Mechanism of natural resistance in chilli against *Chilli Leaf Curl Virus*

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CERTIFICATE

This is to certify that the work embodied in the thesis **"Mechanism of natural resistance in chilli against *chilli leaf curl virus*"** has been carried out at the School of Life Sciences, Jawaharlal Nehru University, New Delhi, 110067. This work is original and has not been submitted so far, in part or full for any other award of degree or diploma of any university.

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Plant viruses have evolved with versatile capabilities of exploiting and modulating host machinery for establishment of successful infection. Like other smart parasites, they modulate cellular processes of host for their replication, transcription, translation and movement (Nelson and Citovsky 2005; Patarroyo *et al.,* 2012). Plants have also developed different kinds of efficient and sophisticated defense machineries which are activated following virus infection (Bendahmane *et al.,* 1999; Van-den-Bosch *et al.,* 2006; Maule *et al.,* 2007). In addition, plants can recognize invading pathogens and can defend against viruses (Aurora-Fraile and García-Arenal, 2010). Therefore, different level of interactions between virus and host determine fate of pathogenesis (Culver and Padmanabhan, 2007; Pallac and Garcia, 2011). If a plant recognizes the viral protein, an incompatible interaction that is unfavorable for the virus might get established (Flor, 1971; Jones and Dangle, 2001). It is also known that plants can recognize the virus, limiting it to the site of infection. A series of complex cascade defense reactions can be induced within the host to limit virus replication and movement (Soosaar *et al.,* 2005; Ishibashi *et al.,* 2007) which ultimately makes a plant resistant (Kang *et al.,* 2005). In some cases, viruses fail to utilize the host proteins required for their replication and movement either due to some mutational changes or absence of the protein which is prerequisite for the successful completion of life cycle (Johansen *et al.,* 2001; Parrella *et al.,* 2002). Transcriptional and post transcriptional gene silencing, methylation and ubiquitination are some of the defense strategies which plant employ to prevent virus multiplication and movement (Alvarez *et al.,* 2010; Latzel *et al.,* 2012). If the viral protein is not recognized by the corresponding protein of host plant, a compatible interaction between the plant and the virus is established. This interaction may be favorable for the virus and plant is considered to be a susceptible host (Flor, 1971).

Chilli *(Capsicum annuum* L.), originated from the south and central America is a member of the family *Solanaceae.* Approximately, 25 wild and 5 domesticated species of chillies are known to exist. Full-grown plants are usually small, bushy and semi perennials in nature. Chilli is one of the important crops used for many purposes and is rich source of

several vitamins (A, C, E) and antioxidant. It is used as intact fruit, grinded powder, value added processed products, capsaicin extracts, oleoresin extracts, and processed pickles etc. Chilli production is affected by a plethora of pathogens including viruses (Maury *et al*., 2005). *Chilli leaf curl virus* (ChiLCV) is one of the devastating pathogens causing severe losses in the production of chilli worldwide. Leaf curl is a complex disease which is characterized by leaf curling, crinkling and puckering, swelling of the veins, stunted growth with reduced number of fruits or no fruits (Park and Fernando 1938; Mishra *et al.,* 1963; Muniyappa and Veeresh, 1984; Hussain *et al.,* 1932). With potential of recombination and fast evolving nature of begomoviruses (Padidam *et al.,* 1999; Martin *et al.,* 2011), ChiLCV has emerged as a severe threat to chilli production. Further, the loss is aggravated by lack of an efficient antiviral strategy. Therefore, it becomes imperative to identify resistance factor(s) and to develop antiviral strategy against ChiLCV.

Despite of losses incurred in chilli production due to leaf curl disease, report of resistance in chilli varieties against ChiLCV is also known. Field screening of more than 300 genotypes indicated that varieties like BS35, GKC29, EC-497636, Punjab Lal and Kalyanpur Chanchal genotypes had various degrees of resistance against ChiLCV (Kumar *et al.,* 2006; Balvir and Daljit, 2009). Some of these varieties are known to be resistant against ChiLCV for more than two decades. These resistant genotypes can serve as potential sources of resistance factor(s) against ChiLCV. Hence, it is of immense importance to study cellular factors conferring natural resistance and to elucidate the molecular mechanism in resistant chilli plants.

ChiLCV is a member of the genus *Begomovirus* within family *Geminiviridae* and possesses a circular ssDNA of around 2.7 kb (Chattopadhyay *et al.,* 2008; Shafiq *et al.,* 2010; Tahir *et al.,* 2011). It is often found to be associated with 1.3 kb satellite DNA β (Chattopadhyay *et al*., 2008, Tahir *et al.,* 2011). ChiLCV genome like other monopartite begomoviruses, encodes six proteins viz., two from viral sense strand and four from complementary of sense strand (Lazarowitz, 1992; Hanley-Bowdoin *et al*., 2000;

Gutierrez, 2000a; Gutierrez, 2000b; Jeske, 2009). These proteins are involved in initiation of replication, activation of transcription, enhancement of replication, encapsidation of genome, nucleocytoplasmic trafficking and cell to cell or long distance movement of virus (Lazarowitz, 1992; Hanley-Bowdoin, 2000). Satellite DNA β encodes a single protein βC1 which is known to be required for pathogenesis, movement and suppressor of gene silencing (Cui *et al.,* 2004; Saunders *et al.,* 2004; Saeed *et al.,* 2005). Chilli varieties may elicit differential response following ChiLCV infection. In a susceptible host / variety, ChiLCV can easily modify and utilize the cellular machinery. Although interaction is complex but a susceptible host always facilitates virus multiplication and movement. In contrary, resistant plants have developed elaborate and effective defense mechanisms to prevent or limit the viral infection.

Several plant-virus interactions leading to resistance have been studied in depth and found to be extremely complex. As a consequence, the mechanisms linked to the inhibition of viral accumulation inside host cells, prevention of movement of virus within the plant as well as activation of plant defense mechanisms, have been partially elucidated. But, identification of resistance factors which are involved in mechanism of resistance against begomoviruses has remained a long lasting challenge. In some cases transcriptional and post transcriptional gene silencing have been found to be associated with recovery of begomovirus infected plants (Raja *et al.,* 2008; Rodríguez-Negrete, 2009; Paprotka, 2011). Some recent research also suggest involvement of methylation (Raja *et al.,* 2008; Yadav and Chattopadhyay, 2011; Paprotka, 2011) and post translational modification of histone in regulation of viral gene expression (Raja *et al.,* 2008). Because of magnitude of losses caused by ChiLCV in chillies and lack of management options, it becomes necessary to identify the mechanism of durable natural resistance in chilli var. Punjab Lal.

Keeping in view the information available in understanding antiviral resistance mechanism, the present work was carried out with the following objectives:-

1. To develop highly efficient infectious clone of ChiLCV.

2. To screen chilli varieties for resistance using highly efficient infectious clone.

3. To identify the resistance factor(s) in chilli against ChiLCV.

4. To elucidate the mechanism of natural resistance in chilli.

2.1 Chilli (*Capsicum annuum*)

Chilli was originated in the South America and known to be domesticated over 5000 year ago. It belongs to the *Solanaceae* family and is mostly cultivated as perennial but in some temperate region as annual crop. Typically, a chilli plant resembles bushy appearance with many branches and can grow up to 75 cm. Several varieties of *Capsicum annuum* have been developed which are being cultivated around the world for production of sweet and hot chillies. More than 30 species of chillies are known to exist among which five species (*C. annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, and *C. baccatum*) are cultivated widely (Kumar *et al*., 2006; Sanatombi and Sharma, 2008).

