“Comparative analysis of host factors regulating phytohormones and viral pathogenicity factor during ToLCV infection.”

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<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<tr>
<td>Bp</td>
<td>Base pair</td>
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<td>cDNA</td>
<td>Complementry DNA</td>
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<tr>
<td>$C_t$</td>
<td>Threshold value</td>
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<td>DCL</td>
<td>Dicer-Like</td>
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<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>Ethidium bromide</td>
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<td>HRM</td>
<td>High Resolution Melting</td>
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<td>JA</td>
<td>Jasmonic acid</td>
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<td>Kb</td>
<td>Kilo base</td>
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<td>L-RNA</td>
<td>Large RNA</td>
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<td>RdRP</td>
<td>RNA dependent RNA polymerase</td>
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<td>RNAi</td>
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<td>siRNA</td>
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Plant development is tightly controlled process regulated at various levels of gene expression. Phytohormones play important roles in plant development through regulating the expression of many genes transcriptionally, and the cis-acting elements have been shown to be involved in specific plant hormone-induced gene expressions (Liu et al., 2009). Their signalling pathways can be effectively controlled by modulation of positive and negative regulators during plant growth and development (Huq 2006). Several miRNAs have been shown to respond to plant hormones (Sunkar and Zhu, 2004; Zhang et al., 2005). The ability of viruses to perturb or interfere with these signalling pathways involving phytohormones and regulatory small RNAs provides opportunities for them to interfere with host gene expression and affect plant growth and development leading to the symptom development (Whitham et al., 2006).

MicroRNAs are endogenous, small, noncoding RNA of ~ 21 nt in length that negatively regulate target mRNAs at post transcriptional level in many eukaryotic organisms (Ambros, 2004). In plants, miRNAs target a wide range of mRNAs encoding transcription factors required for development. Major classes of miRNA-targeted genes include those that encode transcription factors and F-box (a motif that was first identified in cyclin F) proteins, which constitute major plant developmental regulatory networks. These transcription factors and F-box proteins regulate numerous plant developmental processes such as leaf development, patterning and polarity, floral identity and flower development, flowering time, developmental phase transition, shoot and root development, vascular and plastid development and hormone signalling for plant development (Wang et al., 2007). They are involved in many other biological processes such as regulation of miRNA and trans-acting siRNA (ta-siRNA) biogenesis, signal transduction and responses to environmental stress and pathogen invasion (Lu et al., 2008).

Viral infection interferes with plant developmental processes regulated by miRNAs and phytohormones and causes developmental defects in the plant by recruiting geminiviral pathogenicity factor AC4 which is also a RNAi suppressor (Vanitharani et al., 2004).
A structural examination of the EACMCV AC4 protein and those of several other geminiviruses that infect a range of crops shows a conserved consensus N-myristoylation motif which is found to be required for producing disease like symptoms, membrane binding and pathogenicity. Since viral suppressors of viruses differentially regulate small RNAs (siRNA and miRNA) viral pathogenicity and symptom development in the host cannot apply universally (Praveen and Ramesh 2007). AC4 protein encoded by African cassava mosaic virus Cameroon Strain is silencing suppressor found to interact directly with miRNA pathway. AC4 can bind with single-stranded miRNA or siRNA and inhibit miRNA-mediated negative regulation of gene expression in Arabidopsis plants; as a consequence, plants show developmental defects (Chellappan et al., 2005). It was reported that, viruses that produced the most severe symptoms on tobacco under the conditions tested (TMV and ToMV) altered miRNA accumulation to a greater extent than viruses that produced mild symptoms (i.e., TEV and PVY), suggesting that certain disease symptoms depend on miRNA levels and reported that, transgenic plants co-expressing movement protein and coat protein exhibit abnormal development, including phenotypes similar to those exhibited in mutants of A. thaliana that in which miRNA pathways are altered. These results suggested that interference with miRNA-directed processes might be a general feature of pathogenicity (Bazzini et al., 2007). Thus interferences of viral suppressors with miRNA-mediated developmental genes regulation will result in abnormal phenotypes. On the other hand, the interferences of viral suppressors with miRNA-mediated antiviral defense genes regulation will alter plant sensitivity to viral infection, and both may occur simultaneously (Chengguo et al., 2006).

Various studies indicate that molecular evolutionary changes in this pathogenicity factor that accompany changes in the host range leads to mutations (Tan et al., 2005). In plant viruses, genomic variation caused by mutations is enhanced by recombination, pseudo-recombination and acquisition of extra genomic components. Mutation is the commonest kind of viral genomic variation and is a necessary precursor to other types. Among RNA viruses, the typically short replication times, high yields and high mutation rates (caused by RNA-dependent RNA polymerase having an
An estimated error rate of about $10^{-4}$ per nucleotide per replication, and no proof-reading of the product) result in virus cultures consisting of a complex dynamic of mutants termed a ‘quasi-species’ (Harrison 2002). Among vertebrate-infecting dsDNA viruses, which use host DNA polymerase for replication, mutation rates are typically at least a 1000-fold less than in RNA replication, because of the DNA proof-reading activity. However, it is doubtful if this applies to those viruses, such as the geminiviruses, which have circular single-stranded DNA genomes that are replicated by a rolling-circle mechanism and lack of proof reading activity. It has been shown in the large number of begomovirus species (>100) (Fauquet et al., 2003) and the continued reports of new species (Bull et al., 2006), as well as the high degree of genetic diversity within the species (Patil et al., 2005; Stenger et al., 1995), suggest that begomoviruses have a high mutation rate and that they generate highly diverse populations in a short time. Genetic diversity of a single isolate population of Maize streak virus (MSV), a virus species in the genus *Mastrevirus* of the family *Geminiviridae* demonstrated that the population of MSV had a quasispecies structure (Isnard et al., 1998). It has been reported that, sequence diversity in genome of maize streak mastrevirus (family *Geminiviridae*) was as great as for RNA plant viruses (Isnard et al., 1998). Also, among a large set of samples of cotton leaf curl begomoviruses (family *Geminiviridae*) from Pakistan, nucleotide diversity at synonymous positions in genes was even greater than that typical for RNA plant viruses; non synonymous changes were commoner in the replication associated protein gene than in the coat-protein gene (Sanz et al., 1999). However, in contrast to plant RNA viruses, the available information about genetic variability is scant, and essentially no quantitative studies have analyzed the genetic structures and variability of begomovirus populations under controlled conditions. This variability in genetic structures caused by mutation pressure must be a key element of geminivirus evolution (Ge et al., 2007). Host induced genome evolution allows for adaptation of viruses to their hosts. Adaptive evolution depends upon the host species and degree of pathogenicity by the virus variants. Thus rapid evolution of geminiviruses depends on high mutation rate and also attributed by several other factors including increased insect vector populations, and can rapidly evolve in response to changes in the environment and diverse host ranges (Hou et al., 1996).
Adaptation of a virus to a host occurs by variation in the viral genome and increases the virus fitness which correlates with deep changes in the patterns of host’s gene expression, this interplay between the pathogen and the plant shifts as the virus adapts to its host. Adaptation of virus to host as a natural selection might favours viral genomes that avoided plant defense mechanisms as a consequence, increases in the strength of symptoms, virus accumulation and transmissibility have been observed (Agudelo-Romero 2008).

Plant defense mechanism-RNA silencing suggest three important roles: first, it is acting as innate immunity to resist viral epidemics. Second, it controls transcriptional gene expression for systematic developmental processes. In higher plants, a natural role of RNA silencing is to protect against viruses (Covey et al., 1997; Ratcliff et al., 1997; Al-Kaff et al., 1998; Hamilton and Baulcombe, 1999) and the RNA silencing machinery in higher plants is highly elaborated relative to other eukaryotes. In plants, there are three RNA silencing pathways (Baulcombe, 2004). The first pathway primarily associated with defense functions, comprises 21 nt small interfering RNAs (siRNAs) that are processed from double-stranded RNAs (dsRNAs). The source of dsRNAs includes replication intermediates of plant RNA viruses, transgenic inverted repeats, and products of RNA-dependent RNA polymerases (RdRps) etc. The second pathway involves a class of endogenous small RNAs, micro RNAs (miRNAs). Micro RNAs are generated by Dicer-like 1 (DCL1) from miRNA precursors that are transcribed from miRNA genes. Many transcription factors mediating the differentiation of multicellular organisms are regulated via these miRNAs, and severe developmental disturbances are associated with malfunctions of the miRNA pathways (Bartel 2004; Deferis et al., 2006). The third pathway is transcriptional gene silencing (TGS) that is associated with siRNA-directed epigenetic changes targeting de novo cytosine or histone methylation to their homologous DNA sequences to induce chromatin silencing (Xie et al., 2004; Chan et al., 2004, 2006).

In RNA-directed DNA methylation (RdDM), RNA is assumed to bind to complementary DNA sequences and this then leads to DNA methylation along the RNA-DNA duplex (Wassenegger et al., 1994; Pélassier et al., 1999; Pélassier and Wassenegger, 2000). It has been illustrated by Raja et al., (2008) that, genomic and
viral genome targets may be transcribed by an RNA polymerase IVa complex, resulting single-stranded RNA (ssRNA) is converted to dsRNA by complexes containing RDR2. The 21 to 24nt siRNAs processed from dsRNA by DCL3 are loaded into complexes containing AGO4, which subsequently associates with Pol IVb. The AGO4-associated siRNAs target the complex to homologous DNA sequences, where cytosine methyltransferases e.g., DRM and CMT3 proteins are recruited to methylate CpHpH and CpG, CpNpG sequences respectively, the bottom pathway represents PTGS, in which hairpin structured double-stranded RNA is processed into siRNAs by an unknown DICER protein. These siRNAs are used to degrade target mRNA and contribute to RNA-directed methylation independently of AGO4 possibly through another AGO protein (Ziberman et al., 2004), proposing that, RNA-directed DNA methylation driven by inverted repeats utilizes both pathways. It has been reported in a number of organisms, transgenes containing transcribed inverted repeats (IRs) that produce hairpin RNA can trigger RdDM which is associated with 21–24 nucleotide small interfering RNAs (siRNAs) (Tijsterman et al., 2002). Endogenous genomic sequences, including transposable elements and repeated elements, are also subject to RdDM. It has also been shown that, maintenance of non-CG DNA methylation is dependent on AGO4/RDR2/DCL3-the component of RNA-mediated silencing pathway along with DRM DNA methyltransferase (Chan et al., 2004; Cao and Jacobsen 2002). The biological function of RNA-directed and DNA-mediated DNA methylation is incompletely understood. The association of RdDM with both TGS and PTGS may indicate that RdDM is part of a defence system against pathogen attack, which could be limited to plants and fungi. Foreign nucleic acids, such as transgenes, transposons and virus sequences, can be recognized by the host plant and RdDM may serve as a mechanism to inactivate them (Kumpatla et al., 1998; Matzke and Matzke, 1998; Voinnet et al., 1998). This proposal has been supported by the observation that infection with an RNA virus induces RdDM in plants (Jones et al., 1998).

Keeping in mind the importance and crucial role of viral pathogenecity factor-AC4 in regulating plant developmental pathways, following objectives were proposed to study ToLCNDV-AC4, its synergistic role in plant defense, viral adaptation and symptom development.
1. Study of molecular basis of host specific adaptation of Tomato leaf curl New Delhi virus (TOLNDCV) – pathogenecity factor (AC4).

2. Characterization of microRNAs regulating phytohormones during infection.

3. Profiling of transcriptional expression of host genes involved in phytohormonal regulation during infection.

2. BACKGROUND

In plants each developmental process integrates a network of events that are regulated by different phytohormones and interactions among hormonal pathways modulate their
effects and their signaling pathways can be effectively controlled by modulation of positive and negative regulators during plant growth and development (Huq 2006). Several miRNAs have been shown to respond to plant hormones (Sunkar and Zhu, 2004; Zhang et al., 2005). The ability of viruses to perturb or interfere with these signaling pathways involving phytohormones and regulatory small RNAs provides opportunities for them to interfere with host gene expression and affect plant growth and development leading to the symptom development (Whitham et al., 2006). These interactions can be further fine-tuned in the host by viral mutation during its adaptation to hosts by evading plant defense mechanism and this high rate of mutations adds to the complexity of the interaction network which involves stress signalling/host defense pathway, hormone signalling and miRNA mediated regulation in plant development. Though evidence regarding microRNA mediated cross-talk in viral infections is just emerging, it offers an immense opportunity to understand the intricacies of host-pathogen interactions. The journey from first observation of leaf curl virus infection, their interaction with host with respect to hormone signalling during viral infection, role of viral pathogenicity factor in symptom development by modulating host gene expression, adaptation of virus to host ranges and operation of defense mechanism in the plants has been elucidated in a timeline (Fig. 1).

MicroRNAs (miRNAs) are a class of 21–24 nucleotides, endogenous noncoding small ribonucleic acids (RNAs) (Bartel, 2004). It was not until 8 years after the discovery of miRNA that regulatory functions for miRNA were elucidated (Reinhart et al., 2002; Ambros 2003). Since then, numerous studies have significantly expanded our knowledge of the roles of miRNAs in biology and physiology. As of October 2006, the sequences of 4361 miRNAs have been deposited in the miRNA database (miRbase, release 9.0; http://microrna.sanger.ac.uk/sequences/index.shtml). The diversity of the miRNA genes and their expression patterns in different tissues or development stages implies that, miRNAs play versatile roles in plant growth and development (Fig. 2). This is evident from the study of loss of function mutants of enzymes involved in miRNA biogenesis, dcl1 mutant shows reduced expression level of mature miRNAs and exhibits many developmental abnormalities including immature embryos, altered leaf
shape and morphology, delayed floral transition, and female sterility (Park et al., 2002; Reinhart et al., 2002; Liu et al., 2005; Kurihara et al., 2006). Similarly, hst mutant also exhibits pleiotropic abnormalities, such as abnormal leaf and flower morphology, accelerated phase transition and reducing fertility (Bollman et al., 2003).

Leaf development is regulated by modulating the expression of class-III homeodomain leucine zipper (HDZIP) transcription factor genes, which control leaf patterning along adaxial/abaxial axis (Juarez et al., 2004). PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) are three closely related Arabidopsis HD-ZIP transcription factors that are the targets of miR165 and miR166 (Emery et al., 2003; Bao et al., 2004) Altered expression of miR165 and miR166 resulted in leaf developmental abnormalities in Arabidopsis and corn (Juarez et al., 2004). It was recently observed that over-expression of miR165 affects apical meristem formation, organ polarity establishment and vascular development in Arabidopsis (Zhou et al., 2007). In addition miR159/Jaw is found to regulate a subset of TCP transcription factor genes that control leaf development (Palatnik et al., 2003). A recent study revealed that miR164 is another regulator of leaf patterning (Nikovics et al., 2006). These authors showed that the balance between the miR164a and the transcription factor CUP-SHAPED COTYLEDON2 (CUC2) gene controls leaf margin serration in Arabidopsis. Although the pattern of serration is determined first independently of CUC2 and miR164, the balance between co-expressed CUC2 and miR164a then determines the extent of leaf serration. Leaf development, patterning and polarity are also controlled by many other transcription factors, including leucine zipper family, TCP family and MYB family. miR159 shares sequence similarity with miR319/ JAW. The targets of miR159 include two members of MYB transcription factors, MYB33 and MYB65. Palatnik et al., (2003) reported that over expression of a miRNA-resistant version of MYB33 in Arabidopsis causes leaves to curl upwards (Palatnik et al., 2003). Similarly, Arabidopsis transformed with MYB33 containing the mutated miRNA target site show dramatic pleiotrophic developmental defects, including abnormal leaves that were more rounded and upturned at the sides (Millar and Gubler, 2005). reduced leaf size is seen in
Arabidopsis as well as tomato plants in which miR319 control of TCP genes is impaired (Palatnik et al., 2003).

2.0 Micro RNA linking hormone signalling and plant development

The first report linking miRNAs and hormone signaling was that the hyl1-1 mutant displayed impaired responses to auxin, ABA and cytokinin (Lu and Fedoroff, 2000). After this, gibberellic acid (GA) has been shown to modulate miR159 levels during another development (Achard et al., 2004), and auxin induction of miR164 to clear NAC1 mRNA to reset auxin signalling (Guo et al., 2005). Furthermore, miR160 has been shown to regulate expression of an auxin response transcription factor, ARF17 (Mallory et al., 2005) and miR159 is induced by ABA to cleave two MYB factors (MYB33 and MYB101) mRNAs during Arabidopsis seed germination (Fujii et al., 2005). There are many predicted targets for miRNAs include several mRNAs which are involved in hormone responses, such as transport inhibitor response 1 (TIR1), a negative regulator in auxin signalling (Reyes and Chua, 2007; Fujii et al., 2005) and the latest research showed that TCP (TEOSINTE BRANCHED/CYCLOIDEA/PCF), the targets of miR319, control biosynthesis of the hormone jasmonic acid (Schommer et al., 2008) Transcripts encoding transcription factors in the CUC family, which in addition to the their aforementioned role in leaf development mediate auxin-dependent lateral root formation, organ shape and leaf senescence, are targeted by miR164 family members (Sieber et al., 2007). Several lines of evidence link miR319, which targets transcripts encoding a series of TEOSINTE BRANCHED1, CYCLOIDEA, AND PCF (TCP) transcription factors, indirectly to auxin activity. Micro RNA319–TCP3 node is involved in the regulation of miR164 nodes as part of the network that outlines organ growth and shape in Arabidopsis (Koyoma et al., 2007). Seedlings that ectopically express miR319a possess downward-curled cotyledons that are typical of auxin overproducers (Palatnik et al., 2007). In contrast, the constitutive expression of miR319-resistant forms of TCP4 causes phenotypic abnormalities in the plants, which are impaired in auxin transport (Palatnik et al., 2003; Mravec et al., 2008). Within the auxin-signalling pathways, ARFs function as core positive and negative regulators (Guilfoyle and Hagen, 2007). The highly conserved miR167–ARF6/ARF8 nodes also
modulate auxin early response regulators, as well LIPOXYGENASE2 (LOX2) expression (Nagpal et al., 2005). LOX2 acts enzymatically in a dedicated biosynthesis pathway that leads to the hormone jasmonic acid (JA) (Bell et al., 1995), indicating a potential interplay between auxin and JA pathways. miR393 targets transcripts encoding a small subset of F-box-containing auxin receptors, including TRANSPORT INHIBITOR RESPONSE1 (TIR1) (Jones-Rhoades and Batrel 2004; Navarro et al., 2006) TIR1 releases the activity of ARF transcription factors by submitting Auxin/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) repressors to proteolysis (Dharmasiri et al., 2005); reduction of miR393- mediated control of TIR1 transcripts results in phenotypes consistent with elevated auxin sensitivity or increased auxin biosynthesis (Navarro et al., 2006). However, the roles of miRNAs in plant hormone signalling are still largely unexplored and up to now no miRNAs have been identified to be involved in cytokinin or ethylene signalling (Liu et al., 2009). In the line of this work, it was also reported that, viral infection interferes with plant developmental processes regulated by miRNAs and phytohormones and causes developmental defects in the plant by recruiting pathogenicity factor AC4 which is also a RNAi suppressor (Vanitharani et al., 2004).

2.1 Host-virus interactions leading to plant developmental abnormalities
The obligate intracellular nature of viruses provides numerous opportunities for viral proteins and nucleic acids to interact with and influence the activity of host proteins and nucleic acids. As viral proteins and nucleic acids accumulate, they perform their various roles in replication, movement, encapsidation, and suppression of host defenses. In compatible interactions that result in systemic infections, each protein may have multiple functions that enable invasion throughout the plant and, ultimately, transmission to a new host. Frequently, viral proteins accumulate to extreme levels relative to host proteins, which is indicative of their proficiency in hijacking host cellular functions and resources. Accumulation of viral proteins and the accompanying infection is commonly associated with numerous effects in host cells as well as eliciting defects in plant growth and development. However, it is becoming increasingly evident that increases in the intracellular concentrations of viral proteins have many
consequences for host gene expression and metabolism. Some of these effects do not necessarily provide an advantage to the virus but nevertheless have effects on the host. One consequence of viral infection is the altered expression of host genes, which may have several root causes. A major challenge has been to identify host genes with altered mRNA transcription profiles and to decipher how and why the changes are initiated. The ultimate goal is to then use this information to investigate the functions of genes with altered expression profiles in plant-virus interactions (Whitham et al., 2006). **Fig. 3** shows host responses and altered gene expression associated with plant virus infections. Viral infections can also disrupt the functions of regulatory miRNAs, and phytohormone signaling or biosynthesis leading to developmental defects. Thus it outlined some common ways that a broad range of viruses may alter plant gene expression. Incompatible interactions between viruses and hosts have previously served as models for investigating host defense responses.

### 2.2 Role of plant hormones in plant defense responses

Plant hormones play important roles in regulating developmental processes and signalling networks involved in plant responses to a wide range of biotic and abiotic stresses. The identification and characterization of several mutants affected in the biosynthesis, perception and signal transduction of these hormones has been instrumental in understanding the role of individual components of each hormone signalling pathway in plant defence (Bari and Jones, 2009). For example, defense signalling pathway initiated through production of JA-Ile mimic by *Pseudomonas syringae* pv. tomato (Pst) bacteria, triggers the activation of JA-dependent defence responses leading to the suppression of SA-dependent defence responses and promotion of disease symptoms (Cui et al., 2005; Laurie-Berry et al., 2006). Thus, SA and JA/ET defence pathways are mutually antagonistic and suggests that the defence signaling network activated and utilized by the plant is dependent on the nature of the pathogen and its mode of pathogenicity. Several studies have demonstrated that concentrations of JA increase locally in response to pathogen infection or tissue damage and exogenous application of JA induced the expression of defense related genes (Lorenzo and Solano 2005; Wasternack 2007). It was also showed that, during pathogen infection GH3 (early
Auxin responsive gene) is activated to modulate auxin pathway resulting in enhanced disease susceptibility through increasing IAA biosynthesis and de-repressing auxin signaling. Similarly GH3.5 positively modulates the SA pathway to enhance plant defense response through elevating SA biosynthesis, activating SA-induced genes, WRKYs, and basal defense-related genes (Zhang et al., 2007). Host defense response through signalling molecules such as SA, JA and ET alters the plant developmental processes during defense responses against pathogens (Chung et al., 2008). Interactions between defense signalling pathways is an important mechanism for regulating defense responses against various types of pathogens. In the recent years, several components regulating the cross-talk between SA, JA and ET pathways have been identified. However, the underlying molecular mechanisms are not well understood.

Compatible interactions between susceptible hosts and virulent pathogens frequently lead to the expression of defense-related genes. Many incompatible interactions are associated with increased PR gene expression and protein accumulation mediated by salicylic acid (SA) (Gaffney et al., 1993) Further analyses demonstrated that CMV2b was antagonistic to the SA-mediated expression of defense-related genes when expressed from CMV or as a transgene. In Nicotiana species, it is apparent that the induction of SA-dependent defenses is detrimental to compatible interactions with CMV or TMV. Thus, CMV2b may promote virus accumulation in Nicotiana species by antagonizing SA dependent defenses (Whitham et al., 2006).

The abnormal growth forms of virus-infected plants have encouraged experiments that examine the effects of viruses on hormone levels in plants and vice versa (Jameson 2000). While it is difficult to formulate general rules about the effects of viruses on phytohormones, it is clear that abscisic acid, auxin, cytokinin, giberellin, and ethylene levels, alone or in combination, can all be perturbed, depending on the virus-host combination. Recently, direct links between auxin signaling and giberellin levels have been established for TMV and Rice dwarf virus (RDV), respectively. The helicase domain of the TMV 126- and 183-kDa replicase proteins was shown to interact with the Aux/indole 3-acetic acid (IAA) transcription factor IAA26 (Padmanabhan et al., 2005).
A single amino-acid mutation (V1087I) in the helicase domain abolished the interaction between the replicase proteins and IAA26. Even though this interaction was disrupted, the TMV mutant replicated and moved systemically in *Arabidopsis* and tobacco plants at levels equivalent to the wild-type virus. Interestingly, the symptoms of infection were attenuated indicating that TMV replication and movement could be partially uncoupled from symptoms in both *Arabidopsis* and *N. benthamiana*. Thus, this interaction, while contributing to the symptoms, is apparently not required for a successful TMV infection in *Arabidopsis N. benthamiana*. Silencing of IAA26 resulted in plants with phenotypes similar to those infected with TMV, indicating that TMV replicase also causes a loss of IAA26 function. This was verified, in part, by showing that TMV infection redirects IAA26 from the nucleus to the cytoplasm. The ability of TMV to modulate symptoms through interaction with IAA26 was postulated to be due to interference with its normal role in forming heterodimers with auxin-response factors (ARF). It was also documented that *Tobacco mosaic virus* (TMV)-induced disease symptoms, including the loss of apical dominance, stunting and leaf curling, are caused by the inappropriate expression of auxin-related genes, which is mediated through an interaction between TMV replicase and its specific target protein PAP1, a negative regulator of auxin response factor (ARF) (Padmanabhan *et al.*, 2005). In the absence of auxin, Aux/IAA proteins like IAA26 bind to ARF transcription factors and prevent them from modulating the transcription of auxin-responsive genes. However, in the presence of auxin, the Aux/IAA proteins are targeted for degradation, thus freeing the ARF proteins to modulate transcription of their target genes. The TMV replicase thus functions as an auxin mimic by diverting IAA26 from the nucleus and allowing its target ARF to modulate transcription of their target genes. In support of this idea, the authors observed that 30% (20 of 66) of genes that were significantly altered in their expression in response to TMV in *Arabidopsis* (Golem and Culver 2003) also possessed two or more auxin-response elements (ARE) in their promoters region TGTCTC. Genes possessing this motif in their promoters are part of the early response to auxin and are subject to transcriptional control by Aux/IAA and ARF proteins. Five of the 20 genes that respond to TMV and possess at least two ARE were tested for their response to auxin and were shown to exhibit similar patterns of expression in response to auxin and TMV. Because
IAA26 is one of 29 members of a gene family, it will be interesting to see how TMV interacts with other members of this family and if any of these interactions have additional effects on symptoms, host gene expression, or viral infection itself.

In addition to affecting auxin signalling events, viral proteins have also been identified that affect giberellic acid (GA) signalling. One such example is the P2 protein of RDV (Rice dwarf virus), which currently has no known role in the infection process except that it is a symptom determinant. Zhu and associates (2005) demonstrated that P2 interacts with four different ent-kaurene oxidases or oxidaselike proteins by yeast two-hybrid assay. Oxidases ent-kaurene catalyze a step in the synthesis of GA, and ent-kaurene oxidase mutant rice plants are deficient in GA. In addition, this mutant rice possesses a phenotype similar to symptoms caused by RDV infection. The authors demonstrated that RDV infection results in decreased GA levels and symptoms that could be alleviated by GA application. Although host gene expression assays were not performed, it is likely that GA responsive genes are modulated by RDV infection. Despite the role of RDV P2 in disrupting GA levels in rice, the authors were unable to conclude that this interaction has a significant effect on infection, suggesting that it may be coincidental but detrimental to the plant.

