Chapter 1: Introduction
1.1 General Introduction

The world population is increasing at a rapid rate with about 90% of the population residing in developing countries. Adequate amount of food is required for feeding these growing number of populations (Rajyashri and Mohan 2004). However since the early 1990s, there has not only been a reduction in the rate of growth of food production, but the area under cultivation has also reduced, therefore it became indispensable to find new ways of increasing crop yield (Khush 1997). While a lot of work has already been initiated to address this problem the best way to overcome this problem lies in reducing the loss of crop due to various stress factors. Crop yield is greatly affected by various biotic and abiotic factors, resulting in a huge gap between the yield potential and the actual yield, especially in the developing countries with limited resources to combat these problems. Strategies were aimed to develop crop cultivars with greater tolerance to biotic stress such as resistance to pests and pathogens. Development of such varieties is one of the viable means of improving the crop yields in these regions.

Plant diseases are the major sources of biotic stress of crop and damages caused by viruses, bacteria, fungi and insect pests are responsible for enormous economic loss worldwide. The development of disease resistant cultivars is a serious challenge to plant biotechnologist in present time. Resistance is genetically based minimization of the susceptibility of a population over a period of time. It may be in the form of decrease in plants susceptibility to a pathogen or increase in pathogen susceptibility to insecticide. Disease and pest problems often prompted farmers to use excessive applications of agrochemicals, which ultimately brings threat to human health and environment. One of the alternative approaches is development of disease resistant cultivars, however, identification of resistant germplasm always is not possible, and moreover, introgression of the resistance traits to cultivars is laborious and time consuming. This has necessitated the use of advanced molecular biology tools for the fast development of disease resistant crop. For this purpose one need to identify genes responsible for disease resistance. As a prerequisite for such
identification, understanding the disease biology as well as genes responsible for disease development is utmost important.

1.2. Tomato

The tomato (*Solanum lycopersicon*) is a herbaceous fruiting plant. It originated in Latin America and has become one of the most widely grown vegetables with ability to survive in diverse environmental conditions (Rice et al., 1987). The plants typically grow to 1–3 meters (3–10 ft) in height and have a weak stem that often spreads over the ground and climbs over other plants. It is often grown outdoors in temperate climates as an annual. An average common tomato weighs approximately 100 grams. The tomato plant have spread throughout the world following the Spanish
colonization of the Americas. Tomato fruit contains very high amount of vitamins A and C. Tomato was regarded as a top most vegetable by scientists of Technical Advisory Committee of the Consultative Group on International Agricultural Research (CGIAR) (FAO, 1990). Recently, more emphasis is given on tomato production not only as source of vitamins, but also as a source of income and food security. Tomato grows best in fertile, well-drained soils, with pH 6 and ambient temperatures of about 25 °C (Villareal, 1979; Rice et al., 1987).

1.3. Tomato leaf curl disease

In tomato, disease symptoms include upward or inward rolling of margins, interveinal yellowing, vein clearing, enations, crinkling and puckering of the leaves. Infected leaves usually develop thickening of veins and shortening of interveinal distance. Plants become stunted and bushy at late stage of infection and bear few or no fruit.

Symptom development by a Geminivirus depends on the plant type infected. For example, ToLCV (Tomato leaf curl virus) can infect both tomato and tobacco; however, in tobacco it produces milder symptoms (Thierry et al. 2012). Thus, host factors are important determinants of the degree of
symptom severity. Several host factors have been implicated as the interacting partner for virus proteins and having role in symptom severity. AL2 protein, silencing suppressor, from Tomato golden mosaic virus (TGMV) and Cabbage leaf curl virus (CaLCuV) interacts with TIFY4B, a cell cycle regulator of different dicotyledonous plants. AtTIFY4B overexpression delays symptom development probably due to the inhibition of cell cycle, which also affect viral replication. The virus may counteract this host defense response via interaction with and AL2-mediated capturing of TIFY4B protein (Chung and Sunter 2014). Another host factor, SISnRK1 (Solanum lycopersicum Sucrose-Nonfermenting 1-related kinase) could inhibit β-satellite mediated symptom manifestation and virus replication by specific phosphorylation of the βC1 protein (Shen et al. 2011). The nuclear receptor karyopherin α1 mediates viral CP nuclear localization and prevention of its interaction with CP resulted in symptom-less mutant of TYLCV (tomato yellow leaf curl virus) (Yaakov et al. 2011). Interaction of C4 gene of ToLCV-Australia virus and V2 protein of TYLCV (Tomato yellow leaf curl virus) with tomato shaggy-like Kinase (SISK) and SISGS3, respectively was required for the silencing suppressor activity of the virus and symptom appearance (Dogra et al. 2009; Glick et al. 2008). A detailed analysis using Arabidopsis plant depicted that the βC1 protein could interact with AS1 (Asymmetric Leaf 1) to alter leaf development (Yang et al. 2008). In addition, host transmembrane transporter (Permease I-like protein), ubiquitin-(Eini et al. 2009) of viral proteins and manipulation of these proteins’ expression affected symptom development (Eini et al. 2009; Eybishtz et al. 2009).
1.4. Tomato leaf curl virus