The leaves appear as simple and alternate, elliptical or lanceolate, with smooth margins (entire). The flowers are small (around 1.5 – 2.5 cm in diameter) and borne singly or in pairs in the axils. Flowers are usually white (or occasionally purple), bell-shaped (campanulate), often with 5 lobes, and contain 5 stamens. The fruits are berries (pod-like with many-seeded), with no sutures but vary considerably in size and shape.

Chilli is used either as fresh vegetable, or as cooked, or dried to prepare an enormous variety of dishes characteristic of different regional cuisines (Fig. 2.1). It is rich in vitamins like A, C and E. However, it also has numerous medical uses, including skin irritations, topical pain relief for muscle soreness, and rheumatism, and also as an anti-inflammatory agent. Recent medical research reports have also demonstrated antimicrobial and antifungal activity of capsaicin obtained from several *Capsicum* varieties, and ongoing studies are exploring its use in cancer treatment.

Although, India is the major chilli producing country in the world (Fig. 2.2), the productivity is still below the world average. One of the major reasons for low productivity is losses caused due to pest and pathogens. Chilli serves as a host for several pathogens including fungi, bacteria and viruses. Among these pathogens, viruses have emerged as the most serious threat for successful production in India. In India, chilli leaf curl disease (ChiLCD) was reported approximately fifty years ago (Mishra *et al.,* 1963;

Dhanraj and Seth, 1963). Infected plants exhibit several abnormal morphological and physiological modifications which are characteristic symptoms of ChiLCD (Fig.2.3 B)(Puttarudraiah, 1959). At early stage of infection, plants show only noticeable mild leaf curling which in later stage of severe infection exhibit enhanced leaf crinkling, puckering or leaf curl complex (Husain , 1932) (Fig. 2.5). In majority of the cases, deformities of leaves are also associated with the vein clearing (Fernando and Peiris, 1967). Several other abnormal changes like curling of leaf margin, reduction in leaf size, vein clearing have been reported in India, Sri Lanka and USA (Puttarudraiah, 1959). In some other cases, from different region of India, abaxial curling of the leaves accompanied by puckering, thickening and swelling of the veins were observed (Mishra *et al.,* 1963; Muniyappa and Veeresh, 1984). Prominent symptoms such as vein clearing followed by veinal distortion, swelling of veins and veinlets on dorsal side were reported by several workers (Muniyappa, 1980; Ravi, 1991).

In the recent past, Senanayake *et al.* (2007) and Chattopadhyay *et al.* (2008) reported the association of begomoviruses with ChiLCD in India. ChiLCD is wide spread in India and is caused by more than 10 species of chilli-infecting begomoviruses and 6 species of betasatellites (Chakraborty *et al.,* personal communication). Among the chilli-infecting begomoviruses, *Chilli leaf curl Multan virus* has emerged as the predominant species and is also known to act as either major or minor parent during recombination among chilli-infecting begomoviruses (Chakraborty *et al.,* unpublished results).

2.2 *Chilli leaf curl Multan virus* (Genus: *Begomovirus*, Family: *Geminiviridae*)

Geminiviruses are characterized by geminate shaped virions containing single stranded circular DNA which replicate through double stranded DNA intermediate in the nucleus of infected cells (Fig. 2.3D)(Hanley-Bowdoin *et al.,* 2000; Jeske, 2009). Electron microscopy revealed size of virus particles ranging from 18-20 nm in diameter with length of 30 nm (Fig. 2.5) (Lazarowitz, 1992; Bottcher *et al.,* 2004; Shepherd *et al.,* 2010). The twinned geminate shaped capsids with two incomplete T=1 icosahedral

symmetry, is composed of 22 pentameric capsomeres made of 110 capsid proteins (Bottcher *et al.,* 2004).

Geminiviridae comprises four genera which have been categorized on the basis of genome organization, host range and vector transmission (Padidam *et al.,* 1995; Fauquet et al., 2003): *Mastrevirus, Curtovirus, Topocuvirus* and *Begomovirus.*

The members of the genus *Mastrevirus* are monopartite in nature and are transmitted by leafhopper (Howell, 1984) (Fig. 2.4). Most of the mastreviruses are known to infect monocotyledonous plant. The genus *Curtovirus* infect dicotyledonous plant and are transmitted by leafhopper (Fig. 2.4). Genome of this group of geminiviruses is monopartite having a single genomic component. The genus *Topocuvirus* contains only one member, *Tomato pseudo curly top virus* (Fig. 2.4). This virus is monopartite and is transmitted by treehopper. The genus *Begomovirus* constitutes the largest group of this family and may have either monopartite or bipartite genome (Fig. 2.4). They are transmitted by whiteflies and known to infect wide range of dicotyledonous plants (Fig 2.3C). Many monopartite begomoviruses like *Chilli leaf curl Multan virus* (ChiLCV) are also known to be associated with satellite DNA β (Briddon *et al.,* 2003; Chattopadhyay *et al.,* 2008).

2.3 Genome organization of *Begomovirus*

Geminiviruses have small genomes consisting of either one (monopartite) or two circular ssDNA molecules (bipartite) ranging from 2.5 to 3.0 kb in size (Stanley and Townsend 1985; Hanley-Bowdoin *et al.,* 2000). The coding capacity of the viral genomes varies among the different subgroups. Virus encoded proteins are specified by either the virion (V) or complementary (C) sense DNA strands. All geminiviruses contain 5′ intergenic conserved region which forms stem-loop structure that harbors nonanucleotide (TAATATTAC) sequence. This intergenic region is the key genomic structure for viral replication. Monopartite viruses contain one virus genome designed as DNA A whereas bipartite viruses contain two genomic components of similar size designated as DNA A

and DNA B. Satellite DNA molecules of monopartite viruses are smaller in size (approx 1300 bp) and known to encode only single functional protein. They are designated as DNA β or DNA α. Satellite DNAs are required for characteristic symptom development of the disease. Presence of a satellite DNA associated with *Tomato leaf curl virus* (ToLCV-sat) was reported for the first time from Australia (Dry *et al.,* 1997). Recently, satellite DNA 177H1 was found to be associated with *Malvastrum coromandelianum* (Fiallo-Olivé *et al.,* 2012) (Fig. 2.5).

Bipartite begomoviruses consist of two genomic components named as DNA A and DNA B. DNA A encodes for 6 proteins: two proteins i.e. coat protein (AV1) and pre coat protein (AV2) from virion strand and four proteins i.e. AC1 replication initiator protein (AC1), AC2 transcription activator protein (AC2), replication enhancer protein (AC3) and pathogenesis related protein (AC4) - from complementary of sense strand. DNA B encodes for two proteins, one each from virion strand (BV1, nuclear shuttle protein) and complementary sense strand (BC1, movement protein).

Monopartite begomoviruses contain single genome which is analogous to DNA A component of bipartite begomoviruses and encodes for six open reading frames (ORFs). In addition to other functions, AV2 of monopartite viruses also facilitate viral movement. Betasatellite (DNA β), half of the size of viral genome, are known to be associated with monopartite viruses. DNA β requires helper DNA for multiplication and is known to contain one ORF (βC1) encoded by complementary of sense strand (Fig. 2.6).