Taken together, emerging evidence suggests that auxin acts as an important component of hormone signalling network involved in the regulation of defence responses and modulates defence and development responses. However, how auxin levels affect the balance of other hormones and fine tune defence responses specific to different pathogens remains to be discovered (Bari and Jones, 2009). Numerous miRNAs have been predicted or validated to be involved in plant defense. For example miR-139 targets a gene coding for a mucin-like protein carrying a dense sugar coating against proteolysis, which is a pivotal step in pathogen invasion, miR160-3 acts on intracellular pathogenesis- related protein and miR408 provides defense though interaction with the genes coding for a copper ion binding protein (Isam et al., 2007). Over expression of a plant miRNA (miR393) resulted in the increased bacterial resistance (Navarro et al., 2006). Therefore, it is thought that plant miRNA-directed
RNAi or miRNA-specified mRNA destruction determines the balance in plant defense system.

2.3 Viral suppressors interferes with miRNA pathway causes developmental abnormalities

Systemic infection by plant viruses frequently results in disease symptoms that resemble developmental defects, including loss of leaf polarity, loss of proper control of cell division, and loss of reproductive functions (Hull 2001). These and other phenotypes are frequently associated with virus-encoded pathogenicity factors, many of which are suppressors of RNA silencing (Voinnet et al., 1999). RNA silencing functions as an adaptive immune response which restricts accumulation or spread of inducing viruses (Waterhouse et al., 2001). Suppressor proteins encoded by members of different virus families are distinct, suggesting that plant viruses evolved this counter defensive mechanism independently on many occasions (Vaucheret et al., 2001; Tijsterman et al., 2002). RNA silencing suppressors from different plant viruses are structurally diverse. In addition to inhibiting the antiviral silencing response to condition susceptibility, many suppressors are pathogenicity factors that cause disease or developmental abnormalities.

Suppressors from multiple viruses were shown to inhibit microRNA (miRNA) activities and trigger an overlapping series of severe developmental defects in transgenic Arabidopsis thaliana. This suggests that interference with miRNA-directed processes may be a general feature contributing to pathogenicity of many viruses (Chellappan et al., 2004). An intermediate in the miRNA biogenesis/RNA-induced silencing complex (RISC) assembly pathway causes accumulation of miRNA* specifically in the presence of suppressors (P1/HC-Pro, P21, or P19) that inhibited miRNA-guided cleavage of target mRNAs. Both P21 and P19, but not P1/HC-Pro, interacted with miRNA/miRNA* complexes and hairpin RNA-derived short interfering RNAs (siRNAs) in vivo. In addition, P21 bound to synthetic miRNA/miRNA* and siRNA duplexes in vitro. We propose that several different suppressors act by distinct mechanisms to inhibit the incorporation of small RNAs into active RISCs (Chapman et al., 2004). It was also shown that microRNAs (miRNAs) are involved in modulating
plant viral diseases (Dunoyer et al., 2004). Micro RNA-mediated gene silencing serves as a general defense mechanism against plant viruses (Lu et al., 2008). Virus-derived siRNA have been detected in plants infected with various RNA viruses (Szittya et al., 2002) and DNA geminiviruses (Chellappan et al., 2004; Kon et al., 2006). Furthermore, both RNA and DNA viruses encode distinct suppressors of RNA-silencing that target different components of this system (Voinnet 2005). In particular, tombusvirus P19 protein selectively sequesters 21-nt siRNA duplexes (Lakatos et al., 2004). Geminivirus suppressor protein AC4 appears to selectively bind single-stranded sRNAs including miRNAs (Chellappan et al., 2005). The latter observation is consistent with the hypothesis that not only the siRNA but also the miRNA pathway might restrict virus replication, as demonstrated for a mammalian retrovirus (Lecellier et al., 2005).

In line with this idea, most viral silencing suppressors, when over expressed in transgenic plants, interfere with production and/or action of miRNAs, thus leading to various abnormalities of plant development, often resembling viral symptoms (Voinnet 2005). AC4 protein encoded by African cassava mosaic virus Cameroon Strain is silencing suppressor found to interact directly with miRNA pathway. AC4 can bind with single-stranded miRNA or siRNA and inhibit miRNA-mediated negative regulation of gene expression in Arabidopsis plants; as a consequence, plants show developmental defects (Chellappan et al., 2005). It was reported that, viruses that produced the most severe symptoms on tobacco under the conditions tested (TMV and ToMV) altered miRNA accumulation to a greater extent than viruses that produced mild symptoms (i.e., TEV and PVY). suggesting that certain disease symptoms depend on miRNA levels and reported that, transgenic plants that co-expressing movement protein and coat protein exhibit abnormal development, including phenotypes similar to those exhibited in mutants of A. thaliana that in which miRNA pathways are altered. These results suggested that interference with miRNA-directed processes might be a general feature of pathogenicity (Bazzini et al., 2007). Thus interferences of viral suppressors with miRNA-mediated developmental genes regulation will result in abnormality phenotypes. On the other hand, the interferences of viral suppressors with miRNA-
mediated antiviral defense genes regulation will alter plant sensitivity to viral infection, and both may occur simultaneously (Chengguo et al., 2006).

2.4 Role of viral pathogenecity factor during infection

Although exact role of the AC4 (bipartite) and C4 (monopartite) gene products remains unknown, mutation analysis infer atleast one of these (AC4) may play an important role in begomovirus movement or induction of host cell replication machinery (Stanley and Latham 1992). Transgenic plants over-expressing C4 protein develop virus like symptoms (Karke et al., 1998) suggesting that, C4 may signal the alteration of host gene expression independently of other viral genes. Consistent with this hypothesis, other studies have indicated that C4 gene product is targeted to the cell periphery and mesophyll plasmodesmata and may interfere with natural trafficking of endogenous non cell autonomous signaling molecules. As a result C4 may contribute to provide an optimal environment for viral replication and proliferation to further enhance viral symptoms (Rojas et al., 2001)

Plant defence systems are elegant example of how nature can find highly efficient solutions to the problems it faces. Overall, it can be described as a co-evolution of defence and counter defence mechanisms between the host plant and the invading virus (Lu et al., 2008). RNA silencing suppression is a common property of plant viruses. Suppressor proteins are considered as pathogenicity determinants, needed for efficient accumulation found in most viruses. Silencing suppressor proteins show a tremendous structural and sequence diversity that has been explained as an evolutionary convergence toward a common functional necessity (Li and Ding 2006).

PTGS occurs through homology-dependent degradation of RNA and is regarded as a fundamental process related to a wide range of epigenetic phenomena. In plants, PTGS represents an effective defense system against virus infection. On the other hand, plant RNA and DNA viruses have evolved a counter-defense mechanism by encoding suppressors of PTGS, which often contribute to viral pathogenicity (Wezel et al., 2002). PTGS suppressors characterized so far include 2b, HC-Pro, P1, and P19 proteins from cucumoviruses, potyviruses, sobemoviruses, tombusviruses, PVX p25 movement protein, ACMV AC2 (TrAP) (Carrington et al., 2001; Voinnet 2001), and TYLCV-C
C2 protein described here. There is much variation in the extent of suppression of PTGS by different viruses (Voinnet et al., 1999). Indeed, such proteins mediate suppression by targeting different steps in the PGTS pathway (Anandalakshmi et al., 2000; Voinnet et al., 2000). Fondong et al., 2007 reported that, EACMCV-AC4 is an intrinsic membrane binding protein and required for pathogenesis by examining the AC4 sequence for membrane localising signal. They also identified the N-myristoylation motif from begomoviruses and some animal viruses which shared the sequence similarity (Table.1) and proved the membrane binding and pathogenesis function by mutating Gly-2 and Ile-15 of N-myristoylation motif with alanine.

Table.1 Consensus N – myristoylation motif sequences of selected virus and eukaryotic proteins
<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession No.</th>
<th>Consensus Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>East African cassava mosaic Cameroon virus AC4</td>
<td>AF112354</td>
<td>MGNLISTCSS SSKANTSARI</td>
</tr>
<tr>
<td>Sida mosaic Sinaloa virus AC4</td>
<td>YP619889</td>
<td>MGCLISMFSS NSKASSNVPT</td>
</tr>
<tr>
<td>Tomato golden mottle virus AC4</td>
<td>ABF67530</td>
<td>MGSLISTFSS SSKANTTARI</td>
</tr>
<tr>
<td>Cotton leaf curl Bangalore virus C4</td>
<td>YP277440</td>
<td>MGNLISMPLS NLKEKSRSRM</td>
</tr>
<tr>
<td>Okra yellow mosaic Mexico virus AC4</td>
<td>AAY27084</td>
<td>MGNLICTSLS SSRGNDTARI</td>
</tr>
<tr>
<td>Sweet potato leaf curl Georgia virus C4</td>
<td>AAL69644</td>
<td>MGLCTSMLSS SSRVKPNLKT</td>
</tr>
<tr>
<td>Tomato golden mottle virus AC4</td>
<td>AAY27084</td>
<td>MGNLICTSLS SSRGNDTARI</td>
</tr>
<tr>
<td>Potato yellow mosaic Trinidad virus AC4</td>
<td>AAC09257</td>
<td>MGNLISTCLS SSKVNISARI</td>
</tr>
<tr>
<td>Abutilon mosaic virus -HW C4</td>
<td>AAB18174</td>
<td>MGSLISMCSY SSKANSNARI</td>
</tr>
<tr>
<td>Foot-and-mouth disease virus - 3 Capsid</td>
<td>DQ009741</td>
<td>MGAGQSSPAT GSQNQSGNTG</td>
</tr>
<tr>
<td>Human immunodeficiency virus type 1 Gag</td>
<td>AJ293865</td>
<td>MGRASVLIG KLNPEKIR</td>
</tr>
<tr>
<td>Type III effector protein avirulence protein avrB</td>
<td>AAA25726</td>
<td>MGCVSSKSTT VLSPQTSFNE</td>
</tr>
<tr>
<td>Calmodulin-domain protein kinase CDPK isoform 2</td>
<td>AAF76372</td>
<td>MGNACVGPNI SGNFLQTVTV</td>
</tr>
<tr>
<td>Arabidopsis calcium dependent protein kinase</td>
<td>BAB08991</td>
<td>MGNTCVGSPR NGFLQSVSAA</td>
</tr>
</tbody>
</table>

Reproduced from Fondong *et al.*, 2007
2.4.1 *Tomato leaf curl virus* (ToLCV) - A Geminivirus

One of the most important geminivirus *Tomato leaf curl virus* (ToLCV), belongs to genus *Begomovirus* of family *Geminiviridae*. The virus is transmitted by whitefly *Bemisia tabaci*. It causes severe leaf curling, cupping of leaf lamina and overall stunting of growth in tomato (*Solanum lycopersicum*) leading to severe yield loss and ensuing economic losses (Rataul and Brar, 1989; Dougherty et al., 1994). *Tomato leaf curl virus* possesses a bipartite genome, both the components of the genome are essential for causing infection. DNA-A is characterized with 6 ORFs, AC1 encodes a replication-associated protein (Rep) essential for viral DNA replication in association with host DNA polymerase (Hanley-Bowdoin et al., 2000); AC2 encodes a transcription activator protein (TrAP) (Sunter and Bisaro, 1992); AC3 encodes a replication enhancer protein (REN) (Sunter et al., 1990); AC4 in ToLCV has not been attributed with any function in viral DNA replication rather its role in small RNA binding and altering host small RNA metabolism have been demonstrated in related geminiviruses also (Krake et al., 1998; Chellappan et al., 2005). The AV1 and AV2 encode coat protein and pre-coat protein, respectively (Padidam et al., 1996). DNA-B has BV1 and BC1 genes that encode a nuclear-shuttle protein (NSP) and movement protein (MP), respectively (Sanderfoot and Lazarowitz, 1995) (Fig. 4).

Due to the worldwide increases in the population and distribution of the insect vector and global movement of plant materials, begomovirus-induced diseases have become a major constraint on crop production in tropical and subtropical regions (Rojas et al., 2005; Seal et al., 2006). Study on their geographical distribution, has revealed that the begomoviruses affecting tomato in northern India are bipartite and those affecting tomato in southern India are monopartite. These two groups of viruses are
quite distinct in their biological activity and genomic organizations. Irrespective of its genomic nature virus replicates in the host nuclei via double-stranded DNA intermediates using a rolling circle mechanism (Saunders et al., 1991; Stenger et al., 1991). Begomovirus evolution is likely to proceed fastest in the wake of pandemics, which in turn reflect key changes in the factors that affect virus survival and spread. The probable genetic material among viruses with overlapping ranges would enable distinct viruses in the same region to evolve in a concerted manner. Moreover, some of the new variants will be preserved by geographical or biological isolation, and enhanced by further genetic changes of all kinds, to the point where new virus species can be considered to have evolved (Harrison and Robinson 1999).

2.5 Molecular basis of viral genome mutation and host specific adaptation of viruses
Host-induced genome evolution allows for adaptation of plant viruses to their hosts (Rodriguez-Cerezo et al., 1989). Adaptive evolution has been found to be either random or selected, and it depends on the virus and its host species (She et al., 2001). During the last few decades, viral genome evolution has been investigated at the molecular level in two different ways. (i) Viral populations are transferred in parallel to different host species or to the same host in different geographic areas, as evidenced in plant viruses such as *Tobacco mosaic virus* (TMV) (Kearney et al., 1999). (ii) Viral populations are transferred serially in a specific host or to different hosts (or cell lines) with serial passages, as shown in plant viruses TMV, Cucumber mosaic virus (CMV), and *Cowpea chlorotic mottle virus* (Kearney et al., 1999; Schneider et al., 2001). A rapid increase of virus-induced reduction of host fitness is the most general result of serial passage experiments. Such repeated bottleneck events are also expected to cause progressive changes of the original genome sequence (Ebert 1998; Domingo and Holland, 1997). Although viruses exhibit rapid sequence divergence over periods of time and mutations occur in variable positions of viral genomes (Strauss and Strauss 2001), genetic stability has been revealed in plant viruses over time, space, and host species separations (Kearney et al., 1999). This agrees with the view that host-
associated selection results in decreased diversity in viral genome (Garcia-Arenal et al., 2001). It seems that the co-variation leads to reduction in fitness (ability to replicate infectious progeny) of the virus (Liang et al., 2002).

Although the impacts of recombination and pseudo recombination on the evolution of begomoviruses and epidemics of begomovirus-induced diseases have been extensively documented (Garcı´a-Andres et al., 2006), the effects of mutation on begomovirus evolution have thus far not been documented extensively. Unlike RNA viruses, begomoviruses replicate their genomes inside the nucleus by using the host replication machinery (Gutierrez 1999). Thus, these viruses were assumed to have higher replication fidelity and lower rates of mutation accumulation than RNA viruses (Rojas et al., 2005). However, the large number of species (>100) (Fauquet et al., 2003) and the continued reports of new species (Bull et al., 2006), as well as the high degree of genetic diversity within the species (Patil et al., 2005; Stenger 1995), suggest that begomoviruses have a high mutation rate and that they generate highly diverse populations in a short time.

Although recombination is undoubtedly an important evolutionary mechanism in these viruses, it cannot create genetic variation de novo, so background mutation pressure must also be a key element of geminivirus evolution (Ge et al., 2007). Genetic diversity of a single isolates population of Maize streak virus (MSV), a virus species in the genus Mastrevirus of the family Geminiviridae, and demonstrated that the population of MSV had a quasispecies structure (Isnard et al. 1998). However, in contrast to plant RNA viruses, the available information about genetic variability is scant, and essentially no quantitative studies have analyzed the genetic structures and variability of begomovirus populations under controlled conditions. (Ge et al., 2007)

It is generally thought that nucleotide mis-incorporation does not contribute significantly to the genomic variation of small DNA viruses that are replicated by cellular DNA polymerases. This assumption is supported by long-term mutation rates for dsDNA viruses, which are low and comparable to those measured for cellular genes (Bernard, 1994). There are numerous reports of the emergence of geminivirus strains with altered pathogenicity (Seal et al., 2006), indicative of rapid genetic change that has
been attributed to recombination or reassortment among different viral genomes. However, our finding that mutations in the helix 4 motif of the AL1 gene of two distantly related begomoviruses revert at 100% frequency suggests that nucleotide substitutions occur with high incidence and are under strong selective pressure during geminivirus infection. Thus, in agreement with recent report of high mutation rates for other ssDNA viruses infecting vertebrates and bacteria (Shackelton et al., 2005), nucleotide substitution events are likely to contribute to the diversity and rapid evolution of geminivirus ssDNA genomes.

The mutation frequency for a virus is determined by a combination of the intrinsic frequency of mis-incorporation and the capability for mismatch repair, as well as the extent of specific selection or stochastic drift resulting from genetic bottlenecks imposed on the virus population. The high mutation frequency associated with RNA viruses is presumed to be due in large part to their lack of proofreading capability during replication (Roossinck, 1997). In comparison, geminiviruses replicate using their host DNA replication machinery (Gutierrez, 1999). Theoretically, these viruses should have less population variation, however, there is no information about the nature of plant DNA polymerase or the polymerase factors involved in the replication of geminiviruses. In particular, it is not known whether only a subset of cellular DNA replication and/or mismatch repair machinery is activated for geminivirus replication or whether the cellular environment affects the fidelity of those polymerases (Ge et al., 2007). It was reported that DNA methylation inhibits the replication of Tomato gold mosaic virus in tobacco protoplasts (Brough et al., 1992), implying that geminivirus DNA may not be methylated and that the normal mechanisms for mismatch repair probably do not operate during the Tomato gold mosaic virus replication cycle (Inamdar et al., 1992). Thus, it is possible that the mechanisms of mismatch repair may function differently during geminivirus DNA and cellular DNA replication and that the lack of postreplication repair may be responsible for higher misincorporation in the geminivirus progeny DNA (Seal et al., 2006).

An important consequence of high mutation and recombination rates is the continuous production of genetic variation in geminivirus populations. This variability is balanced by a complex set of selection pressures including those associated with
intrinsic properties of the virus, such as the maintenance of essential nucleotide structures and replication signals, and selection pressures to maintain crucial interactions with plant hosts and insect vectors (Astorga et al., 2007). Thus, despite their variation potential, geminiviruses populations exhibit significant genetic stability over time and space, as has been documented for plant RNA viruses that also display high mutation rates (Gibbs, 1999). Nonetheless, the evolutionary potential of geminiviruses needs to be considered in long-term control strategies, because any disease management effort will result in selective pressure on the virus population to adapt to new circumstances (McDonald and Linde, 2002). A recent mathematical analysis of the potential impact of disease control strategies concluded that the use of resistant cultivars with reduced within-plant virus titers puts pressure on the target virus to evolve towards a higher multiplication rate (Van den Bosch et al., 2006). The results reported here demonstrated experimentally that geminivirus variants with residual replication capabilities are under strong selective pressure to generate variants that replicate to high titers. Given the large size and genetic heterogeneity of geminivirus populations and their capacity to rapidly change their genomes by recombination and mutation, it will be necessary to devise resistance strategies that prevent virus replication and not simply reduce it because of the risk of generating more harmful variants that overcome resistance (Astorga et al., 2007).

2.5.1 Virus diversity, host range and host defense
Viruses adapt to their hosts by evading defence mechanisms and taking over cellular metabolism for their own benefit. Alterations in cell metabolism as well as side-effects of antiviral responses contribute to symptoms development and virulence. Sometimes, a virus may spill over from its usual host species into a novel one, where usually will fail to successfully infect and further transmit to new host. However, in some cases, the virus transmits and persists after fixing beneficial mutations that allow for a better exploitation of the new host. This situation would represent a case for a new emerging virus. Thus emerging infectious diseases might be due to changes in the pathogen’s genome that are responsible for adaptation to a new host after spilling over from the
original one which provides an important clue to understand how these changes may alter host’s metabolic and regulatory interactions (Agudelo-Romero et al., 2008).

Variability in the leaf curl viruses, found maximum and minimum similarity at 1700-1899 (200 nt) and 2411-424 (771nt) respectively (Kirthi et al., 2004). The region 2411-424 encompasses the intergenic region and partial ORFs of AC4, AV1 and AV2. Similarly analysis of genetic variation among TYLCV variants suggests high substitution rate of nucleotide compared to background mutation rates were observed in the intergenic regions of TYLCV variants reflecting the rapid mutational dynamics rather than frequent adaptive evolution (Duffy and Holmes, 2008). Thus rapid evolution of geminiviruses depends on high mutation rate and also attributed by several other factors including increased insect vector populations, and can rapidly evolve in response to changes in the environment and diverse host ranges (Hou and Gilbertson, 1996). The host range of geminiviruses is rather narrow and some genes are known to function in host range determination. In monopartite geminiviruses, a small ORF called C4 has been shown to encode a host range gene (Yahara et al., 1998). Three independent studies using site-directed mutagenesis showed that mutations in ORF C4 that do not cause amino acid replacement in ORF C1 do change host range/or disease severity (Jupin et al., 1994). Thus it is highly probable that, products of ORF C4 interact with defence genes of the host, although interaction might be indirect (Yahara et al., 1998). Thus the genetic diversity generated by high mutation rates and frequent recombination allows the rapid evolution of viruses in response to host defenses (Power 2000).

How do plants defend themselves against the evolutionary potential of invading viruses? A variety of defense responses have been reviewed recently (Carrington and Whitham, 1998), but one of the most exciting areas of current research is post-transcriptional gene silencing (PTGS). Recent work on PTGS in plants has provided evidence that this mechanism functions as a general defense against virus invasion. Viral invasion can induce gene silencing and provide cross-protection against secondary virus infection (Ratcliff et al., 1999; Ding, 2000). At the same time, suppression of gene silencing is a general strategy used by a broad range of DNA and RNA plant viruses. Successful virus infection results from a virus’ ability to prevent PTGS-mediated degradation of its genome, either by directly incapacitating the plant’s PTGS response.
or by moving through the plant more quickly than the PTGS response or both (Waterhouse et al., 1999).

2.6 RNA silencing

RNA silencing in plants is considered to be an adaptive defense mechanism against viruses (Voinnet 2001; Waterhouse et al., 2001). RNA-silencing, including posttranscriptional gene silencing (PTGS in plants) and RNA interference in animals and gene quelling in fungi, represents a sequence-specific RNA degradation mechanism directed against invasive nucleic acid molecules (Fire et al., 1998). RNA-silencing, a robust host defense mechanism against plant viruses is generally countered by virus-encoded silencing suppressors. PTGS, a sequence-specific defense mechanism that can target both cellular and viral mRNA for degradation, is widely used as a tool for inactivating gene expression. Three initially unrelated lines of research led to the recognition of RNA-silencing as an important means of defense against viruses (Sharma and Ikegami 2008). The first clue came from studies of transgene-induced RNA-silencing in which attempts to overexpress endogenous genes by introducing additional copies resulted instead in turning off the endogenous gene as well as the transgene (Napoli et al., 1990). The second line of research led to the discovery of pathogen-derived resistance in that RNA-silencing directed against a viral transgene provided resistance to any virus carrying the target sequence (Baulcombe 1996). Thus viruses could be targets of RNA-silencing. A common feature of RNA-silencing involves structured or double-stranded (ds) RNA that is processed into small interfering (si) RNAs of 21–25 nucleotides by the enzyme Dicer, a member of the RNase III family of dsRNA-specific endonucleases. The siRNAs become incorporated into an RNA-induced silencing complex (RISC) via a Dicer associated protein R2D2 that links the initiation and execution of RNA-silencing. It is induced in response to viral replicating form RNA, presumably to degrade the invasive viral genomes. Plant endogenous or introduced genes that have homology with an infecting viral RNA also could be silenced (Jones et al., 1999, 2001). This process is known as virus-induced gene silencing (VIGS). It has been found that DNA viruses, including geminiviruses, also are capable of inducing gene silencing (Chellappan et al., 2004; Kjemtrup et al., 1998).
There are approximately 450 species of plant-pathogenic viruses, which cause a range of diseases. However, plants have not been passive in the face of these assaults, but have developed elaborate and effective defence mechanisms to prevent, or limit, damage owing to viral infection. Plant resistance genes confer resistance to various pathogens, including viruses. The defence response that is initiated after detection of a specific virus is stereotypical, and the cellular and physiological features associated with it have been well characterized. Recently, RNA silencing has gained prominence as an important cellular pathway for defence against foreign nucleic acids, including viruses. These pathways function in concert to result in effective protection against virus infection in plants (Jennifer et al., 2005)

2.6.1 Different pathways of silencing mechanism

There are at least three different pathways in the gene silencing mechanism: cytoplasm siRNA silencing, silencing of endogenous mRNAs by microRNA (miRNAs), and DNA methylation and suppression of transcription (Baulcombe 2004). SiRNA-directed de novo methylation of DNA and histone proteins (e.g. H3K9), leading to transcriptional gene silencing (TGS). The third pathway supports methylation of DNA virus genomes, inhibiting virus replication and/or transcription. That is, RNA-directed methylation (RdDM) is a novel form of defense against DNA viruses.

DNA methylation has been implicated in both gene regulation and transgene silencing in plants, in addition to its known role in genomic imprinting and the control of parasitic elements. Understanding transgene silencing is a challenge that needs to be overcome for the successful exploitation of transgenic plants as a recombinant protein production system. It is clear that understanding the part that DNA methylation plays in silencing will bring this goal much closer (Wassenegger 2000).

2.6.1.1 RNA dependent DNA methylation

The first demonstration of RdDM that RNA can trigger the cytosine methylation of identical genomic DNA sequences came from experiments using an RNA viroid, a short circular infectious RNA species with a high degree of secondary structure, in tobacco (Wassenegger et al., 1994). RdDM involves methylation of DNA homologous to a triggering RNA is methylated de novo (Mette et al., 2000). RdDM occurs at both symmetric cytosine sites (CpG and CpNpG, where N is A, T, C, or G) and at
asymmetric sites (CpHpH, where H is A, T, or C). Cao and associates (2003) proposed that the initial establishment of RdDM requires the enzyme known as domains rearranged methylase (DRM), which is guided by siRNA to the target sequences. The maintenance of CpG methylation is accomplished by methyltransferase, whereas CpNpG and CpHpH methylation is maintained by chromomethylase and DRM redundantly (Cao et al., 2003). siRNA homologous to the promoter region of a target gene induces transcriptional gene silencing (TGS), which is associated with promoter methylation. If siRNA is homologous to the coding region of the target gene, it induces posttranscriptional gene silencing (PTGS), which involves sequence-specific RNA degradation and methylation of the coding region. PTGS and TGS are mechanistically related because both involve the production of siRNA (Sijen et al., 2001).

Wassenegger et al., (1994) engineered tobacco plants to carry viroid-identical DNA sequences in their genomes on integrated transgenes. These target transgene sequences become efficiently cytosine methylated in strains in which the viroid is actively replicating but are not methylated in replication-deficient controls. More recently, several other plant RNA viruses have been shown to trigger methylation of identical DNA sequences during the course of infection (Jones et al., 1998; Jones et al., 1999; Waterhouse et al., 2001). Because RNA viroids and viruses produce only RNA species during their replication cycles, these experiments provide clear evidence that RNA can communicate directly with matching DNA sequences. Clues to the nature of the RNAs that attract the DNA methylation machinery came from the observation that RdDM often occurs together with RNA interference (RNAi) where RNAi both triggered by dsRNA. dsRNAs are cleaved by enzymes of the Dicer family to generate small, 21-26 nucleotide siRNAs. These in turn are taken up by the RNA-induced silencing complex (RISC) to direct degradation of complementary RNA sequences. Such RNAs are also involved in RdDM, which is mediated by three classes of methyltransferase: DRM, CMT and MET1 (Mathieu and Bender 2004) (Fig. 5).