Geminiviridae is a family of plant viruses. There are 325 species at present in this family, subdivided within 7 genera. The major Diseases associated with this family include: yellow mottle, bright yellow mosaic, yellow mosaic, streaks, leaf curling, stunting, reduced yields. They have single-stranded circular DNA genomes encoding all the necessary genes. According to the Baltimore classification they are regarded class II viruses. It is the largest known family of single stranded DNA viruses. Mastrevirus transmission is via various leafhopper species (e.g. maize streak virus and other African streak viruses are transmitted by Cicadulinambila), curtoviruses are the only known topocuvirus species, Tomato pseudo-curl virus are transmitted by treehopper species (e.g. Tomato pseudo-curl virus is transmitted by the treehopper Micrualismalleifera), and begomoviruses are transmitted by the whitefly species, Bemisiatabaci (De Barro et al. 2011; Gotz et al. 2012).

Tomato leaf curl New Delhi virus (ToLCNDV), is a member of begomovirus group. This group of viruses may have monopartite (DNA A) or bipartite (DNA A and DNA B) circular ssDNA genomes of
~2,700 nucleotides each which carries six partially overlapping genes that are bidirectionally organized into two transcriptional units separated by an intergenic region (IR) of 300 nucleotides. The DNA A component encodes six Open Reading Frames (ORFs) namely, AC1(Rep), AC2 (TrAP), AC3(REn), AC4, AV1 (CP), and AV2. BC1 (MP) and BV1 (NSP) (see below for details) proteins are expressed from DNA B. Each genome is encapsidated in an 25- by 30-nm geminate particle (Fig 3). Most of the monopartite geminiviruses need a β-satellite for their infectivity. Satellite DNAs are not commonly associated with bipartite geminiviruses (Nawaz-ul-Rehman et al. 2009).

1.5. Tomato leaf curl virus replication and transmission

Geminiviruses have a small genome and encode only a few proteins. So, their DNA replication cycle depends largely on the use of cellular DNA replication proteins. The strategy used by geminiviruses to replicate their single-stranded DNA (ssDNA) genome involves a first stage of conversion of ssDNA into double-stranded DNA (Fig 5).
(dsDNA) intermediates and, then, the use of dsDNA as a template to amplify viral dsDNA and to produce mature ssDNA genomes by a rolling-circle replication mechanism.

All geminiviruses having an intergenic region where the cis-acting signals are recruited for initiation of rolling-circle DNA replication. Within it, the invariant 9-nt sequence (TAATATT↓AC) contains the site (↓) where the initiation of (+) strand DNA replication was mapped in vivo (Heyraud et al. 1993a; Heyraud et al. 1993b; Stenger et al. 1991) and in vitro (Heyraud-Nitschke et al. 1995). The invariant loop sequence also plays a vital role for viral DNA replication (Revington et al. 1989). The structure of the stem is also important as point mutations within this region will destroy base pairing which will be fatal for viral replication.

The dsDNA is transcribed by host RNA polymerase II leading to production of replication initiator protein (Rep). Rep protein initiates rolling-circle replication by inserting a nick into a viral dsDNA molecule to produce a free 3′-hydroxyl end that primes ssDNA. Viral replication transitions to recombination-dependent replication, which is initiated by homologous recombination between a partially replicated ssDNA and a closed, circular dsDNA to form a looped molecule that serves as a template for both ssDNA and dsDNA synthesis (Fig.5, inset). In the later stage of infection Rep protein represses its own transcription, leading to upregulation of transcriptional activator protein (TrAP) expression, which in turn activates coat protein (CP) and nuclear shuttle protein (NSP) expression. Circular ssDNA can then be encapsidated by CP into virions, which are consumed by whitefly. NSP binds to viral DNA and moves it across the nuclear envelope, where movement protein (MP) transports it across a plasmodesmata.