2.4 Function of *Begomovirus* ORFs

AV1 encodes for structural protein i.e. coat protein (CP) of approximately ~28 KDa which is involved in encapsidation of new viral genome (Stanley and Gay, 1983; Mullineaux *et al.,* 1984; Briddon *et al.,* 1990; Boulton *et al.,* 1993; Zhang *et al.,* 2001). Coat protein can interact with ssDNA or dsDNA (Foulds *et al*., 1993; Liu *et al.,* 1997; Pitaksutheepong *et al*., 2007). Coat protein gets localized in the nucleus and interacts with α importin which indicates that coat protein utilizes the conserved cytoplasmic

trafficking mechanism of the host during virus infection cycle (Liu *et al.,* 1999). CP of monopartite viruses plays crucial role during cell to cell and long distance movement of viral genome (Fig. 2.8) (Boulton *et al.,* 1989; Liu *et al.,* 1997; Pitaksutheepong, 1999, Kotlizky *et al*., 2000). Coat protein is also known to be determinant of vector specificity which is mapped in the central domain of protein (Noris *et al.,* 1998; Hohnle *et al.,* 2001; Liu *et al.,* 2001; Unseld*et al.,* 2001).

Mutation in AV2 (~19 kDa) of bipartite *begomovirus* ToLCNDV (Tomato leaf curl New Delhi virus) exhibited low viral DNA accumulation in infected plants (Padidam *et al.,* 1996, Rothenstein *et al*., 1999). But in the case of monopartite begomoviruses, this functions as movement protein and is involved in intracellular, intercellular and systemic movement of the virus (Fig. 2.8) (Stanley *et al.,* 1992; Hormuzdi and Bisaro, 1993). AV2 localizes in the cytoplasm and cell periphery of the infected cells (Chowda-Reddy *et al.,* 2008; Priyadarshani *et al.,* 2011). Recent studies have identified AV2 as suppressor of plant RNAi machinery (Chowda-Reddy *et al.,* 2008).

AC1 encodes for ~40 kDa multifunctional Rep protein which is required for viral genome replication (Fig. 2.8) (Hanley-Bowdoin *et al.,* 2000). Rep is also known to be involved in modulation of host gene expression (Elmer *et al.,* 1988; Etessami *et al.,* 1991; Saunders *et al.,* 1991). Rep initiates and terminates viral DNA synthesis and induces accumulation of host replication factors in infected cells (Fontes*et al.,* 1994). Rep can specifically bind to double-stranded DNA (dsDNA) at a repeated consensus sequence in the 5′ intergenic region of the viral genome. It cleaves and ligates DNA at conserved nonanucleotide TAATATT↓AC within a hairpin loop of the plus-strand origin (Fig. 2.7) (Lazarowitz *et al.,* 1992; Laufs *et al.,* 1995; Orozco *et al.,* 1997). The liberated 3' hydroxyl of nucleotide +7 (T) becomes available to prime plus-strand DNA synthesis, while the 5' end of the cleaved strand stays covalently attached to Rep protein via an ester bond, presumably to the conserved Tyr-103 (Ilyiana *et al.,* 1992). Recent information suggested role of AC1 in transcriptional gene silencing (Fig. 2.8) (Rodríguez-Negrete *et al.,* 2013). During replication of plus-strand replication, it acts as a DNA helicase to unwind viral

DNA. Apart from its role in viral genome replication, Rep interacts with several host factors involved in DNA replication, for example, retinoblastoma protein (RBR) (Xie *et al*., 1995), elongation factor 2 (E2F) (Arguello-Astorga *et al.,* 2004; Desvoyes, 2005), PCNA (Xie *et al.,* 1996; Ach *et al.,* 1997; Kong *et al.,* 2000), replication factor C (RFC) (Pavlov *et al.,* 2004; Luque *et al*., 2006), RAD51 (Suyal, et al; 2013), histone H3 and a mitotic kinesin (Kong *et al.,* 2000). It interacts with host regulatory factors, including RBR which modulates the plant cell cycle and differentiation (Kong *et al.,* 2000; Egelkrout *et al.,* 2001; Arguello-Astorga *et al.,* 2004; Chen *et al*., 2006), GRIK, a protein kinase involved in several cellular processes from cell cycle to metabolic processes (Kong *et al.,* 2000) and Ubc9, a component of the sumoylation pathway (Castilo *et al.,* 2004).

Begomoviruses express a small protein AC2 (also called as AL2 or TrAP), which transactivates transcription of late viral genes (Fig. 2.8) (Haley *et al.,* 1992; Sunter and Bisaro, 1992; Shivaprasad *et al.,* 2005). Consistent with its function as a transcriptional activator, three conserved domains have been recognized in this protein: a basic domain with a nuclear localization signal (NLS) at the N terminus, a central DNA-binding domain with a non classical Zn-finger motif, and an acidic activator domain at C-terminal end (Hartitz *et al.,* 1999). AC2 from bipartite and monopartite begomoviruses serves as factor for virus pathogenicity and suppression of gene silencing (Voinnet *et al.,*1999; Trinks *et al.,* 2005; Vanitharani *et al.,* 2005; Chowda-Reddy *et al.,* 2008).

The C3 protein (REn), absent in *Mastrevirus,* acts as a replication enhancer during geminivirus DNA accumulation. AC3 expression is driven by strong monodirectional left promoter (Shivaprasad *et al.,* 2005). The role of REn in viral DNA replication requires its interactions with Rep and PCNA (Castillo *et al.,* 2003; Settlage *et al.,* 2005). REn of *Tomato leaf curl Kerala virus* was shown to interact with Rep and enhance the Rep mediated ATPase activity (Pasumarthy *et al.,* 2011). REn interacts with RBR and enhances the virus replication (Settlage *et al.,* 2001).

AC4 ORF is entirely present within AC1 ORF but AC4 expression is driven by the same promoter. Several line of evidences suggested role of AC4 in viral pathogenesis and symptom development. Disruption of the C4 ORF of ToLCV , *Tomato yellow leaf curl virus* (TYLCV) and *Beet severe curly top virus* (BSCTV) resulted in attenuated viral DNA accumulation and symptom development (Rigden *et al.,* 1994; Jupin *et al.,* 1994; Teng *et al.,* 2010) . Recent studies have shown AC4 suppresses posttranscriptional gene silencing (PTGS) (Vanitharani *et al.,* 2005; Fondong *et al.,* 2007). C4 can potentially attach two fatty acids including myristatic and palmitatic acids to Gly-2 and Cys-3, respectively which determine the subcellular localization and pathogenesis of the virus (Fondong *et al.,* 2007, Fondong., 2013).

Sattelite DNA β is comparatively smaller in size (~1.3kb) and contains SCR region, A-rich region and βC1 ORF (Briddon *et al.,* 2003; Briddon and Stanley, 2006; Briddon *et al.,* 2008; Yang *et al.,* 2011). SCR region is of ~200 nt long with hair-loop structure containing TAA/GTATTAC conserved sequence. The SCR region is the key structural component of DNA β for Rep binding and replication. However, DNA β lacks conserved Rep binding canonical iteron sequence and TATA motif which indicates that Rep binding is nonspecific during DNA β replication. Satellite DNA β depends on the helper virus for encapsidation and systemic infection (Briddon and Stanley, 2006). βC1 (~13 kDa) binds to DNA in a size- and sequence-nonspecific manner. Recent studies have revealed that the βC1 protein functions as a suppressor of RNA silencing and is targeted to the nuclei of insect and plant cells (Fig. 2.8) (Cui *et al.,* 2004).

2.5 Mechanism of replication of geminiviruses

Geminivirus DNA follow the mechanism of rolling circle replication (Jeske *et al.,* 2001). With the help of host primase and polymerase, minus strand genome is synthesized to form a dsDNA replicative form (Saunders *et al.,* 1991; Gutierrez, 2000a; Gutierrez, 200b Hanley-Bowdoin *et al*., 2000). The negative strand synthesis is initiated by priming of oligo ribonucletide at intergenic region. Double stranded replicative form (RF) of DNA

may generate single stranded viral genome or transcribed to produce viral proteins (Hanley-Bowdoin *et al*., 2000; Jeske *et al.,* 2001).