The coincidence of RNAi and RdDM has been demonstrated in plant RNA virus systems. For example, when an RNA virus carrying green fluorescent protein (GFP) sequences infects tobacco that expresses GFP from an integrated transgene, GFP siRNAs are produced, GFP transcripts become degraded by RNAi and the GFP DNA
sequences become methylated (Jones et al., 1999; Vaistij et al., 2002). Mechanisms for generating dsRNA in plants. dsRNA can be generated through replication of ssRNA viruses (A) or transcription of inverted repeats (B). It can also be generated from endogenous genes or transposons whose transcripts become substrates for RNA-directed RNA polymerase (RdRP); the action of Dicer-like enzymes can then amplify the effect, generating primers for RdRP (C). Alternatively, transcription from promoters on opposite strands could yield complementary RNAs that undergo intramolecular pairing (Mathieu and Bender 2004) (Fig. 6).

2.6.1.2 RNA-directed DNA methylation as a plant genome defense mechanism

High levels of dsRNA produced by viral infections or highly transcribed transgenes can provoke DNA methylation. For example members of the *Geminiviridae* are true DNA viruses that replicate circular, single-stranded DNA genomes in the nucleus by a rolling-circle mechanism that employs host replication machinery (Hanley-Bowdoin et al., 2004) The double-stranded DNA (dsDNA) intermediates that mediate both viral replication and transcription associate with cellular histone proteins to form mini-chromosomes (Pilartz and Jeske, 2003). Transcripts produced from these mini-chromosomes are subject to PTGS, and geminiviruses and their associated satellites have been shown to encode a variety of proteins that can suppress this defense (Trinks et al., 2005; Vanitharani et al., 2004). In addition, given the role of RNA-directed methylation in silencing endogenous invasive DNAs, it is reasonable to propose that plants might also use methylation as a means to repress transcription and/or replication from a viral mini-chromosome (Bisaro 2006). Fig. 7 depicts a putative pathway for RNA-directed DNA methylation during geminivirus infection, which explains viral genome targets, may be transcribed by an RNA polymerase IVa complex (Pol IVa; containing NRPD1A and NRPD2). Resulting single-stranded RNA (ssRNA) is converted to dsRNA by complexes containing RDR2. The 24-nt siRNAs processed from dsRNA by DCL3 are loaded into complexes containing AGO4, which subsequently associates with Pol IVb (containing NRPD1B and NRPD2). The AGO4-associated siRNAs target the complex to homologous DNA sequences, where cytosine methyltransferases (e.g., DRM1/2) are recruited. Cytosine methyltransferases CMT3
and MET1 are primarily involved in methylation maintenance at CNG and CG sites, respectively. CNG methylation by CMT3 is also linked to H3K9 methylation carried out by KYP2 (Raja et al., 2008).

It was shown that in vitro methylation of geminivirus DNA greatly reduces its ability to replicate in plant protoplasts (Brown et al., 1992), and demonstrated that geminivirus AL2 (also known as AC2 or C2) and L2 proteins can act as silencing suppressors by interacting with and inhibiting adenosine kinase (ADK) (Wang et al., 2005). ADK is required for efficient production of the methyl group donor S-adenosyl methionine (SAM), and the primary defect of ADK-deficient yeast and plants is methylation deficiency (Moffatt et al., 2002). Thus, it is possible that one role of the AL2 and L2 proteins is to counter a methylation-based defense. At least one geminivirus-silencing suppressor protein has been hypothesized to counter this defense by inhibiting methylation reactions (Wang et al., 2003). RNA-silencing in multicellular plants and animals is mediated by 21–24 nt small RNAs (sRNAs) that guide sequence-specific gene regulation, chromatin modification, and defense against viruses. These sRNAs are broadly classified into miRNAs and siRNAs, which have similar chemical structures but differ in function and mode of biogenesis. Production of both types of sRNAs depends on the activity of Dicer proteins (Sharma and Ikegami 2008).

3. MATERIALS AND METHODS
Molecular biological procedures outlined in Sambrook *et al.*, 2000 were followed. Details of commonly used protocols as modified are given in Appendix-I and the composition of buffers and reagents are provided in Appendix-II.

### 3.1 Experimental Material

Viruses used in the study are *Tomato leaf curl New Delhi virus* (ToLCNDV was maintained on tomato using whitefly-mediated inoculation in cage under glass house conditions. Virus infection was confirmed by leaf curl symptoms and by PCR.

#### 3.2 Virus, host plants and whitefly mediated transmission studies

Three weeks old tomato seedling cv. Pusa ruby inoculated with pure culture of ToLCNDV showing typical leaf curl symptoms at 15dpi was used as parental viral stock population. For whitefly mediated virus transmission of parental virus, virus free colonies of *B. tabaci* were established on brinjal plants and in growth chamber at 26°C. In order to transmit the virus to test plants, for inoculation access feeding, whiteflies were reared in groups of 15 to 20 on tomato plants for 24 hr period, which were infected with ToLCNDV pure culture and leaves with maximum number of whiteflies situated were plucked and transferred to healthy test plants and covered with the nylon screen in order to access the viral infection through white flies. All transmission experiment such as acquisition and inoculation access feeding periods took place isolated cages kept in a growth chamber at 26°C with a photoperiod of 16:8 hr (light:dark). This standardized protocol (Caciagli *et al.*, 1995) was followed through out our transmission experiment. To maximize the transmission success, 10 plants were kept for virus transmission per passage.

#### 3.2.1 Serial transmission of virus

In order to study the variability in terms of mutation in symptom determinant ToLCNDV-AC4, three kinds of hosts were selected viz., tomato, tobacco and papaya. Three independent passages of ToLCNDV were maintained by serial transmission of ToLCNDV through whiteflies. All these passages were originally initiated from the parental stock population, so that genetic variation among infected test plants was minimal.
3.2.2 Total DNA extraction and PCR amplification
Total DNA was extracted from infected host samples using DNeasy plant mini kit (Qiagen). The AC4 specific primers corresponding to nucleotide (nt) 2251 to 2427 of ToLCNDV (Accession no.DQ169056) were used to amplify 177bp fragment covering the full length of AC4 ORF. The PCR reaction was performed with 0.5 µl pfu DNA polymerase (5u/µl; NEB).

Primer sequences
Forward primer: 5’ GATGAATTCA CTGGGTCTCCG GCATATCCAT 3’
Reverse primer: 5’ GCTGGATCCCTAGAAC GTCTCCATCTTTGT 3’

PCR profile
Initial denaturation of DNA at 94°C for 4 min.
Denaturation at 94°C for 30 sec
Annealing at 55°C for 40 sec 30 cycles
Primer extension at 72°C for 30 sec.
Final primer extension was at 72°C for 10 min

3.3 HRM analysis, cloning and sequencing
The HRM process consists of performing PCR in the presence of DNA binding dye Evagreen, monitoring the progressive change in fluorescence caused by release of the dye from DNA duplex as it is denatured by increasing the temperature, collecting a high resolution melting curve and identifying the samples with melting curve aberrations indicative of the presence of a sequence variant. Fluorescent intensity as a function of temperature, monitored by LC-480 II instrument can reveal very small change in the melting curve shape when analysed with the LC-480 II® using the gene scanning mode.

To examine the mutation in AC4 gene after three passages through HRM analysis, we chose 177bp AC4 amplicon and PCR products were analyzed by HRM using the 96-well Light Cycler 480® (Roche Diagnostics) using Type-it HRM PCR kit (Qiagen) with a total volume of 25 µl – included 20ng of genomic DNA, 2X HRM PCR master mix containing Evagreen fluorescent dye, 0.7 µM specific forward and reverse
primer of 1 µl each and RNase free water and PCR was performed in a Light Cycler 480 instrument (Roche Diagnostics). Melting data were analyzed by the Light Cycler 480® “Gene scanning” software and detection of AC4 variants were optimized by fluorescence normalization and difference curve analysis. Standard sensitivity and auto group settings were used for all samples. At least one to three representative groups from each type of melting curve variants were selected for sequencing. The obtained melting curves of PCR products of infected samples from all three stages of passage were compared on the difference plots and normalised melting curves of temperature overlaid. In order to avoid the bias that might result from PCR and handing errors, the PCR products from HRM reactions were purified using Minelute® PCR purification kit (Qiagen) and then sequenced (Chromous biotech, India) to confirm the variability obtained from HRM analysis using AC4 forward and reverse primer. Another replica of PCR products were cloned into pGEM-T Easy vector (Promega) and transformed into Escherichia coli DH5α according to the manufacturer’s instructions. About 20-25 clones were randomly picked from each viral population specific to each viral passage and the host. The plasmid DNA was extracted from each clone, quantified and sequenced using either T7 forward and SP6 reverse primers or pair of AC4 forward and reverse primers.

**HRM reaction profile**

Initial PCR activation step at 95°C for 5 min

3 step cycling:

- Denaturation at 95°C for 20 sec
- Annealing at 56°C for 30 sec
- Extension at 72°C for 30 sec

45 cycles

HRM at 65°C for 1 sec (Continuous fluorescence data acquisition, ramp rate 0.02°C/s)

Cooling at 40°C

3.3.1 Determination of variability

The sequencing data was analysed for the presence of mutation by aligning the obtained AC4 sequence data with parental AC4 sequence, that initiated the infection using
BioEdit software. Each progeny clone with one or more base different from the parental sequence was recorded as a mutated clone and each nucleotide change was counted as a mutation. Identical variants detected in the same passage from different host were counted as a single unique variant. The variability of viral population was indicated by the percentage of mutated clones and mutation frequency, which was calculated as the total number of mutations observed in all clones of each population divided by the total number of bases sequenced in the populations.

3.4 Methylation analysis by Bisulfite DNA sequencing.
Bisulfite modifications and sequencing were performed as described (Grunau et al. 2000). Incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged (Fig.8A). Bisulfite conversion (Fig.8B) of DNA was carried out from EpiTech Bisulfite kit (Qiagen). HRM analysis was done with bisulfite converted DNA to monitor bisulfite conversion rate. In order to determine the methylation status in AC1 region, GC rich region of AC1 was selected in which the complete AC4 ORF resides. AC1 specific primers were used to amplify AC1-GC rich region from bisulfite converted DNA from ToLCNDV infected tomato and from lhp-AC4 expressing transgenic tomato. Specific primers designed against converted template were used to amplify the selected AC1 region by PCR. PCR products were separated by electrophoresis in a 1% agarose gel, purified using the QIAquick gel extraction kit (Qiagen), ligated into pGEM-T-Easy (Promega), and introduced into Escherichia coli DH5α cells. Plasmid DNA was prepared from E. coli using a Qiagen Spin miniprep kit (QIAgene) and cloned DNA was sequenced with T7 or SP6 primers. The obtained sequences were analysed for the methylation using BioEdit software.

**Bisulfite conversion thermal cycler conditions**

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>5 min</td>
<td>99°C</td>
</tr>
<tr>
<td>Incubation</td>
<td>25 min</td>
<td>60°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>5 min</td>
<td>99°C</td>
</tr>
</tbody>
</table>
Incubation  85 min  60°C  
Denaturation  5 min  99°C  
Incubation  175 min  60°C  
Hold  Indefinite  20°C  

**Primer sequences**

Forward primer: 5' ATTTTTGGGGGTAAATTTTTTTT 3'  
Reverse primer: 5' AAAAAAACACTTTCCCAATTACA 3'  

**PCR profile**

Initial denaturation of DNA at 94°C for 4 min.  
Denaturation at 94°C for 30 sec
Annealing at 60°C for 40 sec  
Primer extension at 72°C for 30 sec.  
35 cycles  
Final primer extension was at 72°C for 10 min  

3.5 **Plant material and virus infection**

3.5.1 **Plant material, virus infection and temperature treatment**

In the present study we had selected three hosts *viz. Solanum lycopersicum, Carica papaya, Nicotiana tobaccum* to study the effect of ToLCNDV infection on the expression of miRNAs. Seedlings were grown under glasshouse conditions (16 hour photoperiod, temperature at 28°C). Twenty five days old seedlings were kept under netted cage for whitefly mediated ToLCNDV transmission. Sampling and morphological analysis were done 10, 15 and 25 days after infection. Same aged plants corresponding to each time point were taken as control. To study the temperature effect on miRNA expression level, 40 days old control and infected *Solanum lycopersicon, Carica papaya, Nicotiana tobaccum* seedlings were kept in separate chambers (five plants in each chamber) maintaining temperature of 12°C, 25°C, 30°C, 35°C and 40°C provided under the National Phytotron Facility available at IARI, New Delhi.

3.5.2 **PCR/Real-time based detection and quantification of miRNAs**
3.5.2.1 Polyadenylation of miRNAs
Small RNA samples isolated from the infected plants were polyadenylated (Fig.9) at 37°C for 60 min in a 50 µl reaction volume containing 0.5 µg RNA and 1.5 U poly (A) polymerase (NEB). An equal volume of Acid-Phenol: Chloroform was added, mixed and centrifuged. The aqueous phase was carefully removed to a new tube. The poly (A)-tailed small RNA was purified from the sample using a purification filter cartridge provided in the mirVana Probe & Marker Kit (Ambion). Briefly, Binding/Washing Buffer (12 volumes of the sample) was added to the sample and mixed thoroughly. The mixture was applied onto the purification filter cartridge and centrifuged. The cartridge was then washed with 300 µl of Binding/Washing Buffer. Finally, the poly (A)-tailed small RNA was recovered with two sequential elutions using 25 µl elution buffer each time.

3.5.2.2 Ligation of poly (T) tail primer
About 2 µg of the tailed RNA and 1 µg of RTQ primer (containing poly T’s at 3’ end) 5’-CGA ATT CTA GAG CTC GAG GCA GGC GAC ATG GCT GGC TAG TTA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTC C(T25)-3’ were mixed in a 26 µl reaction volume, incubated at 65°C for 10 min, and annealed at 4°C for 20 min.

3.5.2.3 Construction of cDNA library
3.5.2.3.1 Total RNA cDNA library
Total RNA cDNA library was generated by mixing 500 ng of RNA and 1 µg of Random hexamer primer in a 26 µl reaction volume, incubated at 65 °C for 10 min. followed by addition of reverse transcriptase 200U (M-MuLV Reverse transcriptase, NEB), 1 µl dNTP mix (10mM) and 10X buffer in a final reaction volume of 40 µl at 37 °C for 60 min. Reverse transcriptase was inactivated by incubation at 70 °C for 15 min. and 5U RNase H (NEB) was added to remove small RNAs. The sample was purified using the QIAquick spin PCR purification Kit (QIAGEN) in a final volume of 50 µl. The srcDNA concentration was quantified using NanoDrop spectrophotometer (NanoDrop technologies).
3.5.2.4 Small RNA cDNA library

3.5.2.4.1 Polyadenylation of miRNAs
Small RNA samples isolated from the plants were polyadenylated at 37°C for 60 min in a 50 μl reaction volume containing 0.5 μg RNA and 1.5 U poly (A) polymerase (NEB). An equal volume of Acid-Phenol: Chloroform was added, mixed and centrifuged. The aqueous phase was carefully removed to a new tube. The poly (A)-tailed small RNA was purified from the sample using a purification filter cartridge provided in the mirVana Probe & Marker Kit (Ambion). Briefly, Binding/Washing Buffer (12 volumes of the sample) was added to the sample and mixed thoroughly. The mixture was applied onto the purification filter cartridge and centrifuged. The cartridge was then washed with 300 μl of Binding/Washing Buffer. Finally, the poly (A)-tailed small RNA was recovered with two sequential elutions using 25 μl elution buffer each time (Fig.10).

3.5.3 Designing of primers
To investigate the effect of viral infection on expression of miRNAs by real-time PCR, microRNA-specific forward primers and a universal reverse primer along with RTQ primer (Seungil et al., 2006) and U6 snRNA primer were designed using BioEdit software version 5.09.04. (Table 2). The mature miRNA sequences were downloaded from miRNAs Registry database (http://miRNA.sanger.ac.uk) and from previous publications (Li et. al., 2010)

Table: 2 List of primer sequence for miRNA quantification

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Forward Primer (5' → 3')</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR159</td>
<td>TTGGATTGAAGGGAGCTCT</td>
<td>MIMAT0009141</td>
</tr>
<tr>
<td>miR164</td>
<td>GAGAAGCAGGGGCACGTG</td>
<td>MIMAT000185</td>
</tr>
<tr>
<td>miR167</td>
<td>GAAGCTGAGCCAGCAGATG</td>
<td>MIMAT0007917</td>
</tr>
<tr>
<td>miR171</td>
<td>TGATTTGAGGCCGTCGCAATAT</td>
<td>MIMAT0007922</td>
</tr>
<tr>
<td>miR319</td>
<td>CTTGGACTGAAGGGAGCTCC</td>
<td>MIMAT0009145</td>
</tr>
<tr>
<td>miR393</td>
<td>CAAAGGGATCGCATGATGC</td>
<td>MIMAT000934</td>
</tr>
<tr>
<td>miR398</td>
<td>GTTCTCTAGGCTCACC</td>
<td>MIMAT000948</td>
</tr>
<tr>
<td>Extension tail primer</td>
<td>CGAATTCTAGAGCTCGAGGCAGG</td>
<td></td>
</tr>
<tr>
<td>RTQ-UNIr</td>
<td>CGAATTCTAGAGCTCGAGGCAGG</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table: 3 List of primer sequences for mRNAs quantification

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence (5' → 3')</th>
<th>Related miRNA/mRNA</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF8</td>
<td>TGGGAAAGGAAGAGGCTGAA (F)</td>
<td>miR 167</td>
<td>FJ222762.1</td>
</tr>
<tr>
<td></td>
<td>GCGATCCAAGAGATGCGATT (R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MYB</td>
<td>TATTGAGATGCGGAAAGAGTTGC (F)</td>
<td>miR159</td>
<td>AY131231.1</td>
</tr>
<tr>
<td></td>
<td>ATCTGTTCGTCCTGTAATCTTTGC (R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCP1</td>
<td>TCCCATTCCGGCCGCGACCTA (F)</td>
<td>miR319</td>
<td>AJ561196.1</td>
</tr>
<tr>
<td></td>
<td>TGATCCGTACCTCTGGTATTGTGGCA (R)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LoxD</td>
<td>ATGGCACTTGCTAAAGAAATT (F)</td>
<td>TCP</td>
<td>U37840.1</td>
</tr>
<tr>
<td></td>
<td>TCATATCGATACACTATTTGG (R)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F: Forward primer, R: Reverse primer

#### 3.5.4 Detection/quantification of miRNAs

A small RNA specific primer (Table 2) and a universal reverse primer, RTQ-UNIr 5’-CGA ATT CTA GAG CTC GAG GCA GG-3’, were used for amplification of the miRNAs. Conventional PCR and real-time quantitative PCR (Q-PCR) was performed using SYBR Green PCR Master Mix (Roche), 1 μl (25 ng) of the synthesized srcDNAs and 5 μM of the primers in a 25 μl reaction volume on the LC 480 II Real-Time PCR System (Roche). A 3-step PCR protocol (95°C for 10 min, then 40 cycles of 95°C for 15 sec, 50°C for 30 sec and 60°C for 30 sec) was used. The annealing temperature was adjusted according to the Tm of the small RNA. After PCR, an aliquot of 2 μl of the PCR product was analyzed on a 2% agarose gel.

#### 3.5.4.1 Expression levels of transcription factor and LoxD

##### 3.5.4.1.1 Detection of TFs and LoxD transcripts

To investigate the effects of viral infection on expression of miRNAs target mRNAs by real-time RT-PCR, gene-specific primers for ARF8, MYB, TCP1 and LoxD were designed (Table 3). For PCR amplification, the total RNA was isolated from plant samples using Trizol reagent (Invitrogen), reverse transcribed to cDNA using reverse primer and subjected to conventional PCR and RT-qPCR. Primers were validated using gel electrophoresis of PCR amplicons, and by the presence of single peak on the melting curve. The SYBER Green PCR was performed on Roche LC 480II real-time
PCR machine using 25 ng cDNA, 40nM forward and reverse primer each and 1x SYBER Green (Roche) in a 20 µl reaction mix. The real-time experiment was conducted in triplicate and agarose gel analyses were performed to verify the products.

3.5.4.2 Expression levels of miRNAs

3.5.4.2.1 PCR amplification and quantification of miRNAs and transcripts

The cDNA and srcDNA samples (10 ng/µl) were PCR amplified using microRNA and transcript specific forward primer and a universal reverse primer RTQ-UNIr (Table-2). 20 µl reaction volume was having 2 µl of cDNA/srcDNA as template, 1 µl (10 mM) reverse primer, 1 µl (25mM) MgCl₂, 0.25 U Taq DNA polymerase (NEB) and 11.5 µl sterile distilled water (SDW) using PCR protocol (95 °C for 2 min., then 30 cycles of 95 °C for 10 sec., 55 °C for 30 sec. and 72 °C for 30 sec.). PCR amplification was confirmed on 1.5% agarose gel electrophoresis. After PCR confirmation, real time quantitative PCR (RT-qPCR) was performed using 10 µl of 2X Light Cycler® 480 SYBR Green 1 master mix (Roche), 0.8 µl RTQ-UNIr primer (10 µM), 0.8 µl forward primer (10 µM) and 2 µl (10 ng/µl) of cDNA/srcDNA to a final reaction volume of 20 µl on the Light Cycler® 480 II (Roche), using above mentioned PCR protocol. After performing real-time RT-PCR, the relative expression level of all the transcripts and miRNAs were calculated using the comparative 2⁻ΔΔCt method.

3.5.5 Quantification of ToLCNDV-AC4 by real-time PCR

For the quantification of ToLCNDV-AC4, Real time PCR was employed. The oligonucleotide primers were used to perform quantitative real time PCR were designed from ToLCNDV AC4 gene sequence available in GenBank, [Accession no. DQ169056]. The PCR primers i.e. forward 5’ GATGAATTCATGGGTCTCCGCATATCCAT 3’ and reverse 5’ GCTGGATCCCTAGAACGTCTCCATCTTTGT 3’ for 177 bp amplicon were designed using bioinformatic tool ‘Primer3’ programme (http://frodo.wi.mit.edu/). First strand cDNA was synthesised by RT from 1 µg of DNase treated total RNA from each infection period (10, 15 and 25dpi) using first strand cDNA synthesis kit (Qiagen) in a total volume of 20 µl according to manufacturer’s instructions. For the semi-qPCR
amplification, 1 µl of cDNA amplified by PCR (95°C for 5 min, then 95°C for 1 min, 55°C for 30 s, 72°C for 1 min for a maximum of 35 cycles) in 25 µl reaction volume using 10 µM of primers. For qPCR amplification, 25ng of cDNA were amplified using 25 ng cDNA, 0.5 mM forward and reverse primer each and 1X SYBR Green (Roche) in a 25 µl reaction mix. The amplification of all the samples was confirmed by observing the amplification plot. The Ct-value was determine by using instrument’s software and adjusted manually as necessary. The real-time experiment was conducted in triplicate.

3.5.6 Detailed Mathematical Explanation of 2^{−ΔΔCt} method Analysis Method

Comparative C_t (threshold cycle) method is a gene quantitation approach. This involves comparing the C_t values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator (healthy control) and the samples of interest are normalized to an appropriate endogenous housekeeping gene (U6 snRNA: U6 small nucleolar RNA) for miRNA and Actin for transcripts. The comparative Ct method is also known as the 2^{ΔΔCt} method, where

\[ ΔΔCt = ΔCt_{sample} − ΔCt_{reference} \]

In this, ΔC_{t, sample} is the Ct value for miRNAs/transcripts normalized to the endogenous housekeeping U6 snRNA/actin and ΔC_{t, reference} is the Ct value for the calibrator (healthy plant sample as control) normalized to the endogenous housekeeping gene (Livak K.J., and Schmittgen T.D., 2001). For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how ΔC_t varies with template dilution. If the plot of srcDNA/cDNA dilution versus ΔC_t is close to zero, it suggests that the efficiencies of the target and housekeeping genes are very similar.

3.6 Detection of miRNA levels - Northern blotting

3.6.1 RNA isolation
In order to detect the expression of miR159, miR164, miR167, miR171, miR319, miR393, and miR398 through northern blotting, total RNA was extracted from 1 g plant tissue, using Trizol reagent (Invitrogen) according to manufacturer’s protocol Appendix-I. The concentration of RNA was measured using a spectrophotometer NanoDrop, ND-1000 (NanoDrop Technologies). After electrophoresis, the RNA was transferred to positively charged nylon membrane (Millipore) by electroblotting.

3.6.1.1 Denaturing polyacrylamide gel electrophoresis (dPAGE)
Twenty micrograms of RNA was resolved in 17% polyacrylamide gel (a 19:1 ratio of acrylamide to bis-acrylamide, 7 M urea and 20 mM MOPS/NaOH buffer (pH 7.0) Appendix-I, in a BioRad apparatus with 20 cm long plates and 1.5 mm spacers. The gel was poured immediately after adding TEMED and APS and was let to cast at least for 1 h. Pre-run was given for half an hour before loading and leached urea was flushed from the wells just prior to loading. Samples were denatured in 50% deionised formamide with bromophenol blue tracking dye at 95°C for 3 min and placed back on ice until they were loaded. Samples were run at a constant 90 V for 3-4 h at room temperature or by the time bromophenol blue reached near to the bottom of the gel.

3.6.1.2 Transferring RNA by Electroblotting
Once the RNA was run far enough the gel assembly was disassemble and carefully the gel was placed on the top of a pre-wet positively charged nylon membrane (Millipore), in RNase free water (DEPC treated or ultra purified). Gel + membrane were then placed on 3 sheets of 3MM Whatman paper (again pre-moistened with water). Gel+membrane+3MM were then placed on the flat positive electrode of a semi-dry electroblotter. Three pieces of water-moistened 3MM was placed on the top of gel, any bubbles roll out with the help of test tube and the flat negative plate-electrode was placed on that. The RNA was transferred at a constant 20V for 30-60 min at 4°C.

3.6.1.3 Crosslinking
For crosslinking 3MM sheet that has been cut just to be slightly larger than the nylon membrane was wrap in Saran wrap to prevent evaporation and drying of the membrane
and place in an incubator for UV crosslinking. During this time, the RNA becomes crosslinked to the nylon. After this, membrane was taken out for pre-hybridisation and hybridisation process, or dry and store the membrane until use (wrapped in clean Saran wrap, at room temperature in the dark).

### 3.6.1.4 Prehybridization

Prehybridization solution was added at the rate of 0.2 ml/sq cm. The cylinders were then incubated at 38°C for 4h in hybridization oven with gentle rotation.

### 3.6.1.5 Probe preparation (End labelling)

The end labelling reaction was done in 20 µl reaction mixture. The single stranded DNA oligonucleotide complimentary to *Arabidopsis* miRNAs was end labelled with $\gamma ^{32}$P-ATP using T4 polynucleotide kinase (NEB). The 50 ng of DNA template, 2 µl of 10x buffer, 0.5 µl T4 polynucleotide kinase was added into an microfuge tube containing nuclease free H$_2$O to make 20 µl and incubated at 37°C for 1 h. The labelled probe can be stored at -20°C till further use.

### 3.6.1.6 Hybridization

The blot hybridization was performed at 38°C for overnight in hybridization buffer (6x SSC, 5x Denhardt’s solution and 0.2% SDS,), using ssDNA oligonucleotide probe complimentary to *Arabidopsis* miRNAs.