Begomoviruses such as ToLCNDV are acquired by the whitefly while feeding from infected plant phloem as intact virions which pass along the food canal in the insect stylet with other phloem components until they reach the oesophagus (Ghanim et al. 2001a). The first tissue from which virions migrate to the hemocoel is a modified form of the digestive system called the filter chamber (Ghanim et al. 2001b). The fluorescence in situ hybridization (FISH) and transmission electron
microscopy experiments confirmed that most ToLCNDV virions have been observed in the filter chamber, suggesting that it is the major site for viral particle translocation to the hemocoel.

1.6. Initiation of plant leaf formation and leaf pattern formation

Leaf development is a flexible process and is determined by several factors like species, environment and developmental process. The major stages of leaf development are morphogenesis and development. The leaf initiation starts from a group of cells with in the shoot apical meristem called founder cells. In the normal condition the cells in the shoot apical meristem region remains in the indeterminate stage, which is maintained by KNOX family of genes (Moon and Hake 2011). The KNOX group of genes maintains this phenomenon by upregulating cytokinin biosynthesis genes in soot apical meristem. When the KNOX genes were downregulated by the polar auxin hormone, MYB, LOB domain bearing transcription factors, the situation gets reversed. The level of gibberellin (GA) increases significantly than cytokinin resulting in cell elongation. The cell elongation within the group of cells marks the initiation of leaves. When the leaf grows away from the meristem, its shape is determined by growth in three axes, proximal–distal, abaxial–adaxial and medial–lateral. The cells were polarised accordingly depending upon the axis. This distribution of the cells is also critical for the physiology of the plant. For example the adaxial surface cells were responsible for capturing of sunlight however the ventral surface cells were responsible for gaseous exchange to maximise the photosynthesis rate. Similarly the distal part of leaf of pea plant having tendrils providing support to the pea plant. Polarity mutant analysis led to the discovery of complex gene network behind the leaf pattern formation(Moon and Hake 2011). Phavoluta (phv), revoluta (rev) and phabulosa (phb) and HD-ZIPIII are expressed throughout the leaf whereas KAN overexpression causes abaxialization. Another key player in dorsal and ventral pattern determination is miR165/166 which negatively regulates HD-ZIPIII to prevent expression on the abaxial side of the leaf(Mallory et al. 2004; Rhoades et al. 2002). The KAN family genes (ARF3/4) transcription factors determines the abaxial fate and
the tasiRNAs prevent these genes from being expressed adaxially (Chitwood et al. 2009; Fahlgren et al. 2006).

### 1.7. Role of viral proteins and host proteins for generation of developmental defects

Geminiviruses codes for a few genes that are performing the major role in virus replication and movement. For the initiation of replication process the virus has to rely on host polymerases, so the virus somehow hijacks the host transcriptional machinery and directs it to produce host proteins such as those needed for DNA synthesis (Hanley-Bowdoin et al. 2004). Proliferating cell nuclear antigen (PCNA) is a DNA clamp which is a processivity factor for DNA polymerase δ in eukaryotic cells and is essential for replication. However the enrichment of PCNA in the virus infected cells than the uninfected cells confirms the above phenomenon (Egelkrout et al. 2001; Nagar et al. 1995). The transcriptome profiling in virus infected plants indicates preferential activation of cell cycle associated genes expressed during S/G2 phase and also inhabits genes that are active during M/G2 phase (Ascencio-Ibanez et al. 2008). The reason of such behaviour of virus is to promote the upregulation of the genes in S/G2 phase as in this phase the virus can utilize the maximum resource from the host cell for its replication (Ascencio-Ibanez et al. 2008; Lageix et al. 2007).

There are evidences of phenotypic effect in plants when the individual virus proteins were expressed both transiently or by stable transformation. The C1 gene or the Rep protein has been proved to have role in hypersensitive response in *Nicotiana clevelandii*. The expression of C2 gene produces necrotic lesions and huge necrotic venial necrosis on systemically infected leaves in *N. benthamiana*. The over expression of C4 gene also generated virus like symptoms. The expression of V1 and C3 gene resulted severe stunting of *N. benthamiana* plants.
Geminiviral Rep protein is able to interact with the tumor suppressor protein retinoblastoma (Rb) (Ach et al. 1997; Liu et al. 1999; Xie et al. 1995). The C2 gene has been proven to have role to act as a PTGS suppressor. The C3 gene may disrupt the plant Rb control pathway (Settlage et al. 2001) when expressed in an uncontrolled manner in N. benthamiana leading to symptom development.