Replication initiation involves nicking at TAATATTAC located in stem-loop structure of CR region. Following phosphodiester bond cleavage, Rep protein covalently attached to the 5'-terminus via a phosphotyrosine linkage (Laufs *et al*., 1995a; Jeske *et al.,* 2001). The free 3'-OH terminus is exploited as a primer for the synthesis of nascent viral plus strand, which eventually displaces the parental plus strand from the intact minus-strand template. However, polymerization of viral DNA is carried out by coordinate action of host polymerase (Laufs *et al*., 1995b; Gutierrez, 2000a). With the completion of the nascent plus strand, stem loop structure with the origin of replication is regenerated which is subsequently nicked by Rep, this time performing as a terminase to release unit-length viral plus strand (Fontes *et al*., 1994a, Fontes et al., 1994b). Eventually nascent unit-length plus strand is ligated to form new viral DNA unit (Jeske *et al.,* 2001). Subsequently, Rep is transferred to the newly created 5' terminus (Jeske *et al.,* 2001). Initially, the circularized ssDNA is used as template for synthesis of (minus strand) viral DNA, resulting in the amplification of RF. The third stage replication cycle is responsible for the accumulation of nascent viral genomes for encapsidation and transport.

2.6 Strategy of *Geminivirus* transcription

The viral messenger RNAs have been characterized for several geminiviruses (Stanley and Townsend, 1985; Petty *et al.,* 1988; Hanley-Bowdoin *et al*., 1989; Sunter and Bisaro, 1989a; Sunter *et al.,*1989b; Frischmuth *et al.,* 1991; Mullineaux *et al.,*1984). These studies established that geminivirus genomes are transcribed in a bidirectionally and resulting in mRNAs that correspond to both the virion and complementary-sense ORFs. Several reports revealed that the viral transcripts are polyadenylated at 3' end and initiate downstream of either consensus TATA box or initiator elements, indicating that they are transcribed by host RNA PolII. Geminivirus transcription is complex process which frequently generates multiple overlapping RNA species, majority of which are

polycistronic. The transcription strategies and profile of the bipartite begomovirus, *Tomato golden mosaic virus* (TGMV), has been characterized in detail (Petty *et al.,* 1988; Hanley-Bowdoin *et al.,* 1989; Sunter and Bisaro, 1989a; Sunter *et al.,* 1989b). TGMV DNAA acts as a template for production of six viral transcripts, whereas the DNA B is transcribed to generate four RNAs (Frischmuth *et al*.,1993). A single virion sense RNA is transcribed from each genome component, which is translated to give either the coat protein or BR1. In contrast, complementary-sense transcription is more complex process, leading to formation of multiple overlapping RNAs with different 5′ ends but a common 3′end. The polyadenylation sites for complementary and virion-sense RNAs overlap such that they share a few nucleotides at their 3′ ends. The largest transcript of DNA A encodes the entire left side of TGMV DNA A and produces full-length Rep protein. Two of the RNAs are translated to form AC4, which is represented by the first of three ORF on these RNAs. The smaller RNAs specify AC2 from their first open reading and AC3 from a second coding region. No RNA encodes for AC3 as first ORF, indicating that AL3 is produced from a polycistronic mRNA. The polycistronic character and predicted translation properties of various TGMV DNA A complementary-sense RNAs, have been confirmed by translation *in vitro* (Thommes *et al.,* 1994). Other viruses belonging to curtoviruses and monopartite begomoviruses display similar complementary-sense transcription patterns, independent of the number of genomic components (Frischmuth *et al.,* 1991 and 1993; Mullineaux *et al.,* 1984). Unlike TGMV, multiple virion-sense mRNAs have been documented for the single-component viruses, BCTV and TLCV, most likely reflecting the greater complexity of their virion-sense genes (Frischmuth *et al.,* 1993; Mullineaux *et al.,* 1984).

2.7 Mechanism of resistance in plants

Plant viruses are the major hindrance for successful crop production throughout the world. Therefore, there is urgent need for the development of efficient strategies for generating resistance against this group of pathogens. Comprehensive study of plant resistant factor(s) and understanding mechanism of resistance are prerequisite task for the

generation of resistant plants. Broadly, two distinct approaches are undertaken to develop antiviral resistance in plants:-

1. Transgene mediated resistance

2. Natural mechanism of resistance

2.7.1. Transgene mediated resistance

There are several reports of successful development of resistance plants against bacteria, fungi, insects and viruses using transgenes from hetrologous species. Several transgenic approaches have been widely explored and used to develop virus resistant plants. One of the earliest techniques was pathogen derived resistance mediated resistance mechanism. Pathogen derived resistance mechanism involves use of either wild type or mutant version of viral proteins which compete with the normal viral protein during virus infection and inhibit the virus pathogenesis. Transgenic tobacco expressing CP of *Tobacco mosaic virus* (TMV) conferred resistant against TMV (Beachy, 1999). Movement protein (MP) mediated resistance have been demonstrated against TMV. Recently, geminivirus resistance plants have been developed against *Bean golden mosaic virus* using RNAi construct AC1 gene (Bonfim *et al*., 2007). However, PDR is very common method to develop virus resistance plants but there are also several potential risks relate to recombination, heteroencapsidation, gene flow, synergism, effect on non target organisms, and food safety in terms of allergenicity (Fuchs and Gonsalves, 2007).

2.7.2 Natural mechanism of resistance

Plants being sessile are always threatened by several pathogens. To avoid pathogen infection, plants has naturally evolved and are equipped with different sophisticated defense mechanisms. Several resistance and defense related factors have been reported and characterized from wide range of plant species. In nature two types of resistance mechanism have been observed: A) Non host mediated resistance and B) host mediated resistance

2.7.2.1 Non host resistance

Non host resistance encompasses the situation where all genotypes within a plant species show resistance or fail to be infected by a particular virus. This specifically signifies the state where genetic polymorphism for susceptibility to a particular virus has not been identified in a host taxon (Kang *et al.,* 2005). Under natural conditions majority of the plants are usually resistant to several viruses. However, the mechanism of non host resistant is very complex and is largely not known. Its genetic governance is also difficult to interpret (Kang *et al.,* 2005).

2.7.2.2 Host resistance

Host resistance (also known as specific resistance, genotypic resistance, or cultivar resistance) occurs when genetic polymorphism for susceptibility is noticed in the plant taxon that is a few genotypes show heritable resistance to a specific strain of virus whereas other genotypes in the same gene pool are susceptible (Kang *et al.,* 2005). In the case of host resistance, any of the important host factor (required for virus replication, transcription, movement or suppression of host defense) is either mutated or unavailable for virus life cycle. Consequently, virus may or may not multiply, but spread of the virus within the plant is comparatively restricted relative to susceptible hosts, and disease symptoms are either highly localized or not evident (Kang *et al*., 2005).

Following pathogen invasion resistant plants induce defense responses like necrosis, hypersensitive reaction, to restrict the multiplication and spread of pathogen. In some cases pathogen replicates and spreads inside the host without production of visible symptoms. This response of the host is considered as tolerance. The genetics and biology of mechanism of tolerance resistant is very complex and till date is not well understood.