### 3.6.1.7 Washing

The hybridization solution was discarded and the membrane was washed two times with 2x SSC, 0.5% SDS for 30 min at 38°C and last wash at 42°C. The washed membrane was dried on a paper towel.

### 3.6.1.8 Autoradiography

The dried membrane was then placed in a lead cassette and exposed to X-ray film (Kodak) for 18 h at -70 °C. Autoradiograph was then developed as per X-ray films.
manufacturer’s instructions. For repeated hybridization the membrane was stripped with 0.5% SSC, 0.5% SDS for 30 min at 80°C and then with 0.1× SSC, 0.5% SDS for 30 min at 80°C.

3.7 Auxin treatment
Primary shoot cuttings were excised from 5 to 6 weeks old ToLCNDV infected and control *Solanum lycopersicon* plants were used in auxin treatment experiment prior to flowering. Two individual sets of infected shoots were treated with IBA concentration 0.5 mg/L and 2.5mg/L in liquid medium and separate sets of  infected and control shoots were transferred to fresh liquid medium devoid of IBA to compare the effect of auxin treatment and kept in green house condition for root induction. Root parameters were recorded 12 days after incubation. The rooted shoots were transferred to semi sterile substrate (mixture of soil and perlite in the ratio 3:1) for normal growth, three weeks after transfer phenotypic observations were recorded.

4. RESEARCH PAPER I
High-Resolution Melting Curve Analysis to study co-variation in the pathogenecity factor of Tomato leaf curl New Delhi virus

4.1 Abstract:
Tomato leaf curl viruses occur in all tomato growing areas in the world and are considered to be most damaging and highly evolving viruses. We investigated the genetic variation in the pathogenecity factor of Tomato leaf curl New Delhi virus, a prominent virus in South Asian countries. We performed mutation scanning of AC4 gene of ToLCNDV using high resolution DNA melting (HRM) analysis in serial passages to differential hosts *Solanum lycopersicon*, *Nicotiana tobaccum* and *Carica papaya*. Results based on HRM analysis followed by cloning & sequencing showed AC4 is prone to mutation at five different focal points. Most of the mutations (four out of five) found in AC4 region belonged to transition type and were found to be host independent. The transversion mutation at 2296 leading to Ser$^{16}$ to Thr was found to be predominant in solanaeous host and showed high frequency of reversion in the subsequent passaging. Four transition mutations at 2312 leading Ile$^{21}$ to Thr; 2339, 2365 and 2415 leading to Val$^{30}$ to Ala, Thr$^{55}$ to Met and Thr$^{38}$ to Thr respectively (synonymous mutation) were matching with the virus variants of Tomato leaf curl viruses with different degree of symptom severity and pathogenecity at these focal points. Three out of five mutations (one transversions and two transitions) lead to its homology with other begomoviruses at the mutation point. Interestingly two mutations falling in the N-terminal region of AC4 affects the consensus N-myristoylation motif required for membrane binding and pathogencity. The mutation frequency was $10^{-4}$ nucleotide substitution per site (subs/site), which is very close to plant RNA viruses. These observations indicate that pathogenecity factor of Tomato leaf curl virus is important in virus adaptation to different host in serial passages and might be playing a role in evolution. This study also reveals that, AC4 mutation may have structural and functional relationships in host-virus interactions.

**Key words:** Geminiviruses, AC4 mutation, AC4 protein, HRM
4.2 Introduction

Host induced genome evolution allows for adaptation of viruses to their hosts. Adaptive evolution depends upon the host species and degree of pathogenecity by the virus variants. Geminiviruses are devastating viruses of plant that possess single stranded DNA as genome. Some geminiviruses code a small multifunctional protein AC4 whose role in pathogenesis and RNAi has recently attracted attention.

Tomato leaf curl New Delhi virus (ToLCNDV) a member of genus *Begomovirus* and family *Geminiviridae* is a bipartite virus having DNA-A and DNA-B. This virus is present in the form of three variants ToLCNDV (DQ169056), ToLCNDV-Severe (U15015) and ToLCNDV-Mild (U15016), these variants are mostly differ in their pathogenecity response on the common host tomato. The pathogenecity difference of severe and mild strains of ToLCNDV was correlated by a single amino acid change in the AC4 protein. Besides ToLCNDV many other bipartite and monopartite *tomato leaf curl viruses* (ToLCV) are known world wide, all the ToLCV share sequence homology 70-90%. In this study we defined co-variation in pathogenecity factor AC4 of ToLCNDV by mutation scanning between various ToLCV. We are also addressing the similarity of ToLCNDV-AC4 mutation focal points with the other begomoviruses affecting different crops.

The variability in begomovirus genome depends on recombination and mutation rate attributed by several factors including increased insect vector populations, changes in the environment and diverse host ranges (Hou et al., 1996). It is commonly known that, RNA viruses are generally subject to relatively high rates of mutation due to their dependence on error prone DNA dependent RNA polymerases (Van der Walt, 2008) accordingly RNA viruses have been shown to evolve at rates between $10^{-3}$ to $10^{-5}$ substitutions per site per year (Jenkins et al., 2002; Malpica et al., 2002). It is also well documented in DNA viruses especially viruses belongs to family *Geminiviridae* that, mutation is common and frequently occurs in the genome (Harrison 2002). For example mutation frequencies in the order of $10^{-4}$ mutations/site were observed in *Tomato yellow leaf curl china virus* (TYLCCV) in both naturally and experimentally infected tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana* in addition it was also reported that, mutation frequencies in open reading frame (ORF) AC1 that completely overlaps
ORF AC4 was $4.8 \times 10^{-4}$ (Ge *et al.*, 2007). Relatively high rates of nucleotide substitution have also been documented in *Tomato yellow leaf curl virus* (TYLCV) (Duffy and Holmes 2008; Urbino *et al.*, 2008) and Maize streak virus (MSV), these data suggest that geminivirus mutation frequencies are evolving at an unexpectedly rapid rate (Van der Walt *et al.*, 2008) and it is similar to that of RNA viruses (Stenger *et al.*, 1995; Ge *et al.*, 2007) and faster than dsDNA viruses (Bernard *et al.*, 1994; McGeoch and Gatherer 2005). Host induced genome evolution allows for adaptation of plant viruses to their hosts to ever changing environments (Liang *et al.*, 2002; Ge *et al.*, 2007). Adaptive evolution has been found to be either random or selected and it depends on the virus and host species (She *et al.*, 2001). A highly adaptive reversion mutation was detected in 5/8 independent MSV infection within 10 days of inoculation (Shepherd *et al.*, 2006) implying that the virus is capable of adaptive evolution rates rivalling those of even the most rapidly evolving RNA viruses (Van der Walt *et al.*, 2008). During last few decades, viral genome evolution has been investigated at the molecular level in many ways one of such a way is viral populations are transferred serially in a specific host or to a different hosts (Liang *et al.*, 2002) as it is shown in *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) (Kearney *et al.*, 1999; Schneider and Rossinck, 2000). Thus geminiviruses are one of the rapidly emerging groups of plant viruses (Brown and Bird, 1992) and their variation depends ultimately on mutation, is also amplified by various kinds of secondary genome alteration brought about by acquisition of additional DNA molecules, pseudo-recombination or recombination might lead to multiple infection to cause begomovirus epidemics (Harrison and Robinson 1999) which might affect virus survival and spread.

Geminiviral-AC4 is shown to have divergent biological functions. A structural examination of the EACMCV AC4 protein and those of several other geminiviruses that infect a range of crops shows a conserved consensus N-myristoylation motif which is found to be required for producing disease like symptoms, membrane binding and pathogenicity. It was also reported to have RNAi suppressor activity (Vanitharani *et al.*, 2004).

The comprehensive detection of DNA mutation or variants has traditionally relied on re-sequencing, however despite recent advances, re-sequencing is expensive
and time consuming. The simplest, sensitive and specific method for mutation scanning which can reduce the amount of sequencing and potentially increase the efficiency and reduce the cost is HRM (High Resolution Melting) analysis (Wittewer et al., 2003; Reed and Wittewer, 2004). HRM is a new method for DNA analysis introduced in 2002 by collaboration between academics (university of Utah, UT, USA) and industry (Idaho Technology, UT, USA) (Reed et al., 2007).

In this study we scanned mutations in ToLCNDV-AC4 gene during its passage from one specific host to either same host or to a different host, by HRM analysis. In our study we have selected three different hosts for viral passage experiment viz., Solanum lycopersicon, Nicotiana tobaccum and Carica papaya. Our results show that, rapid variation in ToLCNDV-AC4 gene during its passaging to different hosts. We also derived mutation frequency based on sequencing data.

4.3 Materials and methods

4.3.1 Virus, host plants and whitefly mediated transmission studies

Pure culture of ToLCNDV was maintained on three weeks old tomato seedling cv. Pusa Ruby and established typical leaf curl symptoms at 15dpi was used as parental viral stock population. For whitefly mediated virus transmission of parental virus, virus free colonies of B. tabaci were established on brinjal plants were maintained in growth chamber at 26°C. In order to transmit the virus to test plants, for inoculation access feeding, whiteflies were reared in groups of 15 to 20 on tomato plants for 24 hr period, which were infected with ToLCNDV pure culture and leaves with maximum number of whiteflies situated were plucked and transferred to healthy test plants and covered with the nylon screen in order to access the viral infection through white flies. All transmission experiment such as acquisition and inoculation access feeding periods took place isolated cages kept in a growth chamber at 26°C with a photoperiod of 16:8 (light:dark) h. Standardized protocol (Caciagli et al., 1995) was followed through out our transmission experiment. To maximize the transmission success, 10 plants were kept for virus transmission per passage.

4.3.2 Serial transmission of virus
In order to study the variability in terms of mutations in pathogenicity factor ToLCNDV-AC4, three kinds of hosts were selected viz., tomato, tobacco and papaya and three independent passages of ToLCNDV were maintained by serial transmission of ToLCNDV through whiteflies (Fig.11). All these passages were originally initiated from the parental stock population, so that genetic variation among infected test plants was minimal.

4.3.3 Total DNA extraction and PCR amplification

Total DNA was extracted from infected host samples using DNeasy plant mini kit (Qiagen). The primers AC4 F 5’ GATGAATTCA TGGGTCTCCGCATATCCAT 3’ and AC4 R 5’ GCTGGATCCCTAGAACGTCTTTGT 3’ corresponding to nucleotide (nt) 2251 to 2427 of ToLCNDV (DQ169056) were used to amplify 177bp fragment covering the full length of AC4 ORF and part of AC1 coding region. The PCR reaction was performed with 0.5 µl pfu DNA polymerase (5u/µl; NEB). After being preheated at 94°C for 5 min, the reaction mixtures were cycled 30 times at 94°C for 30 s, 55°C for 40 s, and 72°C for 30 s, followed by an extension at 72°C for 10 min.

4.3.4 HRM analysis, cloning and sequencing

The HRM process consists of performing PCR in the presence of DNA binding dye Evagreen, monitoring the progressive change in fluorescence caused by release of the dye from DNA duplex as it is denatured by increasing the temperature, collecting a high resolution melting curve and identifying the samples with melting curve aberrations indicative of the presence of a sequence variant. Fluorescent intensity as a function of temperature, monitored by LC-480 II instrument can reveal very small change in the melting curve shape when analysed with the LC-480 II® using the gene scanning mode.

To examine the mutation in AC4 gene during three passages through HRM analysis, we selected 177bp AC4 amplicon and PCR products were analyzed by HRM using the 96-well Light Cycler 480® (Roche Diagnostics) using Type-it HRM PCR kit (Qiagen) with a total volume of 25 µl – included 20ng of genomic DNA, 2X HRM PCR master mix containing Evagreen fluorescent dye, 0.7 µM specific forward and reverse primer of 1 µl each and RNase free water and PCR was performed in a Light Cycler
480 instrument (Roche Diagnostics). The HRM reaction was same as that of PCR reaction mentioned, in addition included the HRM acquisition for 5 s from 50°C to 95°C to generate melting curves. Melting data were analyzed by the Light Cycler 480® “Gene scanning” software and detection of AC4 variants were optimized by fluorescence normalization and difference curve analysis. Standard sensitivity and auto group settings were used for all samples. At least one to three representative groups from each type of melting curve variants were selected for sequencing. The obtained melting curves of PCR products of infected samples from all three stages of passage were compared on the difference plots and normalised melting curves of temperature overlaid. In order to avoid the bias that might result from PCR and handing errors, the PCR products from HRM reactions were purified using Minelute® PCR purification kit (Qiagen) and then sequenced (Chromous biotech, India) to confirm the variability obtained from HRM analysis using AC4 forward and reverse primer. Another replica of PCR products were cloned into pGEM-T Easy vector (Promega) and transformed into Escherichia coli DH5α according to the manufacturer’s instructions. About 20-25 clones were randomly picked from each viral population specific to each viral passage and the host. The plasmid DNA was extracted from each clone, quantified and sequenced (Chromous biotech, India) using either T7 forward and SP6 reverse primers or pair of AC4 forward and reverse primers.

**Determination of variability**

The resulting data was analysed for the presence of mutation by aligning the obtained AC4 sequence data with parental AC4 sequence that initiated the infection with BioEdit Sequence Alignment Editor Version 5.0.9 (Hall, 1999). The same was used for any other sequence alignment functions during the study until mentioned otherwise. Each progeny clone with one or more base different from the parental sequence was recorded as a mutated clone and each nucleotide change was counted as a mutation. Identical variants detected in the same passage from different host were counted as a single unique variant. The variability of viral population was indicated by the percentage of mutated clones and mutation frequency, which was calculated as the total number of
mutations observed in all clones of each population divided by the total number of bases sequenced in the populations.

4.4 Results

4.4.1 Co-variation in AC4 sequences by HRM analysis
HRM analysis of AC4 sequences (14 melting curves from three different passages) based on the fluorescence differences were grouped to three types of variants. The largest group of AC4 variants (belonging to third stage of passage) were selected as a reference control. Out of 14 high resolution melting curves representing the samples from three stages of viral passage includes tomato, tobacco and papaya; as shown in Fig.11 resulted in 4 unique melting patterns which includes one parental ToLCNDV-AC4 and three variants in second and third passage; variant 2a, 2b and 3a respectively and the melting temperature of parental ToLCNDV-AC4 (82.5°C), variant 2a (82.75°C), variant 2b (83°C) and variant 3a (83.25°C to 83.5°C) (Fig. 12A and B).

4.4.2 Type of mutations in ToLCNDV populations
The variability in ToLCNDV populations is indicated by number and type of mutations occurred in an AC4 gene. All the mutations observed were substitutions, no insertion or deletion mutations were detected. It was found that, transition of C to T found at only one position (2415) as compare to reverse transition which occurred at 3 positions (A to G at 2365th and T to C at 2312th and 2339th position) (Table. 5), suggesting that, these nucleotide positions are most likely the hot spots for mis-incorporation. From the analysis of transitions, it was found that, more than one incident of mutations resulted in the substitution of T to C than any other substitution, these biases in substitution resulted in a higher A/T to G/C substitutions than G/C to A/T substitutions.

4.4.3 Mutation frequency in ToLCNDV populations during its passage
Sequence analysis showed that, the number of clones with atleast one mutation, ranged from 29-36% in passage II and 39-52% in passage III. Variants from passage III exhibited comparatively high nucleotides mutation frequency of 9.8 x 10^-4 mutations/site, approximately five to seven-fold higher than the frequency observed in passage II,
which showed frequency of $4.5 \times 10^{-4}$ and $2.3 \times 10^{-4}$ mutations/site for variant 2a and variant 2b respectively (Table 4). These frequencies are similar to the frequencies observed in geminiviruses like EACMCV (Fondong and Chen, 2011), TYLCCNV (Ge et al., 2007) and TYLCV (Urbino et al., 2008).

<table>
<thead>
<tr>
<th>Variants</th>
<th>Viral passage</th>
<th>% Mutated clones (No. mutated/total no. of clones sequenced)</th>
<th>No. of mutations/no. of bases sequenced</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variant 1</td>
<td>Second</td>
<td>36 (9/25)</td>
<td>2/ (177 x 25)</td>
<td>$4.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>Variant 2</td>
<td>Second</td>
<td>29 (7/24)</td>
<td>1/ (177 x 24)</td>
<td>$2.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Variant 3</td>
<td>Third</td>
<td>43 (10/23)</td>
<td>4/ (177 x 23)</td>
<td>$9.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Variant 4</td>
<td>Third</td>
<td>39 (9/23)</td>
<td>4/ (177 x 23)</td>
<td>$9.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Variant 5</td>
<td>Third</td>
<td>52 (12/23)</td>
<td>4/ (177 x 23)</td>
<td>$9.8 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

**4.4.4 Distribution of mutations in ToLCNDV progeny populations**

To determine the distribution of nucleotide substitution in AC4 fragment, mutations in each progeny of virus population were pooled and a mutation map was generated. In all the passages, viral population showed only point mutations. All four mutations were observed in the population from third passage, which were host independent, identical and uniformly distributed at positions 2312 (ORF AC4-62), 2339 (ORF AC4-89), 2365 (ORF AC4-115) and 2415 (ORF AC4-164). In contrast to this, only one mutation was observed in population from II passage, which was host specific (tomato and tobacco) at position 2296 (ORF AC4-46). All these five point mutations in the AC4 ORF (Fig.13A) changed the amino acid (Fig.13B) encoded in the protein as Ser$^{16}$Thr, Ile$^{21}$Thr, Val$^{30}$Ala, Thr$^{38}$Thr and Thr$^{55}$Met (Fig.14A and B). The first two amino acid changes viz., Ser$^{16}$Thr and Ile$^{21}$Thr specifically occurred at the consensus N-myristoylation motif.
sequence which is conserved in most of the geminiviruses analysed, the last two amino acid changes viz., Val\(^{30}\)Ala, Thr\(^{38}\)-Thr (synonymous mutation) and Thr\(^{55}\)Met occurred at conserved domain of ToLCNDV-AC4 (Table 6 and Fig.14B). Ser\(^{16}\)-Thr mutation was found to be host and passage specific which occurred only in solanaceous hosts during second passage and mutated amino acid threonine was matching with CLCBV-AC4 at the mutation point. This change in amino acid did not affect polarity, whereas Ile\(^{21}\)-Thr occurred in all the hosts (variant 2a, 2b and 3a) during second and third passage was matching with the ToLCNDV-Svr and ToLCNDV-Mild strains at mutation point and affect the polarity (Table. 5), Val\(^{30}\)Ala mutation was unique to ToLCNDV (variant2b and 3a) and did not found matching with any other leaf curl viruses analysed. This mutation did not affect the polarity. Thr\(^{38}\)-Thr was found to be synonymous mutation and did not change the amino acid in AC4 protein. Although Thr\(^{55}\)Met is conserved across leaf curl viruses and found to be matching with ToLNDV-severe strain (Fig.14B).

Table: 6 Consensus sequences of N-myristoylation motif sequences and conserved domains of AC4 protein of selected geminiviruses

<table>
<thead>
<tr>
<th>Name of the virus</th>
<th>Accession no.</th>
<th>N-myristoylation motif sequence</th>
<th>Conserved domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToLCNDV</td>
<td>ADY75700</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLCNDV-Svr</td>
<td>AAA92812</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLCNDV-Mild</td>
<td>AAA92822</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLBV</td>
<td>AAD51290</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLC BV-</td>
<td>AAK19177</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLC BV-</td>
<td>AAL26557</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLCKV</td>
<td>AAW82634</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLC GV-</td>
<td>AAL78670</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLC GV-</td>
<td>AAO25672</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>ToLC GV-</td>
<td>AAM21570</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>TGMV</td>
<td>ABF67530</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
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<td>YP277440</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
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<td>SPLCGV</td>
<td>AAL69644</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
<tr>
<td>PYMTV</td>
<td>AAC09257</td>
<td>MGHRISIFLSKENSNAKIT</td>
<td>MG DSS PQ QHISIR</td>
</tr>
</tbody>
</table>

4.5 Discussion
The AC4 gene variability in ToLCNDV populations were evaluated from HRM analysis and also by cloning, sequencing of viral progeny populations derived from
infecting with single type of ToLCNDV population through whitefly mediated transmission. Serial passage of ToLCNDV in different hosts viz., tomato, tobacco and papaya plants through whiteflies was performed to study the host specific mutations. Our results clearly showed that, ToLCNDV generated variants (w.r.t AC4 gene) rapidly upon its serial passages in three different hosts to generate a heterogeneous populations and based on the mutation distribution and its frequency, the consensus sequence and diversity level in the population were stable as passage of virus was progressed (Table 7). Whereas mutation distribution in the population from each passage was varied. We have determined the genetic diversity of ToLCNDV progeny populations based on the AC4 gene isolated from 20-25 clones from each passage. Results clearly showed that similar to other geminiviruses (Ge et al., 2007; Duffy and Holmes 2008, 2009; van der Walt et al., 2008; Fondong and Chen, 2011) ToLCNDV exhibit frequent substitutions and maintain a heterogeneous population. The levels of genetic variation observed in ToLCNDV in clonal progeny population from III passage and II passage were from $2.3 \times 10^{-4}$ to $9.8 \times 10^{-4}$ substitutions/site which is comparable to the variation reported for plant RNA viruses (Schneider and Rossinck, 2001). Similar kind of mutation frequencies were also observed in TYLCCNV population at different infection periods $4.8 \times 10^{-4}$ for AC1-AC4 overlapping region and $2.6 \times 10^{-4}$ for non overlapping region of AC1 (Ge et al., 2007). It was also reported that a single clone of cassava geminivirus-EACMCV quickly transforms into a collection of mutants upon introduction in to the host (Fondong and Chen, 2011). Higher rate of variations in AC1-AC4 overlapping region were also found in cotton leaf curl virus, and these were thought to be related to the mechanism by which the AC4 gene was generated (by overprinting) and lack of its universal presence in geminivirus (Sanz et al., 1999). It is known that, the high mutation frequency associated with RNA viruses is presumed to be due in large part to their lack of proofreading during replication (Domingo et al., 1994 and 1997; Roossinck, 1997). In comparison, geminiviruses replicate using their host DNA replication machinery (Gutierrez, 1999; Hanley-Bowdoin et al., 2000). Theoretically, these viruses should have less population variation. However, there is no information about the nature of plant DNA polymerase or the polymerase factors involved in the replication of geminiviruses and in particular, it is not known whether only a subset of
cellular DNA replication and/or mismatch repair machinery is activated for geminivirus replication or whether the cellular environment affects the fidelity of those polymerases (Ge et al., 2007). For example it has been suggested (Duffy and Holmes, 2008) that, because geminivirus spends a larger fraction of its infection cycle in the form of ssDNA, host exonucleases, which act on dsDNA, may not repair mutations introduced during replication, or geminivirus genomes might not possess the proper methylation patterns. It has been suggested that, geminivirus genomes recruit a more error prone polymerase from the host’s nucleus for its own replication (Duffy and Holmes 2008).

<table>
<thead>
<tr>
<th>Mutatio</th>
<th>Host</th>
<th>Passage</th>
<th>Reversio</th>
<th>Homology</th>
<th>Homology</th>
<th>Trait/domai</th>
</tr>
</thead>
</table>

Table: 7 Summary of mutations and their role in pathogenesis
Overall we found five nucleotide substitutions in AC4 gene, four were transitions and only one substitution was transversions. The dominance of transitions under natural conditions and under controlled environment is consistent with results obtained in the AC1/AC4 region of TYLCCNV (Ge et al., 2007) and IR/AC1 fragment of EACMCV (Fondong and Chen, 2011). The two possibilities for mis-incorporation of nucleotide in geminivirus genome was explained by Duffy and Holmes (2008): since geminivirus DNA is only transiently double stranded during rolling circle replication, it may not be
suitable for base excision repair, another possibility is substitution biases occur in ssDNA either by spontaneous deamination by the action of deaminating host enzymes, they detected high rates of C to T and G to A transitions that were possibly indicative of increased C and G deamination rates. As deamination rates are probably higher for ssDNA, this was taken to imply that, high begomovirus mutation rates are partially attributable to the considerable fraction of their life cycle spent in ssDNA form. However our results showed higher rate of T to C transitions including A to G transition, this substitution bias might lead to in GC content in viral progeny, the possible mechanism for the over representation of T to C transitions were not known (Fondong and Chen, 2011). We also found T 2296 A reversion in AC4 in III passage, which implies that, a selective host specific pressure might be required for the stabilization of the genome at this focal point (Fondong and Chen, 2011). The point mutations observed in five different sites of AC4 region during II and III viral passage were not found to be responsible for phenotypic expression of symptoms. The observed genetic changes in the pathogenicity factor might be crucial for host adaptations and are evolving under positive selection pressure (van der Wallt et al., 2008). Natural selection may have favoured these mutated sites in the genomes which might be playing some role in defense mechanism as well as in pathogenicity.

4.6 Conclusion:
We have presented evidence from controlled evolution experiment in the pathogenicity factor of ToLCNDV from three different passages using three hosts. Five substitution mutations were observed, two falling at N-myristoylation domain responsible for pathogenicity of the virus and the other three substitutions were in the conserved domain of overlapping AC1-AC4 gene sequence. HRM analysis followed by cloning and sequencing confirmed the sensitivity of HRM analysis and mutation detection. Mutation frequency in different clones suggests that, geminiviral evolving rate are as high as those reported for many RNA viruses. For the first time we show substitution bias for host and severity of symptoms. Co-variation in overlapping AC4 gene, a pathogenicity determinant during ToLCNDV infection suggests its probable role in host specific plant defense mechanism.
Transcriptional changes associated with symptom expression and stress signalling during Tomato leaf curl infection.

5.0 Abstract:
The molecular basis for virus induced symptoms and defense pathways have been longstanding mystery. *Tomato leaf curl virus* induces phenotypic expression of symptoms like stunting, leaf curling and poor root development. These specific developmental disease symptoms results due to transcriptional changes affecting auxin homeostasis and stress signalling in plant cell during viral infection. A comparative transcriptional analysis of certain miRNAs (miR164, miR167 and miR393) and auxin responsive factor 8 (ARF 8) controlling auxin responses, miR159, miR319, miR164 and transcription factor TCP, MYB and lipoxygenase D (Lox D) controlling plant morphogenesis (leaf and root development) and stress signalling was undertaken and it was correlated with disease symptom development. Many of the development disease symptoms and changes in micro RNA levels resemble in ToLCNDV-AC4 expressing transgenic plants, providing a molecular rationale for involvement of viral AC4 in disease development. Virus infected plant shoots respond to auxin treatment with improved root growth and partial recovery in disease like symptoms.

**Key words:** miRNAs, viral suppressor, defense, auxin signalling

5.1 Introduction
Tomato leaf curl New Delhi virus (ToLCNDV) belongs to the family \textit{geminiviridae} having single stranded circular DNA. It infects a broad range of dicotyledonous and monocotyledonous plants and causes various disease symptoms ranging from upward curling of leaves, stunted growth, poor root development etc. These symptom phenotypes are often associated with virus encoded pathogenicity factors, many of which are suppressors of RNA silencing (Vance and Vaucheret 2001).