AL2 protein, silencing suppressor, from Tomato golden mosaic virus (TGMV) and Cabbage leaf curl virus (CaLCuV) interacts with TIFY4B, a cell cycle regulator of different dicotyledonous plants. AtTIFY4B overexpression delays symptom development probably due to the inhibition of cell cycle, which also affect viral replication. The virus may counteract this host defense response via interaction with and AL2-mediated capturing of TIFY4B protein (Chung and Sunter 2014). Another host factor, SlSnRK1 (Solanum lycopersicum Sucrose-Nonfermenting 1-related kinase) could inhibit β-satellite mediated symptom manifestation and virus replication by specific phosphorylation of the βC1 protein (Shen et al. 2011). The nuclear receptor karyopherin α1 mediates viral CP nuclear localization and prevention of its interaction with CP resulted in symptom-less mutant of TYLCV (tomato yellow leaf curl virus) (Yaakov et al. 2011). Interaction of C4 gene of ToLCV-Australia virus and V2 protein of TYLCV (Tomato yellow leaf curl virus) with tomato shaggy-like Kinase (SISK) and SISGS3, respectively was required for the silencing suppressor activity of the virus and symptom appearance (Dogra et al. 2009; Glick et al. 2008). A detailed analysis using Arabidopsis plant depicted that the βC1 protein could interact with AS1 (Asymmetric Leaf 1) to alter leaf development (Yang et al. 2008).

1.8. Developmental genes determining leaf architecture

During vegetative growth of plants leaves are produced in a well organised manner which involves patterning, cell division, expansion, and differentiation. A flattened surface for efficient capture of sunlight. Mechanical support to the lamina (Evert and Eichhorn, 2006). The basic model for explaining vascular patterning is the auxin canalization model, evidences also support the
phenomenon of vascular differentiation by auxin in stem (Donner et al. 2010; Sachs 1991). Auxin is known to be transported in a basipetal manner throughout the plant body from the source of synthesis (Estelle 1998). The shoot apex and young leaf primordia are auxin sinks in which auxin is acropetally transported (Avsian-Kretchmer et al. 2002). Leaves where the polar auxin is inhibited chemically or genetically (silencing PIN1) develop increased vascularization adjacent to the leaf margin (Mattsson et al. 1999; Okada et al. 1991; Steinmann et al. 1999).

Several genes are known for their role in plant development. The EXO (EXORDIUM) gene is known for its function in cell expansion, upon mutation of EXO gene plant showed diminished leaf and root growth and reduced biomass production (Schroder et al. 2009). The overexpression of EXO gene promotes shoot and root growth. In case of compound leaf formation in plants like tomato LYRATE gene helps in leaflet initiation (David-Schwartz et al. 2009).

There are very few vascular development mutants are available as the mutation in the major regions in central regulatory components of vascular development are likely to be lethal. Most of the genetic mutations studied till date includes axr6 (Hobie et al. 2000), bodenlos (Hamann et al. 1999), vascular network defective 1 to 6 (Koizumi et al. 2005; Koizumi et al. 2000), scarface (Deyholos et al. 2000; Sieburth and Deyholos 2006), lop1/tornado1 (Carland and McHale 1996), cotyledon vein pattern 1 and 2 (Carland et al. 2002; Carland and Nelson 2004), ifl1 (Zhong and Ye 1999) and fackel (Jang et al. 2000) show either reduced vascular formation or discontinuous vascular patterning.

Virus movement within the plant takes place between the two cells via plasmodesmata and in distant location by the vascular systems specially phloem. It has been reported (Jin et al. 2006) that downregulation of RPN9 results in increase in phloem and decrease in xylem elements that ultimately leads to blockage in virus movement through phloem. However the systemic movement of virus through phloem is reduced.
1.9. Target gene: TRN1 (TORNADO1) has significant influence in vascular patterning

The gene was identified in *Arabidopsis thaliana* by (Cnops et al. 2006). TRN1 gene codes a protein of 1380 amino acids. The N terminal region containing a putative LRR ribonuclease inhibitor (LRR-RI) subfamily domain. This domain is very close to animal cytosolic nucleotide binding oligomerization domain(NOD-LRR) (Inohara et al. 2005). The LRR domain is followed by an ATP/GTP binding motif.

The EST and genomic sequences of TRN1 gene in monocot, dicot and pine shows it is conserved and already prevailed before the evolutionary angiosperm–gymnosperm split. TRN1 gene is located on the lower arm of chromosome 5 of *Arabidopsis thaliana* (Cnops et al. 2000) and trn1 is allelic to lopped1 (Carland and McHale 1996; Cnops et al. 2000).