2.7.2.2.1 Passive resistance response

Although plants do not have well elaborated and efficient immune system like animal but they have evolved with highly fortified defense mechanism to protect themselves from

different pathogens. Well developed cell wall and cuticle layer are the first layer of non specific passive defense barriers which inhibit the entry and spread of most of the pathogens. Plant cell wall is composed of primary and secondary cell wall. The primary cell wall is polymer of 1,4-linked β-D-glucose with two groups of branched polysaccharides include the pectins (rhamnogalacturonans, homogalacturonans, and substituted galacturonans) and hemicelluloses (Carpita and Gibeaut, 1993). Pectin confers rigidity and resists compression while tensile strength of the cellulose is maintained by cross linking glucans (Carpita and Gibeaut, 1993). Secondary cell wall developed inside the primary cell wall, adds extra strength to the primary cell wall with fortification of strong defense barrier. Since cell wall forms basal passive defense barrier therefore, time and rate of cell wall synthesis, deposition at different developmental stage is also crucial and important for successful infection by the pathogens. However, some structural changes in cell wall also take place following pathogen attack. Fungal infection usually induces the generation of reactive oxygen (Jacobson *et al*., 1996) which triggers the water resistant lignifications of cell wall and inhibits the activity of different cell wall degrading enzymes secreted by fungus. Plant viruses are known to increase the size exclusion limit of plasmodesmata either by reorganization of actin or inhibiting the β-glucuronidase enzyme involved in the callose deposition around the neck of plasmodesmata. Enhanced callose deposition has been demonstrated as defense response against several plant viruses (Iglesias *et al*, 2000; Li *et al.,* 2012).

2.7.2.2.2 Active resistance response

Plants are also endowed with active innate immune system which is activated following recognition of specific conserved microbial-associated molecular patterns (MAMPs). Class of specific plasma membrane bound extracellular or intracellular specific proteins called as pattern recognition receptors (PRRs) recognize the MAMPS and activate the downward defense signaling. MAMPs are essential structures or components that are conserved across diverse classes of pathogens (Fraser *et al*.,1990. This includes oligogalacturonides (Galletti *et al*., 2008), ergosterol (Granado *et al*., 1995), bacterial

flagellin (Felix *et al*., 1999), Pep-13 (Brunner *et al*., 2002), xylanase (Belienet *et al*., 2006), cold-shock protein (Felix and Boller, 2003) and lipopolysaccharides (LPS). In plants, membrane bound receptor like kinases (RLK) plays role of PRR which recognizes broad range of pathogen MAMPs. Structurally, RLK consists of extracellular ligand binding domains, middle transmemebrane domain and intracellular serine/threonin kinase domain. Some receptor like proteins have also been reported which recognizes MAMPs and in association with some other adopter proteins activate defense signaling (Zipfel, 2008). PRR recognizes the MAMPs and activate the defense responses which alters in cytoplasmic Ca^{++} homeostasis, activation of the conserved mitogen activated protein kinase (MAPK), generation of phytoalexins, production of reactive oxygen species (ROS), expression of defense related genes, generation of nitric oxide (NO), synthesis and deposition of callose, closure of stomata and synthesis and spread of antimicrobial elements (Asai *et al.,* 2008; Asai and Yoshioka, 2008; Navarro *et al.,* 2004; Ramonell *et al.,* 2005; Melotto *et al.,* 2006).

2.7.2.2.3 R gene mediated resistance

Several R gene mediated resistant response have been genetically and biologically characterized in plants against various pathogens including fungi, bacteria and viruses. R genes are usually known to confer resistant to a specific pathogen. Structurally, majority of plant R genes belong to NB-ARC-LRR superfamily which contains conserved nucleotide binding domains, region similar to metazoan cell-death genes Apaf-1 and CED4 and leucin rich repeats. ATPase activity of R gene has been reported but the exact function of ATP hydrolysis is not known. N-terminal of NB-ARC-LRR may have either toll interleukin like receptor (TIR) or coiled-coiled domains (CC) (Inohara *et al.,* 2003).

Plant R gene recognizes specific pathogen molecule/elicitor which is called Avr. This R-Avr interaction leads to resistant status of the host (Flor, 1972). Therefore, functions of R gene are implicitly linked to gene for gene hypothesis. An extensively studied R-Avr interaction is N gene mediated resistance in *Nicotiana* sp. against *Tobacco mosaic virus*

(TMV). Mutation in all three domains of R gene fails to confer resistance (Whitham *et al.,* 1996). R gene recognizes replicase of TMV as Avr and triggers the defense response which involves localized cell death, activation of defense gene, production of reactive oxygen species and establishment of salicylic acid mediated systemic acquired resistant (SAR) (Whitham *et al.,* 1996; Erickson *et al.,* 1999a; Erickson *et al.,* 1999b; Marathe *et al.,* 2002). Rx1 is a CC-NB-ARC-LRR type R gene in *Solanum tubersum* which recognizes coat protein of *Potato virus X* as Avr and induces the defense response (Bendahmane *et al.,* 1999). RPM1, RPS2 and RIN4 genes are involved in resistance against *Pseudomonas syringae* pv. tomato (Boyes *et al.,* 1998; Mackey *et al.,* 2003; Belkhadir *et al.,*2004). Although, a particular R gene confers resistance against a specific pathogen, reports indicate that one R gene can also confer resistance against diverse plant pathogens. One of the early reports indicated *Arabidopsis thaliana* RCY1 (resistance to C strain Y1) and HRT (HR to *Turnip crinkle virus*) are genetically allelic and known to encode proteins that share 91% homology but provides resistance to various strain of viruses like *Cucumber mosaic virus* and *Turnip crinkle virus*, respectively (Takahashi *et al.,* 2002).

Following pathogen recognition and activation of defense signal transduction three endogenous signaling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are produced and are involved in eliciting defense mechanism against the pathogen These pathways do not function independently, rather influence each other through a complex network of regulatory interactions.

2.7.2.2.4 Salicylic-acid-mediated defense

Salicylic acid (SA) is the key component produced in response to pathogen attack. SA level gets increased following pathogen recognition and activation of defense signaling. It is also reported that external application of salicylic acid enhanced host plant resistance against broad range of microbial pathogens. R gene mediated resistance response induces the accumulation of salicylic acid which subsequently activates expression of several

pathogen related genes (PR genes) and establishes systemic acquired resistance (SAR) (Shah *et al.,* 2003). It is also demonstrated that *A. thaliana* mutants that are impaired in the synthesis or production of SA like enhanced disease susceptibility1 [eds1], eds4, eds5, phytoalexin deficient4 [pad4] and SA induction deficient2 [sid2]), as well as transgenic plant lines that were unable to accumulate elevated levels of SA because of their expression of the SA-degrading enzyme salicylate hydroxylase (NahG), exhibited enhanced disease susceptibility to a variety of pathogens. Plants with mutations in NON-EXPRESSOR OF PR1 (NPR1) a gene, that functions downstream of SA exhibit enhanced susceptibility to these pathogens (Nandi *et al*., 2003). Salicylic acid (SA) treatment has been demonstrated to inhibit replication of TMV in infected tissue (Naylor *et al.,* 1998). *Arabidopsis* ecotype Dijon (Di-17) showed salicylic acid mediated defense response against *Turnip crinkle virus* (TCV) (Kachroo *et al.,* 2000).

2.7.2.2.5 Jasmonic acid mediated defense

The Jasmonic acid (JA) signaling pathway plays crucial roles in several signaling pathways including plant defenses metabolism and development (Kessler and Baldwin, 2002; Turner *et al.,* 2002; Weber, 2002). Biosynthesis pathways of JA involve a series of oxidation steps from unsaturated fatty acid linolenic acid (18:3), in combination with octadecanoid. The oxidation produces oxide intermediates which subsequently undergo enzyme directed cyclization to generate cyclopentanone 12-oxophytodienoic acid (OPDA, 18 carbon) and its 16 carbon homologue dinor-oxo-phytodienoic acid (dnOPDA). Further, a series of reduction and β-oxidation generate final product, JA (Vick and Zimmerman *et al.,* 1984; Wasternack, 2007; Kazan and Manners, 2008). In cellular milieu and according to need, the JA can combine with various amino acids, to form JA-amino acid conjugates, or methylated to form methyl jasmonate (volatile derivative) (Seo *et al.,* 2001; Staswick and Tiryaki, 2004). A few reports have also indicated hydroxylation and sulfonation of JA (Staswick and Tiryaki, 2004).Jasmonic acid singaling pathway is also influenced with salicylic acid pathway and cross talk between this two determine the defense response processes (Thaler *et al*., 2012).