In recent years it has been shown that microRNAs (miRNAs) regulated gene expression play an important role in plant developmental processes. Various developmental processes like leaf and root development are controlled by many transcription factors, which are regulated by miRNAs. Leaf development, patterning and polarity are controlled by transcription factors, belonging to TCP and MYB family (Wang \textit{et al.}, 2007) regulated by miR319 and miR159 respectively (Palatnik \textit{et al.}, 2003; Millar and Gubler 2005). Similarly lateral root development is regulated by miR164 levels (Guo \textit{et al.}, 2005). Several reports have shown that, many of these miRNA expressions are viral infection responsive (Lang \textit{et al.}, 2011). They are also involved in stress signalling pathway (Wand \textit{et al.}, 2005) and respond to various environmental stresses (Jagadeeswaran \textit{et al.}, 2009; Jones-Rhoades and Bartel 2004) and pathogen invasion (Chapman \textit{et al.}, 2004; Chellappan \textit{et al.}, 2005). Stress responsive JA biosynthesis pathway is regulated by LOX2, which encodes a chloroplast-localized lipoxygenase that catalyses the conversion of a-linolenic acid (18:3) into (13S)-hydroperoxyoctadecatrienoic acid, the first dedicated step in the biosynthesis of the oxylipin JA (Bell \textit{et al.}, 1995). LOX2 is regulated by miR319a through the TCP transcription factors (Schommer \textit{et al.}, 2008).

The phytohormone auxin plays critical roles not only in plant growth (many of which are mediated by the auxin response transcription factors), but is also very crucial for linking stress signalling (Kazan and John 2009). Auxin responses are regulated by ARFs, a class of transcription factors that bind to the promoters of auxin response genes (Nikovics \textit{et al.}, 2006) and this potential interaction between auxin and stress signalling are mediated by certain miRNAs such as miR167, miR319, miR393 and miR164.
Micro-RNA164 down-regulates the auxin signals and reduces lateral root development (Guo et al., 2005). Also, miR393 regulates auxin signalling by targeting transcripts of TIR1 and closely related F-box proteins (Sunkar and Zhu 2004). Transcriptional changes as a result of viral infections leads to the development of symptomatic phenotypes (Bazzini et al., 2007). During viral infection auxin signalling is affected due to disruption of auxin responsive proteins. During infection by Tobacco mosaic virus it is shown that, viral proteins affect/interrupt auxin response proteins. The replicase protein of TMV disrupts the localization and stability of interacting auxin/indole acetic acid (Aux/IAA) proteins, which corresponds to the display of developmental disease symptoms (Padmanabhan et al., 2005). Thus, miRNAs controlling developmental processes and stress signalling form a complex link between the regulatory networks of the host and virus infection.

In the present study, we have analyzed the comparative transcriptional changes of five miRNAs (miR164, miR167, miR159, miR393 and miR319) and some of their target transcription factors like MYB, TCP and ARF8 involved in regulating plant development and stress signalling during viral infection. Besides this we have also compared the levels of defense related transcript LoxD involved in JA biosynthesis. These miRNAs and transcripts were found to be differentially regulated. Interestingly, similar patterns of transcriptional change in relation to these miRNAs and transcripts were found in ToLCNDV-AC4 expressing transgenic plants. Since most of the miRNA and transcription factor studied are auxin responsive, we further demonstrate that exogenous application of auxin during viral infection, resulted in partial recovery of disease like symptoms, suggesting auxin homeostasis is crucial for plant development during infection. The biological significance of auxin signalling in relation to development and stress, during natural viral infection and in transgenic tomato plants expressing viral pathogenicity factor AC4 are discussed.

5.2 Materials and Methods

Plant material and virus infection
Solanum lycopersicum seedlings were grown under glasshouse conditions (16 hour photoperiod, temperature at 25°C). 10-15 days old seedlings were kept under netted cage for whitefly mediated ToLCNDV transmission. Plants were rated for symptom development at 10, 15 and 25 dpi (days after post inoculation). Two biological replications were performed independently for each time point.

5.2.1 Small RNA isolation, Gel blot analysis and quantitative PCR
Leaf samples were collected from healthy and ToLCNDV infected tomato plants at 10, 15 and 25 dpi and quickly frozen in liquid nitrogen and stored at -80°C prior to small RNA isolation. Small RNAs were extracted from 100 mg of leaf tissues using mirVana™ isolation Kit (Ambion) accordingly to manufacture’s instruction. The concentration of small RNA was determined by spectrophotometer NanoDrop, ND-1000 (NanoDrop technologies).

In order to detect the expression of miR159, miR164, miR167, miR319 and miR393 through northern blotting, total RNA was isolated from plant samples using Trizol reagent (Invitrogen). Twenty µg of total RNA was loaded per lane and resolved on a denaturing 17% polyacrylamide gel and transferred electrophoretically to nylon membrane (Millipore). Ethidium bromide staining of polyacrylamide gel was done before membrane transfer to confirm equal loading. The membrane was air dried and UV cross-linked at 1200 joules. The ssDNA oligonucleotides complimentary to miR159, miR164, miR167, miR319 and miR393 were end labeled with [γ-32P]-ATP using T4 polynucleotide kinase (New England Biolabs). Blot was prehybridized for 2 h and hybridized overnight using hybridization buffer containing radiolabeled probe at 38°C. The blot was washed three times, two times with 6X SSC and 0.2% SDS at 30°C for 5 min and once with 6X SSC and 0.2% SDS at 50°C for 10min. The membrane was briefly air dried and then kept at -80°C for autoradiography. RNA gel blot exposed to X-ray film was used to assess relative miRNA levels. The RNA blot analysis was repeated at least twice.

For miR159, miR164, miR167, miR319 and miR393 quantification, small RNA samples isolated from the leaf tissues were poly-adenylated using poly (A) polymerase at 37°C for 45 min. To generate a miRNA cDNA, the tailed miRNA and extension tail
primer (Table: 1) were mixed and incubated at 65°C for 10 min, and annealed at 4°C for 20 min. Reverse transcription was carried out with NovaScript III reverse-transcriptase (Life technologies), dNTP mix and RT-buffer and incubated at 50°C for 60 min. Finally, the reverse transcriptase was inactivated by incubation at 70°C for 15 min and 1.5U of RNase H (NEB) was added to remove the small RNAs. The samples were purified and the small RNA cDNA (srcDNA) concentration was measured using the spectrophotometer (NanoDrop Technologies). The cDNA samples were diluted to the same concentration of 25 ng per μl. miR159, miR164, miR167, miR319 and miR393 specific forward and a universal reverse primer (Table. 2; M&M ) were used for amplification of each of the miRNA. Real-time quantitative PCR (Q-PCR) was performed using 2X SYBR Green PCR Master Mix (Roche), 1 μl (25 ng) of the synthesized srcDNA and 5 μM of the primers in a 25 μl reaction volume at Tm 55°C on the LC480II Real-Time PCR System (Roche) to get threshold cycle (Ct) value. Fold changes in miRNAs were calculated using $2^{-\Delta\Delta Ct}$ equation.

5.2.1 Total RNA isolation and analysis of transcript and transcription factors (TFs)

To investigate the effects of viral infection on expression of miRNA targets mRNAs by real-time RT-PCR, gene-specific primers for ARF8, MYB, LoxD and TCP were designed (Table. 3; M&M) based on mRNA sequences collected from GenBank. For PCR amplification, total RNA was isolated from plant samples using Trizol reagent (Invitrogen), reverse transcribed to cDNA using reverse primer and subjected to conventional PCR and qRT-PCR. Primers were validated using gel electrophoresis of PCR amplicons, and by the presence of single peak on the melting curve. The SYBR Green PCR was performed on LC 480II real-time PCR machine (Roche) using 25 ng cDNA, 0.5 mM forward and reverse primer each and 1X SYBR Green (Roche) in a 25 μl reaction mix. The real-time experiment was conducted in triplicate and agarose gel analyses were performed to verify the products.

5.2.2 Designing of primers
To investigate the effect of viral infection on expression of miRNAs by real-time PCR, microRNA-specific forward primers and a universal reverse primer along with RTQ primer (Seungil et al., 2006), U6 snRNA primer and Actin gene primer were designed using BioEdit software version 5.09.04. The mature miRNA sequences were downloaded from miRNAs Registry database (http://miRNA.sanger.ac.uk) and from previous publications (Li et al., 2010; Sunkar and Zhu, 2004). The primers for amplification of transcripts were also synthesized using software program (http://miRNA.sanger.ac.uk) (Table 2 and 3: showing primers for miRNAs and transcripts).

### 5.2.3 PCR amplification and quantification of miRNAs and transcripts

20 µl reaction volume was having 2 µl of cDNA/srcDNA as template, 1 µl (10 mM) reverse primer 1µl miRNA/transcript specific forward primer, 1 µl (25mM) MgCl₂, 0.25 U Taq DNA polymerase (NEB) and 11.5 µl sterile distilled water (SDW) using PCR protocol (95 °C for 2 min., then 30 cycles of 95 °C for 10 sec., 55 °C for 30 sec. and 72 °C for 30 sec.). PCR amplification was confirmed on 1.5% agarose gel electrophoresis.

After PCR confirmation, real time quantitative PCR (RT-qPCR) was performed using 10 µl of 2X Light Cycler® 480 SYBR Green 1 master mix (Roche), 0.8 µl RTQ-UNIr primer (10 µM), 0.8 µl forward primer (10 µM) and 2 µl (10 ng/µl) of cDNA/srcDNA to a final reaction volume of 20 µl on the Light Cycler® 480 II (Roche), using above mentioned PCR protocol. After performing real-time RT-PCR, the relative expression level of all the transcripts and miRNAs were calculated using the comparative 2-ΔΔCt method.

### 5.2.4 Development of AC4 Gene construct and Agrobacterium mediated transformation

The sequence of AC4 gene from ToLCNDV was amplified using forward primer: 5’(GAT GAA TTC ATG GGT CTC CGC ATA TCC AT)3’ and reverse primer: 5’(GCT GGA TCC CTA GAA CGT CTC CAT CTT TGT)3’ and cloned into pGEM-T Easy vector (Promega). The pGEM-T-AC4 was further fused to cauliflower mosaic virus (CaMV) 35s promoter in a binary vector pCambia 2301 carrying the kanamycin as a selection marker at Bam HI and Hind III sites in MCS. Restriction analysis was done to
confirm the proper cloning of the AC4 gene during various steps of construct formation. The recombinant binary vector pCAMBIA-AC4 was introduced into Agrobacterium tumefacience strain LBA 4404 by freeze–thaw method. Transformation of Solanum lycopersicon plant was performed as described by McCormick 1991 and Selection of transformants was done by screening of the transformed plants using PCR and confirmed by Southern blotting. The protocols were followed as given by Sambrook and Russel (2000).

5.2.5 Auxin treatment
Primary shoot cuttings were excised from 5 to 6 week old ToLCNDV infected and control Solanum lycopersicon plants and were used in auxin treatment experiment prior to flowering. Two individual sets of infected shoots were treated with IBA concentration 0.5 mg/L and 2.5mg/L in liquid medium and separate sets of infected and control shoots were transferred to fresh liquid medium devoid of IBA to compare the effect of auxin treatment and kept in green house condition for root induction. Phenotypic observations were recorded 12 days after incubation. The rooted shoots were transferred to semi sterile substrate (mixture of soil and pearlite in the ratio 3:1) for normal growth, three weeks after transfer phenotypic observations were recorded.

5.6 Results
5.6.1 Viral responsive development of disease like symptoms and transcriptional changes in the levels of micro RNAs and their targets.
One of the characteristic stress responses in the plants is initial appearance of disease symptoms on infection. Tomato seedlings infected with ToLCNDV showed, the characteristic symptom of disease such as curling of young emerging leaves, started appearing from 10th day post whiteflies inoculation (10dpi) with gradual increase in the upward curling of both young and mature leaves with reduction in leaf size, leaf lamina and wrinkling in the leaves was found to be prominent from 15 dpi, as the diseases progressed at 25 dpi the reduced internodal length in the plant appeared to gave stunted
Based on this phenotypic data rating of disease symptom severity was done (Table 8).

**Table: 8 Relative index of disease symptom severity in naturally infected ToLCNDV tomato plants**

<table>
<thead>
<tr>
<th>Virus infection</th>
<th>Symptoms severity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Accumulation of AC4 Transcript (relative units)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 dpi</td>
<td>+</td>
<td>4.27±0.02</td>
</tr>
<tr>
<td>15dpi</td>
<td>+++</td>
<td>4.56±0.12</td>
</tr>
<tr>
<td>25dpi</td>
<td>++++</td>
<td>5.90±0.52</td>
</tr>
</tbody>
</table>

<sup>a</sup> Severity of the disease symptoms in infected plants was based on the degree of stunting, reduction in leaf size and curling of the leaves. Rating scale: +, initiation of curling of leaves; +++, severe leaf curling, reduction in leaf size and stunted growth; ++++, very severe leaf curling giving rosette appearance, extensive stunting and no intermodal growth.  

<sup>b</sup> level of AC4 transcript encoded by ToLCNDV, measured in infected *S. lycopersicon* plants. The values above were obtained from at least three independent experiments.

We also quantified the accumulation of pathogenicity factor ToLCNDV-AC4 in all the three infection periods through semi quantitative and quantitative RT-PCR. AC4 accumulation showed 4.27 fold increase in the levels at 10dpi with a gradual increase to 4.56 folds at 15dpi and attained to maximum up to 5.90 folds at 25dpi (Fig.15F); Most of these stress responses in the plants are mediated by hormone signalling. Stress responsive hormone signalling is regulated by the transcriptional changes. To investigate the interference of viral infections in plant development, transcriptional changes in terms of miRNAs and their target transcription factors and transcripts were studied. In the present study five conserved miRNAs viz., miR159, miR164, miR167, miR319 and miR393 and some of its targets such as MYB, ARF8, TCP and LoxD involved in auxin responses and plant developmental processes such as leaf and root development were selected for expression analysis at 10, 15 and 25dpi during viral infection. All studied five miRNAs were found to be accumulated to a maximum level at 15dpi. Among five miRNAs, miR164 showed maximum accumulation through out the infection period tested (RQ 10.88, 13.36 and 8.8).
Compared to the mock inoculated plants, miR164 and miR393 showed a significant increase at 10dpi in (RQ between 8.4 to 10.8), as compared to miR167, miR159 and miR319 (RQ between 1.6 to 4.3). During 15dpi the profiling of all the five miRNAs showed similar accumulation patterns with higher RQ values. Interestingly the profiling of miR159 and miR319 showed different accumulation pattern at 25dpi and were reduced to 0.61 and 0.15 fold. Although not much significant difference in expression of miR167 and miR393 were observed their accumulation level remained similar (Fig.15H-I). The Q-PCR amplicons of specific small RNA were validated by running them on 1.5% agarose gel which showed the amplicon of ~100 bp size (Fig.15G).

We also investigated the differential accumulation of LoxD involved in stress signalling and TFs MYB, ARF8 and TCP at all the three infectious stages, which is not consistent with the miRNA profiling. Initial increase (at 10dpi) in the level of TCP and LoxD (RQ 1.8 and 5.8 folds respectively) suggests that, stress signalling initiated on the onset of disease and showed subsequent decrease in levels at later stages of the infection. Although accumulation levels of ARF8 and MYB involved in auxin responses and leaf development were found to be relatively low and showed reverse trend. They showed gradual accumulation from 10dpi to 25dpi in accordance with disease symptom development (Fig.15K).

Transcriptional changes in the levels of TCP, MYB, ARF8 and LoxD as compare to mock inoculations showed differential responses. At 10dpi induced levels of TCP and LoxD were found to be three times more than, the accumulation of ARF8 and MYB. Subsequently at 15dpi, there was decrease in the levels of TCP and LoxD (RQ 0.67 to 3.36 folds), whereas the levels of ARF8 and MYB remained more or less constant. Interestingly the levels of ARF8 and MYB showed upward trend at the later stage of infection ie at 25dpi (RQ between 1.9 to 2.08), in contrast to the trend in TCP and LoxD accumulation (RQ 0.06 and 2.4 fold). Our data demonstrated substantial correlation between levels of target mRNAs and the corresponding miRNAs. Taken together we found expression level of MYB, ARF8, TCP and LoxD were significantly
altered as infection period progressed. These results also revealed the selective induction of TFs might differentially alter the miRNA guided gene expression which regulates auxin mediated stress responses during infection. Similarly the Q-PCR amplicons were validated on 1.5% agarose gel for the desired ~200bp size for transcripts (Fig.15J)

5.6.2 ToLCNDV-AC4 responsive development of disease like symptoms in tomato transgenics and transcriptional changes in the levels of miRNAs

To investigate the role of pathogenecity factor AC4 in disease development, we developed transgenic tomato plant expressing ToLCNDV-AC4 gene. Also to establish a link between the development of disease like symptoms and miRNA profiling, we analysed the transcriptional changes in the levels of five miRNAs involved in stress signallling. AC4 expressing transgenic plants exhibited severe phenotypic abnormalities starting from somatic embryogenesis to hardening stages. The typical disease like symptoms appeared in tomato transgenics ranging from reduction in leaf size giving rosette appearance, narrow and needle shaped leaves and stunted growth (Fig.16A-B). To determine whether these developmental abnormalities exhibited in AC4 transgenic are related with the altered expression of miRNAs. We tested the level of the selected miRNAs in AC4 transgenic plants, we found up regulation of selected miRNAs viz., miR164, miR167, miR393, miR319 and miR159 involved in plant development and auxin signallling. These observations gave us an indication that AC4 transgenic mimics not only the phenotype of disease symptoms but also showed similar transcriptional changes in tested miRNAs levels (Fig.16C-D).

5.6.3 ToLCNDV infected plants respond to exogenous auxin treatment

From our previous experiments it was known that, phenotypic symptoms and altered miRNA expression levels were strongly interrelated and most of these miRNAs are auxin responsive. In line with this observation, we have conducted an experiment to determine the response of exogenous auxin on disease like symptoms in virus infected tomato shoots. Different concentrations of exogenous auxin (IBA) showed a dramatic increase in the development of lateral roots in the ToLCNDV infected tomato shoots
within 12 days of treatment. We also measured plant height, no. of leaves, flowers and lateral roots after exposing the infected plants to IBA treatment (Table 9). Even though root length and number of lateral roots were shorter and less respectively to those of control plants (healthy plants) before IBA treatment, IBA treated infected plants showed increase in lateral root length and number and also showed increased plant height, leaf numbers, flowers indicating that IBA treated infected plants were able to recover from phenotypic abnormalities as a result of auxin induced root growth (Fig.17A-L and Table 9). These results indicate that auxin homeostasis plays a role during viral infection.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>^1 Infected plant after IBA treatment</th>
<th>^2 Plants with no IBA treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mg/l</td>
<td>2.5 mg/l</td>
</tr>
<tr>
<td>No. of fully opened Leaves (Avg/plan)</td>
<td>50.91± 4.35</td>
<td>98.12± 6.41</td>
</tr>
<tr>
<td>Plant height (cm)</td>
<td>23.5± 2.8</td>
<td>42.8± 3.96</td>
</tr>
<tr>
<td>No. of lateral roots</td>
<td>32.57± 2.93</td>
<td>56.37± 4.72</td>
</tr>
</tbody>
</table>
Induction of rooting carried out using 0.5 and 2.5 mg/l IBA in liquid medium. Induction of rooting carried out without IBA in liquid medium and these quantitative parameters measured after in vitro transfer of plantlets into the soil.

5.7 Discussion

Although the exact nature of virus induced symptom development with stress signalling responses are generally unknown but they appears to be tightly networked. The viral infection and establishment initiate many transcriptional changes which results in development of disease like symptoms. The cascade of transcriptional changes is regulated by miRNAs. The relationship between miRNAs expression and disease development remain to be explored. Also many viral proteins are known as pathogenecity factors and are involved in causing disease like symptoms (Fondong et al., 2007). The role of these viral proteins in regulating the cascade networking changes in the host during infection is also interesting to study, in order to develop a durable resistance against viruses. In the present study two different cellular responses, one related to plant morphogenesis and other related to stress signalling were chosen to study changes in the selected miRNAs and their targets. We tried to co-relate our results in the form of networking cascade showing relation in plant developmental process and stress signalling. Here we specifically relate the altered miRNA and their targets (miR159, miR164, miR167, miR319 and miR393 and its target mRNAs MYB, ARF8, TCP and LoxD) accumulation with disease like symptom development during early and late phase of infection and in AC4 expressing transgenic tomato plants. For studying two processes (plant development and stress signalling), we selected small RNAs for example miR164 (root development); miR159, miR319 and its target MYB and TCP (leaf development); LoxD (JA defense pathway); miR167-ARF8, miR164 and miR393 (auxin signalling). Our studies revealed that, severity of the symptoms was increase as infection period increases and caused differential alteration in the accumulation of miRNAs under the conditions tested. It was found that, miR164, miR167 and miR393 levels were up regulated during pathogenic processes after viral infection indicating the involvement of these miRNAs in symptoms determination by affecting plant development and auxin homeostasis. Role of miR164 has already being established in regulating auxin signals for lateral root development through its targets NAC1 TF (Guo...
et al., 2005; Xie et al., 2002) and in meristem development, shaping leaves and forming lateral buds through TFs;CUC1/CUC2 (Raman et al., 2008; Sieber et al., 2007). Similarly, miRNA167 and its target ARF6/ARF8 TFs have already been reported in mediating auxin induced gene expression responses (Guifoyle and Hagen, 2002), and in controlling expression of auxin homeostatic enzymes (Mallory et al., 2004; Tian et al., 2004), beside their involvement in root development (Gutierrez et al., 2009; Meng et al., 2010). With respect to miR393, the expression levels were found to be increased at all the infection stages suggests its probable role in maintaining imbalances in auxin homeostasis through the infection processes as it has a role in repressing the auxin signalling through cleavage of its target TIR1 (Navarro et al., 2006).

The other two studied miRNAs; miR159 and miR319 having role in leaf development and JA mediated stress signalling (Palatnik et al., 2003; Millar et al., 2005; Schommer et al., 2008), along with their targets TFs, MYB, TCP and transcript LoxD were found to be differentially regulated at early and late phase of infection. Both of these miRNAs showed accumulation at early stage and decreased levels were observed after onset of the disease suggesting their role in altering leaf development at initial infection stages and their possible involvement in stress signalling pathway (Schommer et al., 2008). Further we found clear induction of LoxD transcript at all infection period suggests the initiation of defense/stress responsive JA biosynthesis pathway during infection.

The auxin and JA signalling share many commonalities (Mockaitis and Estelle, 2008) and are interrelated by a probable network, based on our observations (Fig.18). The JA biosynthetic gene LOX2 (chloroplast encoded lipoxygenase2) regulated by miR319/TCP activity (Schommer et al., 2008). Also miR319/TCP node has been involved in regulation of miR164/CUC node as a part of the auxin signalling network (Somoza et al., 2009). Based on our observations, we propose a network involved in plant development and stress responses during viral infection. These cascades of responses are regulated by miRNAs. The transcriptional changes in the accumulation levels of these miRNAs and their targets can be interpreted in terms of development of disease like symptoms and defense through stress signalling.
Pathogenicity factor/ RNAi suppressor AC4 transgenic mimics leaf curl infection. Function of AC4 was implicated in pathogenicity and development of disease like symptoms (Fondong et al., 2007). Abnormal phenotypes such as stunted growth and narrow rosette leaves developed in ACMV-AC4 transgenics are due to interference of miRNA mediated regulatory functions by the viruses and virus-encoded silencing suppressor (Chellappan et al., 2005). Transcriptional changes in the transgenic tomato plants expressing ToLCNDV-AC4 suggests that AC4 expression mimics, leaf curl infection in terms of phenotypic expression of disease like symptoms and in initiating the stress responses by affecting the accumulation levels of studied five micro RNAs.

Exogenous auxin application resulted in partial recovery in leaf curl symptoms. Auxin levels have direct implications in the development of leaflets, leaf serrations leaf lobes and in root development (Wang et al., 2005; Hay and Tsianta, 2006; Tian and Reed, 1999). Our results on exogenous application of auxins resulted in partial recovery of disease like phenotype probably by affecting the root growth and helping in creating an auxin homeostasis during infection. Besides having role in leaf and root development, involvement of auxin signal transduction pathway in defense responses (Bari and Jones, 2009) may also be contributing in improved development during infection.

5.8 Conclusion
Development of disease like symptoms and stress responses regulated by various miRNAs are very complicated and networked together during infection. The present results on transcriptional changes of selected miRNAs and their targets reinforce the growing awareness of role of regulatory network of miRNAs during infection. Furthermore the role of viral pathogenicity factor (ToLCNDV-AC4) in regulating these changes during the disease indicates the active participation of this viral protein in signal interactions in vivo. In conclusion, ToLCNDV infection in tomato affects (i) transcriptional changes in five different miRNAs and their targets involved in development and stress responses (ii) Viral pathogenicity factors influences these changes and (iii) Exogenous application of auxin during infection results in partial
recovery of symptoms. Further studies are necessary to define links between miRNA regulatory networks, symptom development, hormone based stress signalling. It is expected that these studies will help in better understanding of host virus interface.
Analysis of epigenetic defense against ToLCNDV: An evaluation of role of RNA dependent DNA methylation

6.0 Abstract
RNA silencing is a sequence-specific defense regulating gene expression and involves RNA directed DNA methylation (RdDM), in which DNA homologous to triggering RNA is methylated \textit{de novo}. To analyse RdDM in the pathogenecity factor of ToLCNDV, we compared the methylation pattern in non transgenic and in transgenic tomato plants with a hairpin construct from ToLCNDV-AC4 region during infection. RdDM of cytosine residues of AC4 region specifically occur more in number in Ihp transformed tomato over non transgenic during infection. Methylation was found in cytosine residues falling in putative RNA-DNA duplex and it progressively decreased in the sequence adjacent to the duplex. Methylation results showed abundant levels of methylation in asymmetric cytosines in transformed tomato plants over naturally infected tomato. Given the previous findings that, methylation in cytosine is triggered by RdDM is linked with RNA silencing; we provide a model where by Ihp-AC4 region initiate RdDM for methylation in asymmetric cytosine as a defense response.

Key words: RNA silencing, RdDM, Epigenetic defense
6.1 Introduction

In higher plants, a natural role of RNA silencing is to protect against viruses (Covey et al., 1997; Ratcliff et al., 1997; Al-Kaff et al., 1998; Hamilton and Baulcombe, 1999) and the RNA silencing machinery in higher plants is highly elaborated relative to other eukaryotes and is involved in a number of fundamental processes. These include the regulation of endogenous gene expression via the micro-RNA (miRNA) (Jones-Rhoades et al 2006), trans-acting small interfering RNA (ta-siRNA) (Pulido et al., 2010), and natural antisense siRNA pathways and repression of invasive endogenous DNAs, including repeated sequences and transposons by siRNA-directed chromatin methylation (Baulcombe 2004; Chan et al 2005; Vaucheret 2006).

