCNDV-AV2 with RaLCB and ToLCNDV-AC2 with RaLCB showed RaLCB amplification it can be concluded that AC2 might be influencing directly RaLCB for symptom development there by due to mutation in AC2 the plants did not exhibit any symptoms. AV2 protein might not be interacting with RaLCB thereby AV2 plants showed mild symptom.

6. FUTURE WORK:

Ms. Neha Gupta, under whose direct guidance I have carried out this work will be doing following experiments in future to complete this objective.

a) In order to examine the structural changes of proteins which might be one of reason for the loss of ATPase activity one can done Circular dichroism (CD) spectroscopy. CD spectroscopy provides information about the secondary structure of proteins. For CD spectroscopy the proteins need to be dialysed and concentrated. Further, biological relevance of the ATPase activity will also be investigated.

b) To ensure the interactions between the proteins i.e. AC2, AC4 and AV2 with RaLCB Yeast two hybrid assays will be carried out.

Other experiments can also be done

Protein structure analysis can also be done by X-ray crystallography for that the proteins will be crystallized or by Nuclear magnetic resonance spectroscopy.

In yeast two hybrid assay there are chances of false positive results.

Co-immunoprecipitation is considered as a standard biochemical assay commonly used to identify the interacting protein.

Fluorescence resonance energy transfer (FRET) is a very sophisticated method to analyse protein-protein interaction.

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