Role of JA have been studied and documented in defense response against several pathogens (Jennifer *et al.,* 2012). *A. thaliana* mutants that are impaired in JA production (e.g. fatty acid desaturase [fad]3/fad7/fad8 triple mutants) or perception (e.g. coronatine insensitive1 [coi1] and jasmonic acid resistant1 [jar1]) indicated enhanced susceptibility to various pathogens, including fungi *Botrytis cinerea, Alternaria brassicicola* and *Pythium sp*., and the bacterial pathogen *Erwinia carotovora*. Wheat (*Triticum aestivum*) pretreated with MeJA showed delayed symptom development against *Fusarium pseudograminearum* (Desmond *et al.,* 2005) and enhanced resistance to infection by *Stagonospora nodorum* (Jayaraj *et al.,* 2004). JA and ET application has also been shown to enhance maize resistance to the necrotrophic pathogens Rhizopus microspores and *Colletotrichum graminicola* .

Although less information is available about the role of jasmonic acid against plant viruses. Recently, βC1 of *Tomato yellow leaf curl China virus* (TYLCCNV) interacts with ASYMMETRIC LEAVES 1 (AS1) and ASYMMETRIC LEAVES 2 (AS2), which are known to regulate leaf morphology and development as AS1/AS2 complex. βC1 binds directly to AS1, induced morphological and gene expression changes via AS1dependent but not AS2, and reduced the expression of selective jasmonic acid (JA)-responsive gene (Yang *et al*., 2011).

2.7.2.2.6 Ethylene mediated responses

Ethylene is gaseous hormone involved in seed germination, organ senescence, fruit repining, abiotic and biotic stress. Enhanced ethylene production is an early, active response of plants to perception of pathogen attack and is associated with the induction of defense reactions. Biosynthesis of ethylene from the amino acid methionine is the conversion of S-adenosyl methionine into aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase and which is further converted to ethylene by ACC oxidase (ACO). Pathogen infection is often associated with increased ET production and exogenous application of ET to plants can result in activation of pathogenesis related

protein (PR2, 3 and 5) (Xu *et al.,* 1994). The role of ET in host resistance is limited to particular class of pathogens and not to others. Signal perception and transduction of ET to the nucleus has been elucidated. The signal perception is mediated by ETR1, ERS1 (Group I), ETR2, EIN4 and ERS2 (Group II) (Hua *et al*., 1998a; Hua *et al*., 1998b; Sakai *et al.,* 1998; Hirayama *et al.,* 1999; Rodriguez *et al*., 1999). CTR1 is a serine threonine kinase which functions downstream of the ET perception (Huang *et al.,* 2003). Both receptors and CTR1 are negative regulators of the pathway. EIN2 acts downstream of CTR1 and is a positive regulator of ET pathway (Alonso *et al.,* 1999). EIN2 stabilizes the EIN3 protein which works downstream of it. EIN3 accumulates in response to ET and activates the transcription of ERF1 (Ethylene Response Factor 1). ERF1encodes for a transcription factor whose expression is activated rapidly either by ET or JA and can be activated concomitantly by both the hormones. The PDF1.2 gene expression is blocked in ein2-1 and both jasmonate and ET responses are needed to restore its expression (Penninckx *et al.,* 1998) Similarly, ERF1 activation requires both signaling pathways, mutation in any of them prevents ERF1induction. Moreover, 35S:ERF1line can rescue the defense response defects of coi1 and ein2 (Lorenzo *et al.,* 2003). ein2, ein3or etr1 mutants display more susceptibility to *B. cineria* while ERF1over-expression lines are more resistant to *B. cinerea* (Berrocal-Lobo *et al.,* 2002). ERF1 therefore acts downstream of the intersection between ET and jasmonate pathways.

Ethylene is also known to be involved in defense against plant viruses. TMV infection enhanced the higher accumulation was ethylene which promoted induction of pathogenesis related (PR) genes and formation of necrotic lesions. The incompatible interaction between *C. annuum* and TMV induced expression of CaTin1 (TMV-induced clone 1). Further study revealed CaTin1 probably functions via ethylene dependent pathway because down regulation of ethylene biosynthesis genes resulted in reduced accumulation of CaTin1 gene. CaTin1 tobacco plants indicated elevated level of H_2O_2 and ethylene and also confer resistant to biotic and abiotic stress (Shin *et al.,* 2004).

2.7.2.3. Post transcriptional and transcriptional gene silencing

In susceptible host, geminiviruses are known to replicate efficiently and produce abundant viral transcripts in infected cells of host. However many plant species have evolved with sophisticated and defensive mechanism of RNA interference against plant viruses (Baulcombe, 1999). There are several reports in which plants have executed post transcriptional and transcriptional gene silencing against geminiviruses.

Post transcriptional gene silencing (PTGS) machinery targets the virus transcripts which are produced in the nucleus and transported into the cytoplasm (Vazquez, *et al.,* 2010). After entering into PTGS pathway, virus transcripts are converted into dsRNA by host RNA dependent RNA polymerase (RDRP). Subsequently, dsRNA is sliced into small RNA (siRNA) by DICER like proteins (DCLs) (Vazquez *et al.,* 2004, Meins *et al.,* 2005; Vaucheret, 2006; Vazquez, *et al.,* 2010) which are known to contain RNase III domains. These nascent siRNA duplex is unwound by host helicase to produce single stranded small RNA and subsequently loaded on the RNA induced silencing complex (RISC) which is associated with AGO protein having endonuclease activity (Vauchert, 2006; Mallory *et al.,* 2010). RISC complex encounter the corresponding viral transcript and base pairing between siRNA and mRNA is specific which leads to the degradation of virus transcripts (Vazquez, *et al.,* 2010). The siRNA produced after RISC complex activity are amplified by RDRP (Mourrain *et al.,* 2000; Dalmay *et al.,* 2000) and most probably function as systemic signal for RNA interference. siRNA generated against virus transcripts can also be involve in transcriptional gene silencing (TGS) of viral genome in the nucleus. In nature, plants utilize this mechanism to maintain the heterochromatin region and regulation of transposons. Most probably, during the course of evolution plants are compelled to use this mechanism as a defense weapon against invading viruses. DCL2 is known to produce 24-26 nt siRNA which in association with RDR2 and AGO4 leads to the transcriptional gene silencing (Raja *et al.,* 2008).

In dcl2 mutant plants, accumulation of viral siRNA upon TCV infection is delayed, and these plants showed enhanced susceptibility to TCV. Therefore, DCL2 seems to be involved in the antiviral response to TCV infection (Xie *et al.,* 2004). Silencing of RDR6 in *Arabidopsis* results in enhanced susceptibility to CMV (Dalmay *et al.,* 2000; Mourrain *et al.,* 2000). Comparatively Nt RdRP-1 silenced plants also indicated enhanced susceptibility against TMV (Xie, 2001). It has also been demonstrated that a PVX strain which is unable to spread in tobacco could spread both locally and systemically in NtRdRP1 silenced transgenic plants (Xie *et al.,* 2004). Interestingly *N. benthamiana* encodes NbRdRp1m which is more than 90% identical to NtRdRp1, but produces a truncated protein that does not function properly (Yang *et al.,* 2004). However, *N. benthamiana* is known to be highly susceptible to several plant viruses but, transgenic *N. benthamiana* expressing RdRP from *Medicago truncatula* indicated an enhanced resistance to *Tobamoviruses* (Yang *et al.,* 2004). The truncated RdRP was correlated with the enhanced susceptibility of *N. benthamiana* to viral infection (Yang *et al.,* 2004).