Trn1 (TORNADO1) gene is known to have role in leaf patterning processes such as lamina venation, symmetry determination, and lateral growth. In the trn mutant plants, the leaf venation network complexity decreased drastically: no tertiary veins, incomplete loops and vascular islands were formed. The leaf of trn1 mutant plants laminas were asymmetric and narrow due to severely reduced cell number. This was hypothesised by (Cnops et al. 2006) that the imbalance between cell proliferation and cell differentiation is the major reason for this type of abnormality.
1.10. Stress signalling

Plants are sedentary organisms unable to move from one place to another. Therefore they can’t escape from unfavourable conditions like cold, heat, drought, salinity or pathogen attack. These stresses hamper the plant growth and development leading to decrease in crop productivity. To resist against the environmental stress and biotic stress plants have also evolved defence mechanism by increasing the expression of stress-responsive genes (Niu et al. 2011). The products of these genes can be classified into two groups according to their functions during stress, (Hirayama and Shinozaki 2010). One group contains protein kinases and transcription factors involved in stress-inducible gene expression and further regulation of signal transduction. The other group includes proteins such as osmotin, chaperones, late embryogenesis-abundant (LEA) proteins functioning in direct abiotic stress tolerance.

When plants were exposed to virulent, a virulent and non-pathogenic microbes two types of defence can be triggered first one is systemic acquired resistance(SAR) and the second one is induced systemic resistance(ISR) (Conrath et al. 2002). Systemic acquired resistance (SAR) act through different signalling pathway. SAR is induced by local or systemic increase in SA level within
plants. The upregulation of SA level within plants leads to increase in expression level of pathogenesis related genes like PR1, PR2 and PR5 (Niu et al. 2011; Ward et al. 1991).

ISR is found to be effective in many plant species like bean (*Phaseolus vulgaris*), cucumber (*Cucumis sativus*), tomato (*Solanum lycopersicum*), and the model plant Arabidopsis (*Arabidopsis thaliana*), and is effective against many plant pathogens, including fungi, bacteria, viruses, and even insect herbivores (Van der Ent et al. 2008; van Loon et al. 1998). ISR mediated response is dependent on the JA/ET signalling pathway and NPR1 in Arabidopsis.

There are up to 1500 transcription factors may be involved in plant defence (Li et al. 2004; Riechmann et al. 2000). Among transcription factors, the plant-specific WRKY family transcription factor which are playing the major role in plant defence. During biotic and some types of abiotic stress the WRKY group of transcription factor is upregulated. These group of transcription factors have a very conserved DNA binding domain “WRKYGQK”, which binds at the particular sequence W-Box element “TTGACY” in the upstream region of many defence responsive genes resulting in upregulation of the plant defence genes (Du and Chen 2000; Eulgem et al. 2000).

Geminiviruses typically encode 5 to 7 proteins that interact with many host proteins to reprogram plant cell cycle and transcriptional controls, interfere with cell signalling and protein turnover, and suppress defence pathways. Geminiviruses also display high mutation and recombination rates that allow them to adapt rapidly to new environments and new hosts (Duffy and Holmes 2008; Martin et al. 2011). Virus AL2/C2 proteins are transcriptional activators that are required for expression of coat protein (CP) and nuclear shuttle protein (NSP) genes, which are transcribed late in the infection cycle (Sunter and Bisaro 1992). As a consequence, these AL2/C2 proteins are also called TrAPs, for transcription activation proteins. An Arabidopsis thaliana TIFY family transcription factor, PEAPOD2, has been shown to interact with the AL2 proteins and the CP promoters of Tomato golden mosaic virus (TGMV) and Cabbage leaf curl virus (CaLCuV) (13). Another candidate AL2/C2 partner is the JDK
transcription factor (Lozano-Duran et al. 2011). CP and NSP are required for viral transport and movement, so the expression of AL2/C2 plays the key role in symptom development.
1.11. Objectives

- Identification and expression study of Arabidopsis homologues of tomato leaf proliferation and expansion genes.
- Analysis of variation in steady state mRNA levels of these genes in different stages of virus infection in tomato.
- Analysis of regulatory domains present at the 5'-flanking region of the gene that are significantly regulated during virus infection.
- Determination of which gene of the virus is important for this regulation and significance of this regulation in leaf curling.
1.12. Reference