At present, de novo DNA methylation can be dissected into DNA-mediated DNA methylation (DmDM) and RNA-directed DNA methylation (RdDM). RNA silencing in plants involves RNA-directed DNA methylation (RdDM), in which DNA homologous to a triggering RNA is methylated de novo (Mette et al., 2000; Pelissier et al., 1999). In RNA-directed de novo methylation, RNA is assumed to bind to complementary DNA sequences and this then leads to DNA methylation along the RNA-DNA duplex (Wassenegger et al., 1994; Pélissier et al., 1999). It has been illustrated by Raja et al., (2008) that, genomic and viral genome targets may be transcribed by an RNA polymerase IVa complex, resulting in single-stranded RNA (ssRNA), which is converted to dsRNA by complexes containing RDR2. The 21 to 24nt siRNAs processed from dsRNA by DCL3 are loaded into complexes containing AGO4, which subsequently associates with Pol IVb. The AGO4- associated siRNAs target the complex to homologous DNA sequences, where cytosine methyltransferases e.g., DRM and CMT3 proteins are recruited to methylate CpHpH and CpG, CpNpG sequences respectively, the bottom pathway represents PTGS, in which hairpin structured double-stranded RNA is processed into siRNAs by an unknown DICER protein. These siRNAs are used to degrade target mRNA and contribute to RNA-directed methylation independently of AGO4 possibly through another AGO protein (Zilberman et al.,
2004), proposing that, RNA-directed DNA methylation driven by inverted repeats utilizes both pathways.

siRNA homologous to the promoter region of a target gene induces transcriptional gene silencing (TGS), which is associated with promoter methylation. siRNA is homologous to the coding region of the target gene induces posttranscriptional gene silencing (PTGS), which involves sequence-specific RNA degradation and methylation of the coding region (Bian et al. 2006). RdDM occurs at both symmetric cytosine sites (CpG and CpNpG, where N is A, T, C, or G) and at asymmetric sites (CpHpH, where H is A, T, or C). Cao and associates (2003) proposed that the initial establishment of RdDM requires the enzyme known as Domains rearranged methylase (DRM), which is guided by siRNA to the target sequences. The maintenance of CpG methylation is accomplished by methyltransferase, whereas CpHpH and CpNpG and CpHpH methylation is maintained by chromomethylase and DRM redundantly (Cao et al., 2003).

It also been reported in a number of organisms, transgenes containing transcribed inverted repeats (IRs) that produce hairpin RNA can trigger RdDM which is associated with 21–24 nucleotide small interfering RNAs (siRNAs) (Tijsterman et al., 2002). Endogenous genomic sequences, including transposable elements and repeated elements, are also subject to RdDM. It has also been shown that, maintenance of non-CG DNA methylation is dependent on AGO4/RDR2/DCL3-the component of RNA-mediated silencing pathway along with DRM DNA methyltransferase (Chan et al., 2004; Cao and Jacobsen 2002). It has been also reported that, in the transgenic consisting of AP1 (APETELA1) inverted repeat hairpin which silence the endogenous AP1 gene that, extensive non-CG methylation of target gene which is AGO4 dependent than in AGO4 mutants (Zilberman et al., 2004). In plants, IR-driven RNA silencing also causes extensive cytosine methylation of homologous DNA in both the transgene “trigger” and any other homologous DNA sequences—“targets” (Aufsatz et al., 2002).

The biological function of RNA-directed and DNA-mediated DNA methylation is incompletely understood. The association of RdDM with both TGS and PTGS may indicate that RdDM is part of a defence system against pathogen attack, which could be limited to plants and fungi. Foreign nucleic acids, such as transgenes, transposons and
virus sequences, can be recognized by the host plant and RdDM may serve as a mechanism to inactivate them (Kumpatla et al., 1998; Matzke and Matzke, 1998; Voinnet et al., 1998). This proposal has been supported by the observation that infection with an RNA virus induces RdDM in plants (Jones et al., 1998).

Members of the Geminiviridae are true DNA viruses that replicate circular, single-stranded DNA genomes in the nucleus by a rolling-circle mechanism that employs host replication machinery (Hanley-Bowdoin et al., 2004). The double-stranded DNA (dsDNA) intermediates that mediate both viral replication and transcription associate with cellular histone proteins to form minichromosomes (Pilartz and Jeske 2003). Transcripts produced from these minichromosomes are subject to PTGS, and geminiviruses and their associated satellites have been shown to encode a variety of proteins that can suppress this defense (Wang et al., 2005; Zrachya et al., 2007). In addition, given the role of RNA-directed methylation in silencing endogenous invasive DNAs, it was proposed that plants might also use methylation as a means to repress transcription and/or replication from a viral minichromosome (Bisaro, 2006; Ding and Vionnet, 2007).

During ToLCV infection it has been found that C4 (AC4) transgene showed higher level of symmetric cytosine methylation irrespective of whether these plants were infected with ToLCV or were transformed with C4 and it was reported to be associated with higher level of asymmetric cytosine methylation (Bian et al., 2006).

Tomato leaf curl New Delhi virus (ToLCNDV) belongs to genus Begomovirus of the family Geminiviridae. Tomato leaf curl virus has an ssDNA genome encodes a small ORF AC4 which is overlapped with AC1 ORF region in which AC1 codes for replication initiator protein (Rep or replicase protein) essential for viral DNA replication in association with host DNA polymerase (Hanley-Bowdoin et al., 2000). The ToLCV genome replicates in the nucleus of infected cells (Rojas et al., 2005) by a combination of rolling circle replication (RCR) and recombination-dependent replication (RDR) (Jeske et al., 2001). And Conserved core of the replicase gene, consisting of 330 nucleotides, has been identified (Praveen et al 2005), which overlaps AC4, pathogeneic factor protein responsible for symptom determinant and disease severity (Fondong et al., 2007).
In our study we have analysed and compared the methylation pattern in pathogenecity region of ToLCNDV in Ihp-AC4 transgenic and non transgenic (NT) tomato plants when infected with ToLCNDV.

6.2 Materials and methods

6.2.1 Plant materials
Tomato (Solanum lycopersicon var. Pusa Ruby) were grown under glasshouse conditions at 25 to 30°C. Transgenic tomato (Ihp-AC4) lines developed from our laboratory described previously (Ramesh et al., 2007) carrying a CaMV 35S promoter-driven Ihp-AC4 cassette were used under methylation study. Both non transgenic and transgenic tomato plants were kept under netted cage for the infection of ToLCNDV. Ihp-AC4 construct consists of DNA sequences homologous to 80 bases of ToLCNDV from 2248-2333 which covers partial sequences of AC4 and conserved rep region and inverted repeats of 250 bp was synthesized in vitro with an intron of 85 bp placed between the repeats to generate intron spliced haipin RNA (Ihp RNA-rep) construct (Ramesh et al., 2007)

6.2.2 Bisulfite DNA sequencing
Total DNA was isolated from non transgenic and Ihp-AC4 transgenic which were kept under ToLCNDV infection using DNeasy plant mini kit (Qiagen). Bisulfite conversion of DNA was carried out from EpiTech Bisulfite kit (Qiagen). HRM analysis was done with bisulfite converted DNA to monitor bisulfite conversion rate. In order to determine the methylation status in AC1 gene, GC rich region of AC1 was selected in which the complete AC4 ORF resides. AC1 specific primers were used to amplify AC1 GC rich region from bisulfite converted DNA from ToLCNDV infected tomato and Ihp-AC4 transgenic. Specific primers were designed using Meth software version 5.0 against converted template (Forward primer: 5' ATTTTTGGGGGTTAATTTTTTTT 3' and Reverse primer: 5' AAAAAAAACACTTTTCCAATTACA 3') were used to amplify the selected AC1 region by PCR. PCR products were separated by electrophoresis in a 1% agarose gel, purified using the QIAquick gel extraction kit (Qiagen, Clifton, Australia), ligated into pGEM-T-Easy (Promega), and introduced into Escherichia coli DH5α cells
according to the manufacturer’s instructions. Plasmid DNA was prepared from *E. coli* using a Qiagen Spin miniprep kit (QIAgene) and cloned DNA was sequenced with T7 or SP6 primers (Chromous biotech, India). The obtained sequences were analysed for the methylation using BioEdit Sequence Alignment Editor Version 5.0.9 (Hall, 1999). Details procedure of bisulfite conversion rate and reaction conditions were given in materials and methods 3.4

6.3 Results

6.3.1 Comparison of RdDM in pathogencity region of naturally infected plants/transgenic under ToLCNDV infection

6.3.2 Characterization of pathogencity region

Based on higher GC content (40%-50%), AC1 ORF region from 2113-2509 (AM849548) of 396bp was selected in which it covers full length AC4 ORF (2251-2427) and conserved *rep* region (2365-2621) (**Fig.19A**). AC4 ORF encodes for protein having RNAi suppressor activity (Vanitharani *et al.*, 2004) and pathogenesis function (Fondong *et al.*, 2007) and conserved core of the *replicase* gene, consisting of 330 nucleotides, has been identified by Praveen *et al* (2004). Ihp-AC4 transgenic plants were developed previously in our lab to silence leaf curl virus AC1 and AC4 ORF region. Web based siRNA design tool DEQOR ([http://cluster-1.mpi-cbg.de/Deqor/deqor.html](http://cluster-1.mpi-cbg.de/Deqor/deqor.html)) was used in designing potent siRNAs for the selected pathogenecity region. We found three potent siRNAs in which one of the siRNA (AACGTCTCCGTCTTTGTCGAT) sequence was fall in the AC4 hairpin region, whereas other two siRNAs viz., AAGTCGAAGAATCTGTTATTC and AATCGCCTTAGCATATGCATC were fall in AC4 and AC1 (rep) ORF.

6.3.3 Characterisation of asymmetric and symmetric cytosine methylation
In order to determine RNA directed DNA methylation pattern as plant defense mechanism in ToLCNDV infected non transgenic plants and transgenic Ihp-AC4 plants, and to know whether silencing of AC4 gene in Ihp-AC4 is linked to DNA methylation. Methylation status of pathogenecity region in both infected transgenic and non transgenic plants was determined by bisulfite sequencing. In the procedure, bisulfite treatment converts unmethylated cytosines to uracil while methylated cytosine remains unchanged. To ascertain C-T conversion, HRM analysis was carried out and confirmed the C-T conversions based on the highly distinct melting curves of bisulfite treated DNA with that of non bisulfite treated DNA (Fig. 19B and C). The converted DNA then was cloned and subjected to sequencing. Three individual clones from each sample were sequenced and the sequences were aligned to check methylation status (Fig. 20A).

Total of ninety four cytosine residues are present in the selected pathogenecity region, out of which twenty seven of symmetric cytosine (CpG, CpNpG where N is A, T, C, or G) and sixty seven of asymmetric cytosine (CpHpH residues where H is A, T or C) were found. We found higher level total cytosine methylation in Ihp-AC4 transgenic (69.1%) than in NT plants (44.6%). It was found that, fifty three and twenty eight number of asymmetric cytosine residue were methylated in Ihp-AC4 transgenics (around 79.1%) and non transgenic plants (around 42%) respectively (Fig. 20A), Where as there was no significant difference in methylation level of symmetric cytosines in transgenic (46%) and non transgenic plants (53.8%). Methylation level was observed in the RNA-DNA duplex region of three potent siRNA fallen in the pathogenecity region in both transgenic and non transgenic plants. Significant level of asymmetric cytosine methylation in RNA-DNA duplex region (70.2%) was found in transgenic plants, where as non transgenic plants showed only 12.6% of methylation (Fig. 21A). All asymmetric cytosine residues (four) were methylated in RNA-DNA duplex of Ihp region of transgenic plants, however none of the asymmetric cytosine residue was found to be methylated at Ihp region of AC4 in non transgenic plants (Fig. 21B). Where as no significant difference in the methylation of symmetric and asmmetric cytosine was observed at RNA-DNA duplex excluded from Ihp region (Fig. 21C & D; Table 10).
6.4 Discussion

Several reports have shown that DNA methylation in transcriptional regulatory regions is associated with gene silencing (TGS) in plants and viruses (Kinoshita et al. 2007; Raja et al., 2008; Bian et al. 2006). Evidence has been shown that DNA methylation within, or near sequences of, a positive cis-element (enhancer) interferes with the binding of a cognate transcription factor to this cis-element, which in turn causes TGS (Deng et al., 2001). We show that cytosine residues in ToLCNDV- pathogenicity region are methylated in infected transgenic and NT plants and that methylation is significantly more in Ihp-AC4 plants that display enhanced resistance. Bisulfite sequencing revealed differential methylation status in transgenic and NT plants, firstly it showed greater proportion of asymmetric cytosine methylation than symmetric cytosines in Ihp-AC4 as it was previously observed within C4 ORF of Tomato leaf curl virus DNA from N.benthamiana as well as in CaLCuV IR region from A. thaliana plants (Bian et al., 2006; Raja et al., 2008) thus it shows that, asymmetric CpG sequences might potential targets of methylation for defensive purposes. The difference in the methylation status in both transgenics and non transgenic plants might be due to differential response for viral infection. For example early response of the Ihp-rep transgenic to virus infection might initiate silencing process to methylate cytosine residue this could be due to production of transgene derived aberrant transcript might converted into dsRNA by RdRP reaction and dsRNAs were processed into small siRNAs which triggered RdDM to methylate homologous viral DNA (Fig.22) (Shibuya et al., 2009). Thus it suggests that, transgene triggered silencing mechanism (viral DNA methylation) is efficient enough to maintain the resistance as a heritable epigenetic trait by maintaining methylated cytosine sites. This hypothesis is supported by Molinier et al (2006) and Boyko et al., (2007) who showed the inheritance of an epigenetic signal generated by exposure of Arabidopsis plants by bacterial and virus infection.

6.5 Conclusion:

In the present study we observed RNAi triggered DNA methylation at the asymmetric cytosines as a silencing mechanism by the host. In a transgenic tomato expressing Ihp-AC4, RNAi triggered methylation of more number of asymmetric cytosine can be
linked to gene suppression mechanism by the host, over symmetric methylation for genome maintenance.
Host specific expression of micro RNAs during leaf curl virus infection: in response to viral pathogenicity

7.0 Abstract:

Micro RNAs (miRNAs) play vital role in regulating plant growth and development by regulating various cellular processes. Viral infections dramatically affect these cellular processes and cause symptoms. However the underlined mechanisms of symptom development are not yet understood. Recent studies have shown that, miRNA mediated regulation of cellular processes is involved in host virus interactions and might be playing a crucial role in symptom development. Although knowledge about the miRNA controlled gene expression in different plant species is still limited. Here we studied expression profiling of seven miRNAs viz., miR159, miR164, miR167, miR171, miR319, miR393 and miR398, and their target mRNAs viz., MYB, ARF8, TCP and LoxD in three different hosts viz., Solanum lycopersicum, Nicotiana tobaccam and Carica papaya during leaf curl virus infection. Compared with the mock inoculated plants the expression levels of investigated miRNAs and mRNAs were enhanced in all the hosts by different degree. In addition the obvious up regulation of several miRNAs and target mRNAs can be correlated with the phenotypic expression of symptoms and accumulation of pathogenicity factor AC4.

Key words: miRNA, temperature, defense response
7.1 Introduction:

MicroRNAs (miRNAs) are a class of ~22 nt noncoding regulatory RNAs which are universal in eukaryotes. They act as negative regulator of gene expression by targeting mRNAs for cleavage or translational repression (Bartel 2004). miRNAs regulate various aspects of plant development such as leaf, root and flower development via their posttranscriptional control on the expression of many transcription factors and F-box proteins. This control the regulatory network of plant development is an important level of regulation, which ensures a proper development of a plant.

Leaf development, patterning and polarity are controlled by many transcription factors, including leucine zipper family, TCP family and MYB family. Asymmetry is a hallmark of plant leaf structure (Wang et al., 2007). A recent study revealed that miR164 is another regulator of leaf patterning (Nikovics et al., 2006). These authors showed that the balance between the miR164a and the transcription factor CUP-SHAPED COTYLEDON2 (CUC2) gene controls leaf margin serration in Arabidopsis. Although the pattern of serration is determined first independently of CUC2 and miR164, the balance between coexpressed CUC2 and miR164a then determines the extent of leaf serration. The regulatory role of miR319 (also called JAW) in leaf development has come to light in a developmental mutant screen in Arabidopsis thaliana. A dominant jaw-d mutant exhibits a phenotype of uneven leaf shape and curvature. In these mutant plants, the expression of the miR319a/JAW gene is elevated. In addition, these plants show a reduced accumulation of five TCP mRNAs, each of which has complementary sites to miR319, TCP genes and the miR319/JAW family are found in a wide range of plant species, suggesting that miRNA-mediated control of leaf morphogenesis is conserved across different plant species with very different leaf forms (Palatnik et al., 2003). Micro RNA 159 shares sequence similarity with miR319/JAW. The targets of miR159 include two members of MYB transcription factors, MYB33 and MYB65 and was reported that over expression of a miRNA-resistant version of MYB33 in Arabidopsis causes leaves to curl upwards (Palatnik et al., 2003). Similarly, Arabidopsis transformed with MYB33 containing the mutated miRNA target site show dramatic pleiotrophic developmental defects, including abnormal leaves that were more rounded and upturned at the sides (Millar and Gubler, 2005). 35S-miR164 transgene
exhibit vegetative organ fusion and decreased root branching as found in plants with reduced levels of NAC1. In contrast, loss-of-function in miR164 mutants accumulates higher levels of NAC mRNA, resulting in more lateral root formation (Guo et al., 2005). Evidence indicates the potential role of miR393 in lateral root formation in Arabidopsis. The transcription factor NAC1, known to be targeted by miR164, acts downstream of TIR1 (the target of miR393) to transduce auxin signal to promote lateral root development (Xie et al., 2000). Considered together, the regulatory pathway miR393–TIR1–NAC1, which likely interacts with the miR164–NAC1 pathway, may participate in lateral root development in Arabidopsis (Meng et al., 2010)

Several miRNAs have been shown to respond to plant hormones; these include miR159, miR160, miR164, miR167 and miR393 (Sunkar and Zhu, 2004; Zhang et al., 2005). Auxins are growth regulators involved in virtually all aspects of plant development. Auxin signalling is initiated by the binding of auxin to its receptor, transport inhibitor response 1 (TIR1). The binding of auxin to TIR1 promotes the interaction between TIR1 and short-lived nuclear transcriptional regulators termed Auxin/Indole-3-acetic acid (AUX /IAA) proteins, leading to the degradation of these proteins by ubiquitin-related machinery. AUX/IAA proteins are repressors of auxin response factors (ARFs); the degradation of the AUX/IAA proteins releases ARFs from this repression, permitting the formation of hetero- or homodimers that subsequently control the expression of auxin-responsive genes by binding to the auxin response elements (AREs) in the promoter region. ARFs, TIR1 and other closely related F-box proteins are predicted or have been shown to be the targets of miRNAs in plants (Mallory et al., 2005; Jones-Rhoades and Bartel, 2004; Rhoades et al., 2002; Sunkar and Zhu, 2004). It was shown that miR167 causes the degradation of ARF8- and ARF6-encoded mRNAs (Wu et al., 2006; Ru et al., 2006). miR167 may also repress ARF6 expression at the level of translation. Scare-like family gene (SCL6-II, SCL6-III and SCL6-IV) the targets of miR171 are a class of specific transcriptional factors containing GRAS domain that controls several developmental processes, including root growth (Helariutta et al., 2000) gibberellin signal transduction pathway (Silverstone et al., 1998)
Several reports have also been shown that, many of the miRNA expressions are viral infection responsive (Lang et al., 2011; Bazzini et al., 2007). They are also involved in stress signalling pathway and respond to various environmental stresses (Jagadeeswaran et al., 2009; Jones-Rhoades and Bartel 2004) and pathogen invasion (Chapman et al., 2004; Chellappan et al., 2005). First role of miRNA was described by Jones-Rhoades and Bartel (2004) who showed increased expression of miR395 under abiotic stress (sulphate starvation). After the discovery of miR395, several miRNA were identified involved in abiotic stress. For example, miR399 is highly induced under phosphate starvation (Liu et al., 2010). It was shown that, miR393 and miR398 were strongly induced by cold (Zhou et al., 2008) and miR393 was strongly upregulated by dehydration, high salinity and abscisic acid (ABA) treatment; miR319c was only induced by cold; miR397b and miR402 were slightly upregulated in all stress treatment; however, miR389a was downregulated in all type of stress in Arabidopsis (Sunkar et al., 2006) and showed increased expression of miR398a in Arabidopsis plants exposed to ambient temperatures (23°C) (Lee et al., 2010). Stress responsive JA biosynthesis pathway is regulated by LOX2, which encodes a chloroplast-localized lipoxygenase that catalyses the conversion of α-linolenic acid (18:3) into (13S)-hydroperoxyoctadecatrienoic acid, the first dedicated step in the biosynthesis of the oxylipin JA (Bell et al., 1995). LOX2 is regulated by miR319a through the TCP transcription factors (Schommer et al., 2008).

Tomato leaf curl virus (ToLCV) belongs to the family Geminiviridae and genus Begomovirus and is transmitted through white fly (Bemisia tabaci) to infect broad range of dicotyledonous and monocotyledonous plants. Such infection causes various disease symptoms ranging from upward curling of leaves, stunted growth and poor root development etc. The development of these disease symptoms require specific interaction between viral and plant components to disrupt developmental processes Culver and Padmanabhan (2007). ToLCV is now being recognized as one of the most important threats to tomato crop in both tropical and subtropical parts of the world. Although tomato is its natural host, ToLCV is known to infect other Solanaceous
species as well, thereby adding to its devastating effects. Out of all the variants *Tomato leaf curl New Delhi virus*, the ToLCNDV is most abundant in this part of north India and is mainly responsible for destruction of tomato and other crop plants in our country (Pandey *et al.*, 2009). The organization of ToLCV has bipartite genome having two circular single-stranded DNA components called DNA-A and DNA-B. DNA-A component consists of small ORF called AC4 which encodes for pathogenecity factor protein required for pathogenesis function. Recently it has been showed that the consensus N-myristoylation motif of bipartite begomovirus – AC4 protein is critical for membrane targeting and pathogenicity. It is therefore likely that AC4 requires the attachment of myristate at Gly-2 and palmitate at Cys-3 to bind to the plasma and cytosolic membranes from which it interacts with host factors to induce virus-like symptoms. Thus AC4 pathogenicity is dependent on the consensus N-myristoylation motif, this protein can provide useful information on the ability of virus to counter the defense of the plant and cause a disease (Fondong *et al.*, 2007). These virus encoded pathogenecity factors which are associated with the symptom determination in the plant are also known to act as suppressors of RNA silencing (Vance and Vaucheret 2001). AC4 protein encoded by African cassava mosaic virus Cameroon Strain is silencing suppressor found to interact directly with miRNA pathway. AC4 can bind with single-stranded miRNA and inhibit miRNA-mediated negative regulation of gene expression in plants; as a consequence, plants show developmental defects (Chellappan *et al.*, 2005). Nucleic acid binding with assay revealed the ability of AC4 to bind single-stranded mi159 but not miR159-miR159* duplex both *in vitro* and *in vivo*. These results indicate that AC4 protein has the ability to bind with single-stranded miRNA unspecifically to interfere with miRNA functions.

To determine temperature and host specific expression of miRNA and to ascertain their role in symptom development, we had selected *Tomato leaf curl New Delhi virus* (ToLCNDV) which has wide host ranges. In our study we had selected two of hosts belonging to solanaceae family (tomato and tobacco) and one host belonging to caricaceae family (Papaya). To study temperature and host specific expression of miRNAs, seven miRNAs (miR159, miR164, miR167, miR171, miR319, miR393 and
miR398) and some of the TFs (MYB, ARF8 and TCP1) and transcript (LoxD) regulated by these miRNAs were selected which are involved in hormone signalling, defence pathway, leaf and root development.

7.2 Materials and Methods

7.2.1 Plant material, virus infection and temperature treatment

*Solanum lycopersicum, Carica papaya, Nicotiana tobaccum* Seedlings were grown under glasshouse conditions (16 hour photoperiod, temperature at 28°C). Fifteen days old seedlings were kept under netted cage for whitefly mediated ToLCNDV transmission. Sampling and morphological analysis were done 10, 15 and 25 days after infection. Same aged plants corresponding to each time point were taken as control. To study the effect of temperature on miRNA accumulation in infected tomato plants, 25 days old control and infected *Solanum lycopersicum, Carica papaya, Nicotiana tobaccum* seedlings were kept in separate chambers (five plants in each chamber) maintaining temperature of 12°C, 25°C, 30°C, 35°C and 40°C provided under the National Phytotron Facility available at IARI, New Delhi.

7.2.2 AC4 transcript analysis through semi-quantitative and quantitative RT-PCR

To allow the amplification of AC4, the primers were designed in the coding sequence of expected amplicon length of ~177bp. First strand cDNAs were synthesised by RT from 1 μg of DNase treated total RNA from each infection period (10, 15 and 25dpi) using first strand cDNA synthesis kit (Qiagen) in a total volume of 20 μl according to manufacturer’s instructions. For the semi-qPCR amplification, 1 μl of cDNA amplified by PCR (95°C for 5 min, then 95°C for 1 min, 55°C for 30 s, 72°C for 1 min for a maximum of 35 cycles) in 25 μl reaction volume using 10 μM of primers. For qPCR amplification, 25ng of cDNA were amplified using 25 ng cDNA, 0.5 mM forward and reverse primer each and 1X SYBR Green (Roche) in a 25 μl reaction mix. The real-time experiment was conducted in triplicate. AC4 quantification was done in all the three hosts infected by ToLCNDV including the infected tomato plants kept under different temperatures.
7.2.3 Small RNA isolation
To quantify the miRNAs viz., miR159, miR164, miR167, miR171, miR319, miR393 and miR398, leaf samples were harvested from healthy and ToLCNDV infected plants at different infection period (10, 15, and 25 dpi) and temperature (12°C, 25°C, 30°C, 35°C and 40°C) and quickly frozen in liquid nitrogen and stored at -80°C prior to small RNA isolation. Small RNAs were extracted from 100 mg of leaf tissues using mirVana™ isolation Kit (Ambion) accordingly to manufacture’s instruction. The concentration of small RNA was determined by spectrophotometer NanoDrop, ND-1000 (NanoDrop technologies).

7.2.4 Generation of small RNA cDNA library
Small RNA cDNA (srcDNA) library was constructed according to protocol (Seungil et al., 2006). Small RNA isolated from leaf tissues were polyadenylated at 37 °C for 45 min. in 50 µl reaction volume containing 0.3 µg RNA and 5 U E.coli poly (A) polymerase (NEB). The poly (A) tailed small RNA was purified from sample using a purification cartridge provided in mirVana Probe and Marker Kit (Ambion) as per manufacturer’s protocols.

To investigate the effect of ToLCNDV infection and temperature treatment on expression of miRNAs by real-time PCR, microRNA-specific forward primers and a universal reverse primer along with RTQ primer and U6 snRNA primer were designed using BioEdit software version 5.09.04. (Table. 2; M&M). The mature miRNA sequences were downloaded from miRNAs Registry database (http://miRNA.sanger.ac.uk). Primer sequences were validated using gel electrophoresis of PCR amplicons and by the presence of only single peak on the thermal dissociation (Tm) curve generated by the thermal denaturing protocol, which followed each real time PCR run.

Small RNA cDNA (srcDNA) library was generated by mixing 500 ng of poly (A) tailed RNA and 1 µg of extension tail primer in a 26 µl reaction volume, incubated at 65 °C for 10 min. followed by addition of reverse transcriptase 200U (M-MuLV Reverse transcriptase, NEB), 1 µl dNTP mix (10mM) and 10X buffer in a final reaction
volume of 40 µl at 37 °C for 60 min. Reverse transcriptase was inactivated by incubation at 70 °C for 15 min. and 5U RNase H (NEB) was added to remove small RNAs. The sample was purified using the QIAquick spin PCR purification Kit (QIAGEN) in a final volume of 50 µl. The srcDNA concentration was quantified using NanoDrop spectrophotometer (NanoDrop technologies).