2.7.2.4 Role of epigenetic and post translational modification in plant defense

Epigenetic modifications involve functionally relevant modifications to the genomic DNA which do not involve a change in the nucleotide sequence and some of them are usually inheritable (Vaillant *et al.,* 2007). Eukaryotic DNA carrying genetic informations in association with specific number and type of basic histones proteins are organized in a regulatory structure of chromatin (Luger *et al.,* 1997). All genetic information of DNA never gets expressed at any given time but some of the genes are expressed while other remains silenced. This specific gene expression mechanism implies efficient fine tuning between DNA and histone modifications without changing the primary gene sequences, the phenomenon known as epigenetic. During gene expression, RNA Polymerase II in coordination with other regulatory factors accesses the tightly packaged DNA to activate the promoter for gene expression (Struhl, 1999). Epigenetic modification prevents this kind of complex to access the DNA hence silences the gene expression.

Covalent attachment of methyl group at C5 of cytosine carried out by enzymes called DNA methyltransferases and is involved in many cellular processes, such as silencing of transposable elements and pericentromeric repeats, X-chromosome inactivation and genomic imprinting, etc (Zhu *et al*., 2007; Ahmad *et al.,* 2010). Cytosines can be found to be methylated in three sequence contexts; 5' CpG 3', 5' CpHpG 3' and 5' CpHpH 3'. Cytosine in CpG and CpHpG are called symmetric cytosines and in CpHpH is called asymmetric cytosine (Fig. 2.10). Four classes of cytosine methyltransferases are known to occur in plants: MET1, CMT3, DRM and DNMT2 (Fig. 2.9, Table 2) (Ghoshal *et al*., 2006;Xie *et al*., 2006; Huang *et al;* 2006). They show specific expression patterns and levels in various tissues and different developmental stages and differential activity on cytosines in different sequence contexts (Huang *et al.,* 2010). Majority of cytosine methyltransferase possess four important domains; a binding domain for SAM, a binding site for the DNA target, a catalytic domain that catalyzes the methyl transfer reaction and a genome targeting domain (Fig. 2.9) (Mahfouz, 2010).

2.7.2.4.1 RNA dependent DNA methylation (RdDM)

The RdDM is complex process which requires production of siRNAs by coordinate function and interaction of array of specific proteins and RNA. The dsRNA is pre-requisite substrate for the production of siRNA. In the case of PTGS, siRNA is produced from inverted repeats or overlapping transcription by RNA Polymerase II. Generally, ssRNA is produced by RNA Polymerase IV which is subsequently converted into dsRNA by the action of RDRP2 (RNA dependent RNA polymerase 2). This dsRNA acts as substrate for DCL3 and the product is methylated by HEN1 and loaded onto AGO4. The entire complex of siRNA/AGO4 interacts with RNA Polymerase II transcripts or DNA to guide methylation. RNA Polymerase IV produces precursor transcript which can either generate or amplify the siRNA signal cascades.

Enhanced susceptibility was observed in *Arabidopsis* methyltransferase mutants drm1 drm2, cmt3, and kyp2; the methylation pathway mutants ddm1,ago4, and nrpd2a

(polymerase IV); and the methyl cycle mutants adk1and adk2 against *Cabbage leaf curl virus* (CaLCV) and *Beet curly top virus* (BCTV) (Raja *et al.,* 2008). This result provided strong genetic evidence for involvement of virus DNA methylation as innate immunity against CaLCV and BCTV. In another set of experiments, RDR2 and DCL3 mutant indicated moderate increase in symptom severity which suggested activation of siRNA dependent methylation machinery following CaLCV and BCTV (Raja *et al.,* 2008).

Table 2.1: List of the methyltransferases from *Arabidopsis thaliana*

Classification	Protein name	Function and Target sequence
MET1 family	AtMET1	Maintenance : CG probably CHG; single-copy DNA , rRNA, and centromeric repeats
	AtMETIIa	Maintenance ?
	AtMETIIb	Maintenance ?
	AtMETIII	Maintenance ?
CMT family	A tCMT1	Nonessential
	A tCMT2	Associated with heterochromatin
	A tCMT3	Maintenance: CHG at and transposons in heterochromatin
DRM family	AtDRM1	*De novo* : CG,CHG,CHH Maintenance : CHG,CHH
	AtDRM2	*De novo* : CG,CHG,CHH Maintenance : CHG,CHH
	A tDRML	Putative unknown
Dnmt 2 family	AtDnmt2	PutativeRNA?

Adapted from Huang *et al*. (2010)

The siRNA directed DNA methylation was also reported in the case *of Mungbean yellow mosaic India virus* (MYMIV) in *Glycine max* (soyabean) resistant plant. Bisulphite sequencing result from of MYMIV intergenic region (IR) and detection of siRNA against (IR) region confirmed the involvement of transcriptional gene silencing as defense mechanism in resistant soyabean plants (Yadav and Chattopadhyay, 2011). *Solanum lycopersicum* and *Nicotiana benthamiana* plants infected with *Tomato yellow leaf curl China virus* (TYLCCNV) produced several kinds of v-siRNA including those

involved in virus DNA methylation. Generation of this v-sRNA was confirmed by Solexa-based deep sequencing method (Yang *et al.,* 2011). Interestingly, sense and anti-sense specific siRNAs (22 nucleotides) were detected in *S. lycopersicum* and *N. benthamiana* plants. Only TYLCCNV infected plants generated siRNA which targets preferentially AV2 and 59 nucleotides from N terminus of AV1 where as TYLCCNV and betasatellite coinfection generated siRNAs against AC2/AC3 overlapping region including 39 nucleotides from C terminal of AC1 (Yang *et al.,* 2011).

Table2.2: Methylation-deficient mutants are hypersusceptible to geminivirus infection gene function and mutation(s).

Methylation pathway components	Relative severity of disease
nrpd2a(Pol IV)	+++
ago4	+++
ddm1	+++
Cytosine methyltransferases	
drm1 drm2	++++
cmt3	++++
met1	++
H3K9 methyltransferase,kyp2	+++
Methyl cycle enzymes	
adk1	++++
adk2	+++
mmt	+
DCL and RDR enzymes	
dcl2	+++
dcl3	++
dcl4	+
dcl2 dcl3 dcl4	++
rdr2	+
rdr6	+

Severity of disease symptoms in mutant *Arabidopsis* plants was based on the degree of stunting and floral deformation relative to wild-type plants of the appropriate ecotype (Col-0, Ler-0, or Ws-2) according to the following rating scale:+, typical, wild-type symptoms; ++, moderately enhanced; +++, severe; ++++, very severe with extensive stunting. Pol IV, polymerase IV (Raja *et al.,* 2008).

2.7.2.4.2 Post translational modification of histone

Structural unit of nucleosome formation involves association of DNA which wrap around two molecules of each histone proteins (H2A, H2B, H3, H4) (Bavykin *et al*., 1998; Luger *et al.,* 1997). Linker histone H1 together with some other proteins finally participates in the packaging of nucleosome into the chromatin (Luger *et al.,* 1997). Histones are conserved proteins with core globular domain and N terminal domain protruding outside which can interact with other nucleosomal proteins and cellular regulatory factor which in turn influences the nucleosome structure and gene regulation. The N-terminal domain of histones and some specified globular domain can be modified by acetylation, methylation, phophorylation, ubiquitination, glycosylation, and sumoylation and ADP ribosylation by post translational machinery (Table 2.3, Fig. 2.12).