7.2.5 PCR amplification and quantification of miRNAs

The srcDNA sample (10 ng/µl) was PCR amplified using microRNA specific forward primer and a universal reverse primer RTQ-UNIr. 20 µl reaction volume was having 2 µl of srcDNA as template, 1 µl (10 mM), reverse primer µl (10mM), MgCl2 1 µl (25mM), 0.25 U Taq DNA polymerase (NEB) and 11.5 µl sterile distilled water (SDW) using PCR protocol (94 °C for 10 min., then 30 cycles of 94 °C for 15 sec., 55 °C for 30 sec. and 72 °C for 30 sec.). PCR amplicon were analysed on 1.5% agarose gel electrophoresis to confirm.

After PCR confirmation, real time quantitative PCR was performed using 10 µl of 2X Light CyclerR 480 SYBR Green 1 master mix (Roche), 0.8 µl RTQ-UNIr primer (10 µM), 0.8 µl forward primer (10 µM) and 2 µl (10 ng/µl) of srcDNA to a final reaction volume of 20 µl on the Light CyclerR 480 II (Roche), using above mentioned PCR protocol. The real-time experiment was conducted in triplicate and agarose gel analyses were performed to verify the products. After performing real-time RT-PCR, the relative expression level of all the miRNAs were calculated using the comparative $2^{-\Delta\Delta C_t}$ method.

7.2.6 Synthesis of cDNA and quantification of transcript and transcription factors (TFs)

To investigate the effects of viral infection and temperature on expression of miRNAs target mRNAs by real-time RT-PCR, gene-specific primers for ARF8, MYB, LoxD and TCP1 were designed (Table. 3; M&M) based on mRNA sequences collected from GenBank. For PCR amplification, total RNA was isolated from plant samples using Trizol reagent (Invitrogen), reverse transcribed to cDNA using reverse primer and subjected to conventional PCR and qRT-PCR using same protocol as used for miRNA quantification. Primers were validated using gel electrophoresis of PCR amplicons, and
by the presence of single peak on the melting curve. The SYBR Green PCR was performed on LC 480II real-time PCR machine (Roche) using 20 ng cDNA, 0.8 mM forward and reverse primer each and 10 µl of 2X Light Cycler® 480 SYBR Green 1 master mix (Roche) in a 20 µl reaction mix. The real-time experiment was conducted in triplicate and agarose gel analyses were performed to verify the products.

7.3 Results
7.3.1 Transcriptional changes in response to symptom expression and AC4 accumulation during leaf curl virus infection

7.3.1.1 Phenotypic expression of symptoms

Tomato and tobacco seedlings infected with ToLCNDV showed characteristic disease symptoms- initiation of curling of emerging leaves at 10dpi, as infection period increases to 15dpi, there was gradual increase in curling of both young and matured leaves with reduction in leaf size, including to this tobacco plants showed puckering of leaves and at 25dpi reduced internodal length appeared to give stunted appearance (Fig.23B-D; Fig.24B-D). Whereas ToLCNDV infected papaya plants did not show any symptoms development in tested inoculation periods (Fig.25B-D). Based on phenotypic data, symptom development was recorded and compared over time after infection with ToLCNDV in three different hosts (tobacco, tomato and papaya) at 10, 15 and 25dpi (Table 11).

<table>
<thead>
<tr>
<th>Plants</th>
<th>Infection period</th>
<th>Symptom severity</th>
<th>Accumulation of AC4 suppressor (relative units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solanum lycopersicon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana tobaccum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carica papaya</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table: 11 Variation in severity of disease symtom in *Solanum lycopersicon*, *Nicotiana tobaccum* and *Carica papaya* infected by ToLCNDV
<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Time</th>
<th>Rating</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tobaccum</td>
<td>10dpi</td>
<td>++</td>
<td>3.04±0.03</td>
</tr>
<tr>
<td></td>
<td>15dpi</td>
<td>++++</td>
<td>3.23±0.14</td>
</tr>
<tr>
<td></td>
<td>25dpi</td>
<td>++++++</td>
<td>5.22±0.44</td>
</tr>
<tr>
<td>Solanum lycopersicon</td>
<td>10dpi</td>
<td>+</td>
<td>4.27±0.02</td>
</tr>
<tr>
<td></td>
<td>15dpi</td>
<td>+++</td>
<td>4.56±0.12</td>
</tr>
<tr>
<td></td>
<td>25dpi</td>
<td>+++++</td>
<td>5.9±0.52</td>
</tr>
<tr>
<td>Carica papaya</td>
<td>10dpi</td>
<td>-</td>
<td>1.14±0.04</td>
</tr>
<tr>
<td></td>
<td>15dpi</td>
<td>-</td>
<td>1.26±0.11</td>
</tr>
<tr>
<td></td>
<td>25dpi</td>
<td>-</td>
<td>2.65±0.40</td>
</tr>
</tbody>
</table>

**Rating scale:** +, initiation of leaf curling; ++, initiation of leaf puckering and curling; ++++, severe leaf curling, reduction in leaf size; +++++, severe leaf puckering and curling of younger leaves giving uneven shape appearance; ++++++, very severe leaf curling giving rosette appearance and stunted growth with shoots appearing erect; +++++++, severe leaf puckering and curling of older and younger leaves with uneven leaf shape and severe reduction in leaf size. *Carica papaya* plants did not show any disease symptom at three infection stages studied. Relative accumulation of AC4 suppressor encoded by ToLCNDV was quantified in infected tobacco, tomato and papaya plants. The values obtained above were from at least three independent experiments.

**7.3.1.2 Host and temperature specific accumulation of pathogenecity factor-AC4**

We quantified accumulation of pathogenecity factor-AC4 in all the three hosts at all infection periods through Q-PCR. The accumulation of AC4 in papaya plants were significantly less at 10dpi (1.14 folds), 15dpi (1.26 folds) and 25dpi (2.65 folds) compared to tobacco and tomato plants. In both, tomato and tobacco plants the accumulation of AC4 increased gradually from 10dpi (4.07 and 3.04 folds respectively) to 15dpi (4.56 and 3.23 folds respectively) and attained to maximum at 25dpi (5.9 and 5.29 folds respectively). Taken together, AC4 accumulation was found to be similar in
tobacco and tomato than papaya which is directly related to phenotypic symptom development in the plants. (Fig. 23E; 24E; 25E)

7.3.1.3 Micro RNA profiling
To investigate the effect of virus infection in tobacco, tomato and papaya plants in terms of its ability to induce miRNA accumulation, seven conserved miRNAs viz., miR159, miR164, miR167, miR171, miR319, miR393 and miR398 and some of their targets such as MYB, ARF8, TCP and LoxD involved in hormone responses and plant developmental processes of leaf and root development were selected for expression analysis at 10, 15 and 25dpi during viral infection. The results revealed host specific accumulation of miRNAs for example in symptom developed tobacco and tomato plants we found gradual increase in the accumulation of all the seven miRNAs as infection progresses from 10dpi to 25dpi, whereas in asymptomatic papaya plants we found gradual decrease in the accumulation of miRNAs from 10dpi to 25dpi.

At 10dpi in both tobacco and tomato leaves ToLCNDV infection induced significant alteration in miRNA accumulation demonstrated by ~.47 to 4.7 fold increase in miR159, miR164, miR167, miR171, miR319, miR398 and miR393. At 15 dpi in tobacco plants, the expression levels of miR159, miR164, miR167 and miR171 were notably increased from 0.9 to 1.9 folds and accumulation of miR319, miR393 and miR398 tends to remain unaltered as compare to 10dpi, whereas in tomato plants miR159 and miR171 showed decreased accumulation of 0.05 and 0.65 folds and increased accumulation (between 3.8 to 5.2 folds) of miR164, miR167, miR319, miR393 and miR398 were observed. At 25dpi, accumulation level of all the miRNAs were increased both in tomato and tobacco plants, in which significant increased accumulation of miR159, miR164, miR167 and miR398 were found (Fig.23F; 24F).

It was observed phenotypically in papaya plants that, ToLCNDV infection did not induce symptom development in the inoculation period we monitored, under this condition, increased accumulation level of miR159, miR164, miR167, miR319 and miR398 were observed (3 to 9 folds) at 10 dpi and gradually accumulation level of miR159, miR167, miR171 and miR319 were decreased in subsequent inoculation periods that is at 15 and 25dpi (between 0.02 to 5.52 folds) while expression of miR164,
miR393 and miR398 showed differential expression levels (Fig.25F). Thus our results revealed that, most of the tested miRNAs showed symptom based and host specific responses to ToLCNDV infection.

**7.3.1.4 Differential expression of mRNA targets of selected miRNAs**

In order to investigate the possible effect of miRNAs on their potential targets during ToLCNDV infection in tobacco, tomato and papaya plants; we examined the transcript level of LoxD and level of TFs viz., ARF8, MYB, TCP1. Our study revealed that in most of the cases negative correlation between the expression of miRNA and that of respective target gene has been observed for example, increased accumulation of miRNA showed decreased accumulation of target gene. In tobacco and tomato the initial increase (at 10dpi) in the level of TCP and LoxD (between 1.8 folds to 5.5 folds) suggests that, stress signalling initiated on the onset of disease and showed subsequent decrease in levels at later stages of the infection (Fig.23G; 24G). Although accumulation levels of ARF8 and MYB involved in auxin responses and leaf development were found to be relatively low at initial stages, their level of accumulation was gradually increased at later stages. Although in papaya plants, initial increase in LoxD accumulation it was observed, significant level of accumulation was not found. TCP1, ARF8 and MYB accumulation showed reverse trend in most of the infection period tested as compare to that of infected tomato and tobacco plants, suggesting that, miRNAs may selectively regulate their targets depending upon the severity of the infection (Fig.25G).

**7.4 Temperature specific response in AC4 and miRNAs accumulation in tomato plants**

Since tomato is a natural host for leaf curl virus infection, we have analysed temperature specific accumulation of AC4 and miRNAs in tomato plants. In general we observed abundant accumulation of AC4 and miRNAs at ambient temperatures between
$25^\circ$C to $30^\circ$C, below and above $10^\circ$C of ambient temperature we found drastic decrease in accumulation level of AC4 and miRNAs. Quantification of pathogenicity factor-AC4 in infected tomato plants subjected to different temperatures *viz.*, $12^\circ$C, $25^\circ$C, $30^\circ$C, $35^\circ$C and $40^\circ$C was done through Q-PCR detection. Temperature specific accumulation of AC4 was observed in infected tomato plants. Significantly lower accumulation of AC4 was found at $12^\circ$C (0.71 folds), there after steep increase in the AC4 accumulation of 10.45 folds was found at $25^\circ$C and attains maximum accumulation up to 13.47 folds at $30^\circ$C. As temperature increases to $35^\circ$C and $40^\circ$C, AC4 accumulation was found to be decreased to 8.02 and 6.05 folds respectively (*Fig.26A*).

In order to examine alteration in accumulation of selected miRNAs, analysis by Q-PCR was performed to detect whether they can be regulated by temperature stress after 24hr treatment in infected tomato plants, we examined the miRNA accumulation and compared the miRNA expression changes at different temperatures, we found that, the tested miRNAs (miR159, miR164, miR167, miR171, miR319, miR393 and miR398) showed differential expression in response to different temperature treatment. Generally Increased accumulation of miRNAs were found at $25^\circ$C and $30^\circ$C as compared to $12^\circ$C, $35^\circ$C and $40^\circ$C in which it showed decreased accumulation of tested miRNAs. At all the temperature tested ($12^\circ$C, $25^\circ$C, $30^\circ$C, $35^\circ$C and $40^\circ$C) it was observed that, no significant difference in level of accumulation of miR159 and miR171. At $12^\circ$C and $35^\circ$C miR164 and miR167 showed decreased accumulation (between 1.5 to 2.3 folds) whereas miR319 and miR398 showed increased accumulation (between 3.4 to 6.9). Significant increase in the level of miR164, miR167, miR393 and miR398 were observed at $25^\circ$C and $30^\circ$C (5.6 to 10.17 folds), where as miR319 showed differential level of accumulation (between 2.4 to 4.4 folds). Where as at $40^\circ$C all the five miRNAs (miR164, miR167, miR319, miR398 and miR393) showed decreased accumulation level (between 0.09 to 4.1 folds) as compared to level of accumulation at $25^\circ$C and $30^\circ$C (*Fig.26B*).

We also analyzed the profiling of TFs *viz.*, ARF8, MYB, TCP1 and LoxD transcript. Our results revealed differential accumulation of these mRNAs at different temperatures tested and was not consistent with the miRNA accumulation. Although there was no significant difference in accumulation of TCP1 (between 0.5 to 1.5 folds) and LoxD
(0.12 to 0.51 folds) at 12°C, 25°C, 30°C and 35°C, significant increase in their levels was found at 40°C (between 2.1 to 2.4 folds). Compared to all temperatures significant increase in the level of ARF8 was found at 40°C, where as MYB showed differential accumulation in all the temperatures tested (Fig. 26C).

7.5 Discussion
In the present study two different stresses (virus infection and temperature stress) and their impact on transcriptional changes (miRNAs and their targets) in the hosts has been studied and we also studied symptom development and transcriptional changes upon virus infection were host specific. We observed expression of symptoms were directly proportional to accumulation of AC4 in three different hosts, which in turn directly correlated with the miRNA accumulation. Plant development is governed by cellular processes and these cellular processes are regulated by miRNAs. In our study we showed alteration in miRNAs were host specific, thus during infection altered miRNAs disturbed the plant cellular processes which appeared in the form of symptom development. Host-viral interactions can be studied by studying the viral pathogenicity factor-AC4 and the host miRNAs, to study this interaction we selected three hosts viz., Solanum lycopersicum, Nicotiana tobacca and Carica papaya and seven miRNAs viz., miR159, miR164, miR167, miR171, miR319, miR393 and miR398 and some of their targets such as MYB, ARF8, TCP and LoxD. Since tomato is a natural host for leaf curl virus infection, we studied role of temperature in accumulation of AC4 and miRNAs and results showed that, ambient temperature is most suitable for viral infection and accumulation of AC4 which ultimately results in the changing profile of miRNAs.

It was found that, miR164, miR167 and miR393 levels were up regulated in symptom developed tobacco and tomato than in asymptomatic papaya plants indicating the involvement of these miRNAs in symptom determination by affecting plant development and auxin homeostasis. It has been shown that, miR164 and miR167 were demonstrated to be involved in root cap formation, lateral root development, or adventitious rooting through the auxin signal further transduced by their downstream ARF (auxin response factor) targets (Meng et al., 2010). It was also reported that the
miR164-mediated auxin signaling was required for normal lateral root development. The study showed that one TF NAC1 (NAM/ATAF/CUC1) was targeted by miR164, and NAC1 transduced auxin signal for lateral root emergence. Thus, auxin-induced expression of miR164 and subsequent mRNA clearance of NAC1 provide a homeostatic mechanism to down-regulate auxin signals for lateral root development (Guo et al., 2005). With respect to miR393, evidence indicates the potential role of miR393 in lateral root formation in Arabidopsis. The transcription factor NAC1, known to be targeted by miR164, acts downstream of TIR1 (miR393 target) to transduce auxin signal to promote lateral root development (Xie et al., 2000) Considered together, the regulatory pathway miR393–TIR1–NAC1, which likely interacts with the miR164–NAC1 pathway, may participate in lateral root development (Meng et al., 2010). It was found with respect to miR171 that, no significant changes in its accumulation level in all the three hosts, suggesting that, miR171 expression is independent of virus infection. It was reported that, Scare-like family gene (SCL6-II, SCL6-III and SCL6-IV) the targets of miR171 are a class of specific transcriptional factors containing GRAS domain that controls several developmental processes, including root radioactive growth (Helariutta et al., 2000) gibberellin signal transduction pathway (Silverstone et al., 1998)

The other two studied miRNAs; miR159 and miR319 having role in leaf development and JA mediated stress signalling (Palatnik et al., 2003; Millar et al., 2005; Schommer et al., 2008), along with their targets TFs, MYB, TCP and transcript LoxD were found to be differentially regulated at early and late phase of infection. Both of these miRNAs showed accumulation at early stage and decreased levels were observed once the disease is set, suggesting their role in altering leaf development at initial infection stages and their possible involvement in stress signalling pathway (Schommer et al., 2008). Further we found clear increased induction of LoxD transcript at early infection period in solanaceous hosts as compared to papaya host suggests the initiation of defense/stress responsive JA biosynthesis pathway during infection.

We also found increased and decreased level of miR398 in symptom developed tomato, tobacco plants and non symptom developed papaya plants these observations
suggest a role of miR398 in virus infection might be associated with oxidative stress induced by virus infection. Consistent with this evidence, it has been reported that, decreased accumulation of miR398 causes increase accumulation of its target CSD1 and CSD2 in Arabidopsis seedlings exposed to high light, high Cu\textsuperscript{2+} or high Fe\textsuperscript{3+} (Sunkar et al., 2006) and bacterial (P. syringae) infection (Jagadeeswaran et al., 2009).

It was also found that, abundance of miRNA accumulation in infected tomato plants under ambient temperatures where AC4 accumulation was also found to be more. This miRNA abundance was also correlated with the increased accumulation level of miRNAs in symptom developed tomato plants at 25dpi, suggesting that biotic stress (viral infection) and abiotic stress (temperature treatment) signalling pathway may partially overlap. It was shown that, many of the ambient temperature responsive miRNAs (miR159, miR171, miR167, miR319 and miR398) were up regulated in Arabidopsis (Lee et al., 2010). Although ambient temperature (non stress temperature) which influences the metabolic reactions and morphogenetic processes and mediates the plant growth and development, from our study it was clear that the same temperature is also suitable for virus infection and accumulation of pathogenicity factor-AC4 which might be interfering in normal regulatory pathway of miRNAs and playing role in symptom development.

7.6 Conclusion:
Based on the various observations regarding miRNA and some of its target expression levels, during viral infection and temperature stress treatment, following statements can be hypothesized. First, miRNA expression is highly organized and having correlation with symptom development. Second, miRNA expression patterns are correlated with AC4 accumulation which is maximum at ambient temperature. This observation can link pathogenicity factor-AC4 with the up regulation of most of the miRNA tested. Third, decrease in virus load/AC4 accumulation at higher and lower temperature compared with ambient temperature indicate that, temperature might be playing crucial role in host-virus interaction. Finally we conclude that, this study will provide further insight to investigate a complex network between hosts, virus and temperature and cross talk between biotic and abiotic signalling pathways.
The molecular basis for virus induced symptoms and defense pathways have been longstanding mystery. It is known that, viral infections disrupt the functions of regulatory small RNAs, such as micro RNAs, and phytohormone signalling or biosynthesis leading to developmental defects. Thus it outlined some common ways that a broad range of viruses may alter plant gene expression. Incompatible interactions between viruses and hosts have previously served as models for investigating host defense responses.

To know the host transcriptional changes associated with symptom expression and stress signalling during Tomato leaf curl infection, a comparative transcriptional analysis of certain miRNAs (miR164, miR167 and miR393) and auxin responsive factor 8 (ARF 8) controlling auxin responses, miR159, miR319, miR164 and transcription factor TCP, MYB and lipoxygenase D (Lox D) controlling plant morphogenesis (leaf and root development) and stress signalling was carried out. It was further correlated with disease symptom development. The auxin and JA signalling share many commonalities (Mockaitis et al. 2008) and are interrelated by a probable network, based on our observations (Fig.18). The JA biosynthetic gene LOX2 (chloroplast encoded lipoxygenase2) regulated by miR319/TCP activity (Mockaitis et al. 2008). Also miR319/TCP node has been involved in regulation of miR164/CUC node as a part of the auxin signalling network (Somoza et al. 2009).

Based on our observations, we propose a network involved in plant development and stress responses during viral infection. These cascades of responses are regulated by miRNAs. The transcriptional changes in the accumulation levels of these miRNAs and their targets can be interpreted in terms of development of disease like symptoms and defense through stress signalling. Many of these development disease symptoms and changes in micro RNA levels resemble in ToLCNDV-AC4 expressing transgenic plants, providing a molecular rationale for involvement of viral pathogenecity factor - AC4 in disease development. Our results on exogenous application of auxins resulted in partial recovery of disease like phenotype probably by affecting the root growth and helping in creating an auxin homeostasis during infection. Besides having role in leaf and root development, involvement of auxin signal transduction pathway in defense
responses (Bari et al. 2009) may also be contributing in improved development during infection.

It is expected that these studies will help in better understanding of host virus interface. Host virus interaction also enhanced by increasing host ranges for viruses. On the basis of this, we studied host specific expression of micro RNAs during leaf curl virus infection: in response to viral pathogenicity factor –AC4 by studying the expression profiling of seven miRNAs and their target mRNAs viz., miR159, miR164, miR167, miR171, miR319, miR393 and miR398, and their target mRNAs viz., MYB, ARF8, TCP and LoxD in three different hosts viz., Solanum lycopersicum, Nicotiana tobaccam and Carica papaya during leaf curl virus infection. We observed expression of symptoms were directly proportional to accumulation of AC4 in three different hosts, which in turn directly correlated with the miRNA accumulation. Plant development is governed by cellular processes and these cellular processes are regulated by miRNAs. In our study we showed alteration in miRNAs were host specific, thus during infection altered miRNAs disturbed the plant cellular processes which appeared in the form of symptom development. It was found that, miR164, miR167 and miR393 levels were up regulated in symptom developed tobacco and tomato than in asymptomatic papaya plants indicating the involvement of these miRNAs in symptom determination by affecting plant development and auxin homeostasis (Meng et al., 2010; Guo et al., 2005; Xie et al., 2000; Palatnik et al., 2003; Millar et al., 2005; Schommer et al., 2008).

Since tomato is a natural host for leaf curl virus infection, we studied role of temperature in accumulation of AC4 and miRNAs and results showed that, abundance of miRNA accumulation in infected tomato plants under ambient temperatures where AC4 accumulation was also found to be more. This miRNA abundance was also correlated with the increased accumulation level of miRNAs in symptom developed tomato plants at 25dpi, suggesting that biotic stress (viral infection) and abiotic stress (temperature treatment) signalling pathway may partial overlap. It was shown that, many of the ambient temperature responsive miRNAs (miR159, miR171, miR167, miR319 and miR398) were up regulated in Arabidopsis (Lee et al. 2010). Although ambient temperature (non stress temperature) which influences the metabolic reactions and morphogenetic processes and mediates the plant growth and development, from our
study, it was clear that the same temperature is also suitable for virus infection and accumulation of pathogenicity factor-AC4 might interfering normal regulatory pathway of miRNAs which in turn appears in the form of symptom development.

From these studies we suggest that, viruses adapt to their hosts by evading defense mechanisms and taking over the plant cellular metabolism for their own benefit and these antiviral responses contribute to symptom development. In the line of this, we studied molecular basis of host specific adaptation of tomato leaf curl New Delhi virus pathogenicity factor-AC4 by anlysing its variability/mutation during serial passaging of virus into three different hosts viz., tomato, tobacco and papaya.

An important consequence of high mutation and recombination rates is the continuous production of genetic variation in geminivirus populations. This variability is balanced by a complex set of selection pressures including those associated with intrinsic properties of the virus, such as the maintenance of essential nucleotide structures and replication signals, and selection pressures to maintain crucial interactions with plant hosts and insect vectors (Astorga et al., 2007).

*Tomato leaf curl New Delhi virus* (ToLCNDV) is a member of family *Geminiviridae* posses bipartite genome, having small ORF called AC4 in DNA-A genome which is responsible for pathogenesis and symptom development. To establish a role of pathogenicity factor-AC4 in viral genome evolution and its adaptation to different hosts, the AC4 gene variability in ToLCNDV populations were evaluated from HRM analysis and also by cloning and sequencing of viral progeny populations derived from infecting with single type of ToLCNDV population through whitefly mediated transmission. Serial passage of ToLCNDV in different hosts viz., tomato, tobacco and papaya plants through whiteflies was performed to study the host specific mutations. Our results clearly showed that, ToLCNDV generated variants (w.r.t AC4 gene) rapidly upon its serial passages in three different hosts to generate a heterogeneous populations and based on the mutation distribution and its frequency, the consensus sequence and diversity level in the population were stable as passage of virus was progressed. From our study it was clear that, mutation frequency observed in ToLCNDV in clonal progeny population from III passage and II passage were (2.3 x 10^{-4} to 9.8 x 10^{-4} substitutions/site) comparable to the mutation frequency reported for plant RNA viruses.
Theoretically, these viruses should have less population variation. However, there is no information about the nature of plant DNA polymerase or the polymerase factors involved in the replication of geminiviruses and in particular, it is not known whether only a subset of cellular DNA replication and/or mismatch repair machinery is activated for geminivirus replication or whether the cellular environment affects the fidelity of those polymerases (Ge et al. 2007).

Thus the genetic diversity generated by high mutation rates and frequent recombination allows the rapid evolution of viruses in response to host defenses (Power 2000). Overall we found five nucleotide substitutions in AC4 gene, four were transitions and only one substitution was transversions. The dominance of transitions under natural conditions and under controlled environment is consistent with results obtained in the AC1/AC4 region of TYLCCNV (Ge et al., 2007) and IR/AC1 fragment of EACMCV (Fondong et al., 2011). These observed genetic changes in the pathogenecity factor might be crucial for host adaptations and are evolving under positive selection pressure (van der Wallt et al., 2008). Natural selection may had favoured these mutated sites in the genomes which might be playing some role in defense mechanism as well as in pathogenecity. Thus the genetic diversity generated by high mutation rates and frequent recombination allows the rapid evolution of viruses in response to host defenses (Power 2000).

RNA silencing is one of the host defense mechanism which is a sequence-specific process of regulating gene expression, this also involves RNA directed DNA methylation (RdDM) in which DNA homologous to triggering RNA is methylated de novo. To analyse RdDM in the pathogenecity factor of ToLCNDV, we compared the methylation pattern during natural infection and in transformed tomato plants with a hairpin construct from ToLCNDV-AC4 region (Ihp-AC4). RdDM of cytosine residues of AC4 region specifically occur more in number in Ihp transformed tomato over natural infection. Methylation was found in cytosine residues falling in putative RNA-DNA duplex and it progressively decreased in the sequence adjacent to the duplex. Methylation results showed abundant levels of methylation in asymmetric cytosines in transformed tomato plants over naturally infected tomato. Thus it shows that, asymmetric CpG sequences might be potential targets of methylation for defensive
purposes. Over all our results suggests that, Ihp-AC4 triggered RdDM mechanism in virus efficient enough to maintain the resistance in Ihp_AC4 transgenic as a heritable epigenetic trait by maintaining methylated cytosine sites. This hypothesis is supported by Molinier et al (2006) and Boyko et al (2007) who showed the inheritance of an epigenetic signal generated by exposure of Arabidopsis plants by bacterial and virus infection.
Plant developmental processes are tightly controlled and regulated at gene expression. Plant hormones play a critical role in regulating these processes. In plants, micro RNA (miRNA) targets wide range of mRNAs involved in various developmental processes including hormone signalling. During viral infection various developmental processes are affected leading to symptom development by affecting host defense mechanism.

RNA silencing in the plants involves RNA directed DNA methylation (RdDM) as an epigenetic defense mechanism, in which DNA homologous to a triggering RNA is methylated \textit{de novo}. The AGO4 associated siRNAs (produced from viral genome or from transgene construct) target the complex to homologous DNA sequences, where cytosine methyl transferases proteins are recruited to methylate asymmetric (CpHpH where H is A, T or C) and symmetric (CpG, CpNpG where N is A, T, G or C) cytosines. In response this host defense, during viral infection genetic diversity is generated by high mutation rates and frequent recombination allows the rapid evolution of viruses. Host induced genome evolution allows for adaptation of plant viruses to their hosts which is found to be either random or selected depending on the virus and host species. Since leaf curl viruses (geminiviruses) uses host replication machinery for the replication and spends a larger fraction of its infection cycle in the form of single stranded DNA, where host exonucleases acts on double stranded DNA which may not repair the mutations introduced during replication and leads to higher rate of mutations which are similar to that of RNA viruses.

Viral encoded pathogenecity factor/RNAi suppressor plays a significant role during infection. In the present study we have analysed host-virus interations in relation to \textit{Tomato leaf curl New Delhi virus} pathogenecity factor-AC4 with respect to seven miRNAs involved in hormone signalling, plant defense, root and leaf development. We also compared the miRNA profiling in three different hosts \textit{viz.}, tomato, tobacco and papaya during ToLCNDV infection along with ToLCNDV-AC4 transgenic tomato.

The results and the inference in the present study are described as:
1. We found accumulation of all the studied miRNAs in three different hosts \textit{viz.}, tomato, tobacco and papaya also in ToLCNDV-AC4 transgenic tomato plants.
2. Phenotypic expressions of symptoms were appeared after 15 days of post inoculation in tomato and tobacco although papaya appeared as a symptomless carrier.

3. Interestingly AC4 transgenic tomato plants mimic ToLCNDV infection in terms of phenotypic expression of symptoms.

4. Comparisons of miRNA accumulation in three different hosts suggest a possible link between miRNA accumulation and phenotypic expression of symptoms.

During ToLCNDV viral infection in tomato stress signalling pathway in relation to TF TCP and lipoxygenase D (LoxD) were studied in order to establish a link between auxin regulation and symptom development.

1. It was observed that, down regulation of auxin stress signalling pathway during viral infection.

2. *In vitro* application of auxin to the infected plant partially helps in the recovery of symptoms suggesting a probable role of auxin in disease development.

Molecular basis of host specific adaptation of virus was studied with respect to viral pathogenicity factor AC4 and host epigenetic defense – RNA directed DNA methylation (RdDM).

1. Asymmetric cytosine methylation was found to be more prominent in transgenic tomato plants expressing Ihp-intron spliced hairpin from AC4 region as compared to natural infection. This observation suggests the role of RNAi mechanism in triggering host epigenetic defense responses.

Genetic variation in ToLCNDV-AC4 gene sequence was studied using serial passaging experiment in three different hosts *viz.*, tomato, tobacco and papaya by a sensitive High Resolution Melting (HRM) curve analysis. Co-variation study in AC4 by mutation scanning suggests the occurrence of mutation in N-myristoylation domain responsible for symptom severity. The mutation frequency was very close to as plant RNA viruses. The occurrence of AC4 mutation during passaging may have some structural and functional relationship in host virus interactions.
ABSTRACT

Viruses exclusively depend on host cell machinery for their propagation and survival, hence they modulate the host gene expression to suit their needs. Thus the range of interactions between various host and viral factors at the host virus interface are interesting to study. These interactions can be fine tuned in the host by modulating gene expression through triggering defence mechanism and allowing epigenetic modifications. In the viral genomes high rate of mutations add to the complexity of these interactions and help in developing adaptations to different hosts. In the present study, we analysed host adaptability of Tomato leaf curl New Delhi virus by mutation studies in the pathogenecity factor-AC4 during passaging through three different hosts. The N-myristoylation domain responsible for pathogenecity was found to be more prone to mutations. AC4 was also found to play an important role in stress signalling by regulating miRNAs in auxin biosynthesis. RNAi triggered epigenetic modifications in the AC4 gene was found to be prominent host defense pathway. AC4 was also found to be linked with phenotypic expression of symptoms by its role in accumulation of seven studied miRNAs involved in plant developmental processes in three different hosts. Collectively AC4 a pathogenecity factor/RNAi suppressor was found to be crucial for its role in regulating host gene expression, by stress signalling pathway. Host response to infection was studied by RNAi triggered epigenetic modification in this viral gene.
वियागु अपने पुलिस एवं उत्सुक रखकर हेतु पूर्णक्षण आतंतिक कोडिका की योजना पर
निर्माण होते हैं। इसलिए वे आतंतिक जीन-अभिव्यक्ति को अपनी आयस्तताओं के अनुसार
अधिनियम नियम हैं। इस प्रकार वे, आतंतिक वियागु अंतररुप पर कई आतंतिक एवं वियागु
संबंधी कारों के बीच अन्यत्र किया जा सकता है। इसके विपरीतिचिदं को
आरम्भ कर तथा प्रयोजन परिपत्रों की त्वरितता के गठन से जीन अभिव्यक्ति के अधिनियम
द्वारा आतंतिक ने इन अन्यत्र किया जा सकता है। वियागुओं की जीनी
संरचनाओं में उपर्युक्तों की उच्च दर इन अन्यत्र किया जा सकता है। इसके विपरीती
देते है तथा विभिन्न आतंतिकों में अनुसूचितों के विकास में सहायक होते हैं। प्रतिलक अध्ययन में
हमने तीन मिश्र-मिश्र आतंतिकों से परिपत्रों के दौरान रोगनकता कारक –ए सों के साथ दर्जन
तन अध्ययनों द्वारा टायटर के पर्याप्त नई विलय वियागु की आतंतिक-अनुसूचित आतंतिक का
विलय किया। रोगनकता के लिए उत्तरदायी एवं-माध्यमिक परियोजना डीमन, उपर्युक्तों हेतु
अधिक सुधार पाया गया। अधिकांश नृत्य संस्कृति में एवं आई, एवं एक
के निर्माण द्वारा ए सों 99 प्रतिलक सम्पर्क में भी एक महत्वपूर्ण भूमिका निभाता है। इसी सों 99
जीन में एवं आई द्वारा प्राप्त विकास परिपत्र, आतंतिक रक्षा पावने में विशिष्ट रचना रचने
वाले पाये एवं रोगनकता के लक्षण प्रस्तुत अभिव्यक्ति के साथ भी सों 99 का रंगप किया
पाया गया किंतु तीन मिश्र-मिश्र आतंतिकों में पालन विकास संबंधी प्रतिलकों में अभिनियम,
विलय किया गया एवं आई, एवं ए के संरचन में भूमिका थी। सामाजिक रूप से, इस सों 99, एक
एक रोगनकता कारक/आर ए आई, दमनक, प्रतिलक संरचन पावन द्वारा आतंतिक जीन
अभिव्यक्ति के प्रति निर्माण के आतंतिक आवश्यक रूप से एक रोगनकता कारक/आर ए आई, दमनक,
P्रतिलक संरचन पावन द्वारा आतंतिक जीन
अभिव्यक्ति के प्रति निर्माण के आतंतिक आवश्यक रूप से एक रोगनकता कारक/आर ए आई, दमनक,


Kurihara, Y., Takashi, Y. and Watanabe, Y. 2006. The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. *RNA* **12**: 206-212.


microRNAs in rice, implying their regulation roles in plant hormone signalling.  


Patil, B.L., Rajasubramaniam, S., Bagchi, C. and Dasgupta I. 2005. Both Indian cassava mosaic virus and Sri Lankan cassava mosaic virus are found in India and exhibit high variability as assessed by PCR-RFLP. *Arch Virol* **150**: 389–397.


Raman, S., Greb, T., Peaucelle, A., Blein, T., Laufs, P. and Theres, K. 2008. Interplay of miR164, *CUP-SHAPED COTYLEDON GENES* and *LATERAL*


1. Test Plants

Viruses used in the study are *Tomato leaf curl New Delhi virus* (ToLCNDV was maintained on tomato using whitefly-mediated inoculation in cage under glass house conditions. Virus infection was confirmed by leaf curl symptoms and by PCR and All transmission experiment such as acquisition and inoculation access feeding periods took place isolated cages kept in a growth chamber at 26°C with a photoperiod of 16:8 hr (light:dark).

2. Polymerase Chain Reaction (PCR) Amplification

Specific primers were synthesized from Sigma. Sequences of NSs gene primer was based on sequence of GBNV (R.K.Saritha and R.K.Jain, 2007) and sequences of HcPro gene primers were based on sequence of PRSV available in NCBI database.

The PCR reaction mixtures were prepared as follows, for all of the PCR reactions

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume required (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DNA/cDNA (25 ng/µl)</td>
<td>2</td>
</tr>
<tr>
<td>Forward primer (200 ng/µl)</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer (200 ng/µl)</td>
<td>1</td>
</tr>
<tr>
<td>10 X PCR buffer</td>
<td>2</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1</td>
</tr>
</tbody>
</table>
Taq DNA polymerase 1
Nuclease free water 12
Total 20

After PCR reaction was over, 1 µl of amplified product was subjected to electrophoresis in 1% agarose gel to observe the DNA fragment of predicted size.

3. Agarose Gel Electrophoresis
2 µl of the amplified PCR product was mixed with 4 µl of 6× loading dye and sterilized water to make up the volume to 20 µl and were loaded onto wells in 1% agarose gel, prepared in 1× Tris-acetate-EDTA (TAE) buffer containing EtBr (0.5 µg/ml). Electrophoresis was carried out at 60 V for 20-30 min. An aliquot (500 ng) of 1 kb DNA ladder (MBI Fermentas) was similarly mixed with dye and electrophoresed and run along with the samples to serve as molecular size marker.

4. PCR Purification of Amplified Product
The remaining PCR product left after checking the amplified product by agarose gel electrophoresis was purified by promega PCR purification kit following the manufacturer’s protocol. This protocol is designed to purify single or double stranded DNA fragments from primers, nucleotides, polymerase and salts present in PCR products mixture. The purified PCR product was subjected to electrophoresis in 1% agarose gel, to check its purity. Then product was there after used for cloning purposes.

5. Cloning of amplified product in pGEM-T Easy Cloning Vector
pGEM-T Easy vector (3015 bp) from Promega is convenient system for the cloning of PCR products. Successful clones of the above mentioned inserts in the pGEM-T Easy vectors is identified by colour screening on indicator plates containing ampicillin, X-gal and IPTG, as the vector contains the ampicillin resistance marker gene and the insert interrupts the coding sequence of β-galactosidase thus producing white colonies upon overnight incubation at 37°C temperature.
5.1. Optimization of Insert: Vector Molar Ratios

1:3 ratio of the vector to DNA insert provided good result. The concentration of PCR product was estimated on comparison to DNA mass standards on agarose gel.

5.2. Ligation of PCR Product to pGEM-T Easy Cloning Vector DNA

Ligation reactions were carried out between vector and PCR product. The reaction mix prepared for the purpose was as follows:

- pGEM-T Easy vector (50 ng/µl) 1 µl
- PCR amplified product (50 ng/µl) 3 µl
- 2X Ligation buffer 5 µl
- 10 mM ATP 1 µl
- T4 DNA ligase (3U/µl) 1 µl
- Total 10 µl

The ligation mixtures were incubated at 4°C for 18 hours.

5.3 Preparation of Competent Cells

The competent cells were prepared by CaCl2 method described by Sambrook and Russel, 2000.

- 50 ml Luria Broth (LB) was inoculated with overnight grown culture of DH5α strain of *Escherichia coli* and incubated at 37°C for 1 h and 15 min. with constant shaking at 200 rpm in a shaker incubator till the bacterial growth as measured by optical density reached 0.3 O.D. at 600 nm.
The culture was then aseptically transferred to 40 ml sterile screw capped tubes and kept on ice for 10 min.

The culture was centrifuged at 5000 rpm for 10 min. at 4°C in a Sigma 3K30 centrifuge to obtain the cells as pellet.

The cells were resuspended gently in 10 ml ice cold 0.1 M MgCl₂ solution and centrifuged at 5000 rpm for 10 min. at 4°C.

The pellet was resuspended in 10 ml ice cold 0.1 M CaCl₂ solution and kept on ice for 1 h.

The cells were recovered by centrifuging at 5000 rpm for 10 min. at 4°C and the pellets were resuspended in 1 ml of chilled 0.1 M CaCl₂ and used for transformation after keeping on ice for 1 h.

**5.4 Transformation of Competent Cells**

- 200 µl competent cells were added to 20 µl of each of the ligation mixtures in two separate sterile microfuge tubes and were gently mixed and kept on ice for 1 h.
- The competent cells were given heat shock at 42°C for 90 sec. 1 ml of LB medium was then added and the transformants were allowed to grow at 37°C for 1 h in shaker incubator at 200 rpm.
- Two sets of 200 µl of serially diluted cell suspensions were aseptically plated on Luria Agar (LA) plates separately containing ampicillin, X-gal and IPTG (50 µl of 50 µg/ml ampicillin, 100 µl of 2 per cent X-gal and 10 µl of 0.1 M IPTG in 50 ml LA).
- The plates were incubated overnight at 37°C.

**5.5 Selection of Transformants**

The transformants were selected on the basis of blue/white colonies. The white colonies were selected and subsequently streaked on LA Plates (master plates) containing IPTG, X-gal and ampicillin.
6. Rapid Screening for the Recombinant Clones by Colony PCR Method

From the master plates colonies were picked up randomly and screened by polymerase chain reaction, using the gene specific primers. In this case of colony PCR, a single colony was taken in each reaction mix in lieu of DNA sample.

Following was the colony PCR reaction master mix:

- Forward primer (200 ng/µl) 10 µl
- Reverse primer (200 ng/µl) 10 µl
- 10X PCR buffer 20 µl
- 10 mM dNTPs 10 µl
- *Taq* DNA polymerase (5U/µl) 5 µl
- Sterile distilled water 145 µl
- Total 200 µl

Aliquot of 20 µl of master mix was taken in ten different PCR tubes and the white colonies were taken in each tube. The tubes were then placed in the same thermal cycler. The temperature profile and cycle were same as used in amplification of gene.

7. Isolation of Recombinant Plasmid DNA by Miniprep Method

- The Recombinant plasmid from the positive clones was isolated following the modified alkaline lysis method (Sambrook and Russel, 2000).
- Selected white colonies of positive clones, found positive in colony PCR reaction were individually inoculated in 2 ml of LB medium containing ampicillin (50 µg/ml) in sterile capped culture tubes.
- Tubes were then incubated overnight at 37°C at 200 rpm in a shaker incubator.
- The overnight grown bacterial cells were then transferred to 1.5 ml sterile eppendorf tube and cells were harvested by centrifuging in a table top centrifuge for 1 min. Care was taken to remove the medium adhering to the cell pellet.
- The pellet was resuspended in 100 µl of solution I and mixed vigorously by vortexing.
The 200 µl of freshly prepared lysis solution (solution II) was then added and mixed gently.

150 µl of ice cold solution III was added next and mixed gently with lysed cell suspension and the mixture was kept on ice for 15 min.

The chromosomal DNA and the bacterial cell debris were removed by centrifuging at 10,000 rpm for 20 min, at 4°C in a table top centrifuge (Sigma 112).

The supernatant was again centrifuged for another 20 min at 10,000 rpm at 4°C to remove any unwanted bacterial debris as pellet.

The supernatant was collected and equal volume of phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added. It was vortexed well, centrifuged in a tabletop centrifuge for 15 min. at room temperature. The clear aqueous phase was transferred to fresh eppendorf tube.

The DNA in aqueous phase was precipitated by adding 0.8 volume of isopropanol and kept on ice for 10 min.

The mixture was then centrifuged at 15000 rpm for 20 min at 4°C.

To the pellet 200 µl of 70% ethanol was added. The tube was rotated well so that the pellet from the wall gets suspended in 70% alcohol. This ensures removal of adhering salts by 70% alcohol. DNA was then pelletized by centrifuging at 15000 rpm for 5 min.

The pellet was finally suspended in 30 µl sterile double distilled water.

8. Release of Inserts with Restriction Enzyme

Recombinant plasmids from positive clones were subjected to digestion with NotI restriction enzyme. This enzyme was so chosen because the restriction site of this enzyme is present in both side of insertion site of vector but not present in the insert. Restriction mix was incubated at 37°C for overnight for complete digestion. Restriction was done to release the insert and also to know the insert size. The reaction mixtures were prepared for each of the restriction digestion as follows:

Recombinant plasmid DNA (2 mg/ml) 10.0 µl
10X reaction buffer 2.5 µl
Restriction enzyme (Not I) (10U/µl) 0.5 µl
Sterile double distilled water 12.0 µl
Total 25.0 µl

After restriction digestion, the products were electrophoresed on 1% agarose gel. Fragment size was assessed in comparison with 1 kb DNA ladder loaded as molecular weight marker on to the same gel along with the DNA samples.

**8.1 Gel Purification of Restricted Product**

Released insert were excised from the agarose gel and purified using Qiagen Gel Purification kit following manufacturer’s instructions.

**9.0 Cloning of bisulfite converted DNA**

**9.1 Screening for recombinant clones by colony PCR**

The colonies obtained were screened for the presence of the insert by colony PCR with gene specific primers to screen for the presence of entire cassette.

The mixtures were then placed in thermal cycler separately (ERICOMP Power Block II System). Following were the temperature profile and cycles performed

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total DNA (25 ng/µl) (plasmid or Plant genomic DNA)</td>
<td>2 µl</td>
</tr>
<tr>
<td>Forward primer (200 ng/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Reverse primer (200 ng/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>12 µl</td>
</tr>
<tr>
<td>Total</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

(ERICOMP Power Block II System)
<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation of DNA</td>
<td>94ºC</td>
<td>4 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94ºC</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>58ºC</td>
<td>40 sec</td>
<td></td>
</tr>
<tr>
<td>Primer extension</td>
<td>72ºC</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Final primer extension</td>
<td>72ºC</td>
<td>5 min</td>
<td>1</td>
</tr>
</tbody>
</table>

After PCR reaction was over, 1 µl of amplified product was subjected to electrophoresis in 1% agarose gel to observe the DNA fragment of predicted size.

10. sRNA isolation-Trizol method

- Grind tissue 100mg in liquid nitrogen
- Wait for 1 minute. Add 1 ml Trizol reagent
- Keep for 5 minutes at room temperature in mortar itself
- Transfer in 1.5ml eppendorf
- Add 200µl chloroform, mix well
- Keep at room temperature for 15 minutes
- Spin at 13000rpm for 15 minutes at 4ºC
- Transfer aqueous phase to fresh tube
- Add 0.5ml isopropanol, mix well
- Store at room temperature for 15 min
- Spin at 13000 rpm for 15 minutes at 4ºC
- Discard supernatant
- Add 500µl of 70% chilled ethanol and spin again at 13000 rpm for 15 min at 4ºC
- Discard supernatant
- Dry pellet for 10-15 minutes in incubator at 37ºC
- Add 50µl DEPC treated H₂O
- Incubate at 60ºC for 10 minutes, and store the RNA at -20º/ -80ºC.

14. Northern blotting

14.1 dPAGE recipe for 17% gel
Acrlyamide 40% 6.4 ml
Urea 6.3 ml
3-(N-morpholino) propanesulfonic acid MOPS (1M) 750 µl

Keep at gentle shaking at 37°C
N, N, N', N'-tetramethylethylenediamine (TEMED) 7.5 µl
Ammonium persulfate (APS) 25% 30 µl
DEPC treated water to make 15 ml

Run at 90 V for 3-4 hr.

14.2 Pre-hybridization Solution
Denhardt’s 5×
SSC 6×
SDS 0.2 %

14.3 Probe prepration
DNA or RNA substrate 0.1-20 pmol
γ-32P-ATP 1-40 pmol
10× Kinase Buffer 1 µl
T4 PNK (10 U/µl) 1 µl
Nuclease-free water to make 10 µl

Incubate the 5’ end labeling reaction for 1 hr at 37°C.
Store radiolabeled nucleic acids at -20°C or -80°C.

15. Reverse Transcription
1. RNA and specific reverse primer were mixed in eppendorf tube:
   Total RNA 5 µg
   Reverse primer (50 ng/µl) 2 µl
   DEPC H2O to 10 µl

2. Incubate the samples at 65°C for 5 min and then on ice for at least 1 min.
3. Prepare reaction master mixture. For each reaction:
   10× RT buffer 2 µl
   25 mM MgCl2 4 µl
10 mM dNTP mix 1 μl
0.1 M DTT 2 μl
RNAase OUT 1 μl

4. Add the reaction mixture to the RNA/primer mixture and RNase free H2O to make 49 μl, mix briefly, and then place at room temperature for 2 min.

5. Add 1 μl (200 U) of RT to each tube, mix and incubate at 25°C for 10 min.

6. Incubate the tubes at 42°C for 50 min, heat inactivate at 70°C for 15 min, and then chill on ice.

7. Add 1 μl RNase H and incubate at 37°C for 20 min.

8. Store the 1st strand cDNA at -20°C until use for real-time PCR.

16. Real-time PCR

1. Normalize the primer concentrations and mix gene-specific forward and reverse primer pair. Each primer (forward or reverse) concentration in the mixture is 5pmol/μl.

2. A real-time PCR reaction mixture 25 μl. Prepare the following mixture in each optical tube.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA (20 ng)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>10x TaqMan buffer</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>MgCl2 (25 mM)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>dNTPs (10mM)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Primer F (10 μM)</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>Primer R (10 μM)</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>TaqMan Probe (10 μM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Taq Polymerase (5U)</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Reverse Transcriptase (20U)</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>RNase inhibitor (20U)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Rnase free water to make 25 μl</td>
<td></td>
</tr>
</tbody>
</table>

Set up the experiment and the following PCR program on Real-time PCR.

50°C 2 min, 1 cycle
95°C 10 min, 1 cycle
55°C 30 s, 40 cycles
72°C 10 min, 1 cycle

3. After PCR is finished, check the Ct values remove the tubes from the machine. The PCR specificity is examined by 2% agarose gel using 5 µl from each reaction.

APPENDIX II

Common Reagents, Buffers and Media Used

Antibiotics

Ampicillin

Stock solution (50 mg/ml) of the antibiotic was made in double distilled water, filter sterilized (through 0.22 micron filter) and distributed into 200 µl aliquots and stored at -20°C. It was used at a concentration of 50 µg/ml.

Plasmid Isolation Buffers

Solution I (Resuspension buffers)
- Tris HCl pH (8.0) 25 mM
- Glucose 50 mM
- EDTA 10 mM

Solution II (Lysis buffer)
- NaOH 0.2 N
- SDS 1%

Solution III (Neutralization buffer) pH 4.8
- Sodium acetate 3 M

Agarose gel Electrophoresis Reagents
DNA Molecular Weight Marker

One kilobase (1 kb) DNA ladder of MBI Fermentas was used as marker. The ladder is formed by fourteen DNA fragments of 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3.5 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 0.75 kb, 0.5 kb and 0.25 kb. 100bp DNA ladder of Pro mega consists of 11 fragments of 100 bp to 1 kb and 1.5 kb.

Buffer

Ligation buffer 10X

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl (pH 7.8)</td>
<td>0.5 M</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.5 M</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>500 µg ml⁻¹</td>
</tr>
</tbody>
</table>
40% Acrylamide  Dissolve 380 g Acrylamide and 20 g bisacrylamide in 800 ml 
(19:1)  distilled water the volume was made upto 1 L with distilled water. Heat the solution to 37°C to dissolve the chemicals, Filter the solution with a nitrocellulose filter and store in dark bottle.

MOPS (1M)  20.9 g MOPS dissolve in 80 ml distilled water adjust pH 7.5 with 1 N NaOH, and bring volume to 100 ml with distilled water.

Wash solution  6× SSC

0.2% SDS

0.1% DEPC  1 ml of DEPC mixed with 1 L MQ water and stirred it with treated water spatula for 5-10 ml.

Linking solution  Prepare a solution of 1-methylimidazole by diluting 245 μl of 12.5 M stock in 9 ml RNAse-free water. Adjust the pH to 8.0 with 1M HCl (usually about 300 μl) Add 0.753 g of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide). Make up volume to 24ml to make a working solution of 0.16M EDC in 127mM 1-methylimidazole (pH 8).

**Commonly used stock solution**

**0.1 M Adenosine triphosphate (ATP)**  60.0 mg of ATP was dissolved in 0.8 ml of distilled water. The pH was adjusted to 7.0 with 0.1 N NaOH and volume made up to 1 ml with distilled water. The solution was dispensed into small aliquots and stored at -70° C.

**1 M CaCl₂**  54.0 g of CaCl₂.2H₂O was dissolved in 200 ml of pure water. The solution was sterilized by passing through a 0.22 micron filter and stored in 1 ml aliquots at 4° C.

**0.5 M EDTA (pH 8.0)**  186.1 g of ethylenediamine tetra acetic acid disodium salt 2H₂O was added to 800 ml of distilled water, stirred vigorously on a magnetic stirrer, pH was adjusted to 8.0
with NaOH (20.0 g of NaOH pellets). Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.

**Ethidium bromide (10 mg ml⁻¹)**

1.0 g of ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The solution was transferred to a dark bottle and stored at room temperature.

**Phenol : chloroform : isoamyl alcohol**

Buffer saturated phenol, chloroform and isoamyl alcohol were mixed in the ratio of 25:24:1. The equilibrated mixture was stored under a layer of 0.01 M Tris-HCl (pH 7.6) at 4°C in dark glass bottle.

**IPTG (Isopropyl-β-D-thiogalacto-pyranoside)**

A solution of IPTG was made by dissolving 2.0 g of IPTG in 8 ml of distilled water. Volume was made upto 10 ml with distilled water and sterilized by filtration through a 0.22 µ disposable filter. The solution was dispensed into 1 ml of aliquots and stored at -20°C.

**1M MgCl₂**

203.3 g of MgCl₂.6H₂O was dissolved in 800 ml of distilled water. The volume was made upto 1L, dispensed into aliquots and sterilized by autoclaving.

**3M Sodium acetate (pH 4.8)**

408.1 g of NaOAc.3H₂O was dissolved in 800 ml of distilled water. The pH was adjusted to 4.8 with glacial acetic acid. Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.

**5M NaCl**

233.8 g of NaCl was dissolved in 800 ml of distilled water, volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
10 N NaOH

Dissolve 400 g of NaOH in 800 ml of distilled water and make up the volume to 1 L with distilled water.

1M Tris-HCl

121.1 g of Tris base was dissolved in 800 ml of distilled water. pH was adjusted to the desired value by adding concentrated HCl (for pH 7.4, HCl 70 ml; for pH 8.0, HCl 42 ml). The solution was allowed to cool down to room temperature before making final adjustment to the pH. The volume was made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.

X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside)

The stock solution was made by dissolving X-gal in dimethyl formamide to make a 20 mg/ml solution and stored at -20°C.

11. Materials, Source and Catalogue Number of Chemicals and Reagents

<table>
<thead>
<tr>
<th>Materials</th>
<th>Source</th>
<th>Catalogue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td></td>
<td></td>
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<td>SM 0311</td>
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**Other enzyme**

dNTP mix
Mu-MLV reverse transcriptase
Poly(A) polymerase
RNase H
Taq polymerase
T4 DNA ligase
Phusion Taq polymerase
Lightcycler Probe master

**Other reagents**

Acrylamide
Ammonium persulfate
Bis-Acrylamide
Dimethylaminopropyl-ethyl-carbodiimide
D-galactopyranoside (X-gal)
Isopropyl-thiogalacto-pyranoside (IPTG)
Formamide
Methylimidazole
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