These modifications alter DNA histone interaction and structural integrity of nucleosome that affect the expression of gene regulation. Thus coordinated covalent changes in histone modifications can influence multitude of cellular process, transcription, replication, DNA repair and cell cycle regulation. Some of the histone modifications are specifically responsible of repression of gene regulation where as some covalent modifications facilitate the recruitment of proteins for transcription hence they involve in the activation of gene expression.

2.7.2.4.2.1 Histone acetylation

Positive charges of lysine at specific site of histones can be neutralized by acetylation. Several reports have suggested that histone acetylation alter the interaction between DNA and histones and which form more open chromatin structure which is easily accessible for transcription factors, activators and co-activators. Specific histone acetyltransferases (HATs) execute the function of acetylation of histones (Fig. 2.13).Chromatin immunoprecipitation using H3 acetyl antibody revealed acetylation of H3 in IR region of CaLCV in *N. benthamiana* and *A. thaliana* (Fig. 2.11) (Raja *et al.,* 2008). Histone

acetylation is the hall mark for the activation of gene expression. Acetylated histone in virus minichromosome suggested that virus also utilizes the same mechanism for viral gene expression.

Table 2.3: Histone modification enzymes and their function.

Type	Gene	Full name	Enzyme specificity
Histone acetyltransferases			
GNAT	AtGCN5/HAG1	General control non-respressible 5	H3K14, H3K27
	AtELP3/ELO3/HAG3	Elongator component 3	H3K14
MYST	HAM1/HAM2	Histone acetyltransferase of the myst family 1/2	H4K5
CBP/p300	AtHAC1/PCAT2	Acetyltransferase of the cbp family 1	H3, H4, H2A, H2B
TAF1/TAFII250	TAF1/HAF2	TATA-binding protein associated factoR 1	H3, H4
Histone deacetylases			
RPD3/HDA1	AtHD1/AtHDA19/RPD3A	Histone deacetylase 1	H3K9, H4K5, H4K8, H4K12, H4K16
HD2-like	AtHDA6/RPD3B	Histone deacetylase 6	H3K4, H4K5, H4K12
	AtHDA18	Histone deacetylase 18	Not determined
	AtHD2A/HDT1	Histone deacetylase 2A	H3K9
	AtHD2B/HDT2	Histone deacetylase 2B	Not determined
	AtHD2C/HDT3	Histone deacetylase 2C	Not determined
	AtHD2D/HDT4	Histone deacetylase 2D	Not determined
SIR2	AtSRT1	Sirtuin 1	Not determined
	AtSRT2	Sirtuin 2	Not determined
Histone ubiquitination			
E2	UBC1/UBC2	Ubiquitin carrier 1/2	H2B
E3	HUB1/HUB2	Histone monoubiquitination 1/2	H2B
Histone deubiquitination			
	UBPs	Ubiquitin-specific protease 26	H2B
Histone methyltransferases			
SET domain group	SDG2/ATXR3	Set domain group 2	H3K4me3
	SDG27/ATX1	Set domain group 27	H3K4me2/3
	SDG30/ATX2	Set domain group 30	H3K4me2
	SDG25/ATXR7	Set domain group 25	H3K4me3, H3K36me2
	SDG4/ASHR3	Set domain group 4	H3K4me2, K36me3
	SDG8/ASHH2	Set domain group 8	H3K36me2/3
	SDG26/ASHH1	Set domain group 26	H3me
Histone demethylases			
LSD1-type	FLD	Flowering locus D	H3K4me
	LDL1/LDL2	LSD1-LIKE 1/2	H3K4me2
Jumonji (Jmj)	MEE27/JMJ15	Maternal effect embryo arrest 27	H3K4me1/2/3
	JMJ14	Jumonji protein 14	H3K4me1/2/3
	ELF6/JMJ11	Early flowering 6	H3K9me3
	REF6/JMJ12	Relative of early flowering 6	H3K9me3, H3K27me2/3
	IBM1/JMJ25	Increase in bonsai methylation 1	H3K9me2

Adapted from Berr *et al*. (2011).

2.7.2.4.2.2 Histone phosphorylation

Histone phophorylation are associated with chromosome activation of transcription, condensation, segregation, sporogenesis, gametogenesis, apoptosis and DNA damage repair. DNA damage and repair process involve phosphorylation of histones. Specifically, phosphatidylinositol-3-OH kinases causes the phophorylation of H2A whereas histone H2B phophorylation is carried out by Mst1 (mammalian sterile-20-like kinase) in mammals and the sterile-20 kinase in yeast. H3S10 and H3S28 are phosphorylated by conserved Aurora kinases (Fig. 2.13). In yeast, Histone H4S1 is phosphorylated during sporulation by Sps1 (a member of the sterile-20 family of kinases). Histone H2B (on Ser14 in human, Ser10 in yeast) phosphorylation is associated with meiotic chromosome condensation. DNA damage is also known to trigger histone H2A phophorylation at S129 and S139 in yeast. H2A and H2B are frequently phosphorylated but Histone H4 and H3 also phosphorylated but their role is different. Histone H3 phosphorylation at serine 10 (S10) is signature for activation of gene expression in mammals. In plants, phosphorylation of histone H3 has been observed at serine 10/28 and threonin 3/11 during mitosis and meiosis cell cycle. Phosphorylation of H3T3/T11 in plants is required for the condensation of chromosome during mitotic and meiotic cycle.

2.7.2.4.2.3 Histone methylation

Histone H3 and H4 are mono-, di- or trimethylated on lysine residues 4, 9, 27, 36 and 79 of histone H3 and K20 of H4. SET domain containing histone methyltransferases catalyze the methylation of H3 and H4. Set1 and Set 2 are known to methylate H3K4 and H3K36, respectively. However, most of the lysine methylations (H3K9, H3K27 etc.) are associated with repression of gene silencing. Interestingly, H3K4 methylation is excuted by COMPASS (Complex of Proteins Associated withSet1) and is hallmark of activation of gene expression (Sun and Allis, 2002; Wood *et al.,* 2003; Lee *et al.,* 2007; Cao *et al.,* 2008). H3K79 is methylated by DotI methyl transferase and responsible for induction of

DNA damage response and telomere gene silencing (Tatum and Li, 2011). In eukaryotic system arginine residue can also be methylated which is absent in yeast (Nicholson and Low, 2000; Zurita-Lopez *et al*., 2012). Two proteins viz., coactivator arginine methyltransferase (CARM1) and the protein arginine methyltransferase (PRMT1) are crucial for the methylation of arginin residues of H3 and H4. In *Arabidopsis,* H3K4Me3 methylation is specifically catalyzed by conserved TRITHORAX1 (ATH1) proteins. Experiment demonstrated that ATX1 directly interact with promoter of FLC and methylates H3K4. ATH1 mutant showed reduced level of H3K4Me3 but there was no affect on H3K4me2 level. However, comparative study revealed that ATX1 mutant showed 6-8% H3K4me2 methylation and ~15% in H3K4me3 methylation which indicated involvement of some other methyltransferases in H3K4 methylation. Several reports on *Arabidopsis* and rice suggested association of H3K4Me3 exclusively with active region of the genome where as H3K4me1/2 was observed associated with both active and inactive genomic region. However, lysine residues in histone are known to be methylated frequently. But Arginine is also methylated by specific protein arginine methyltransferases (PRMTs) and this modification regulates transcription, nuclear transport RNA processing, signal transduction and DNA-damage repair (Shen *et al.,* 1998; Gary and Clarke, 1998, Nicholson *et al.,* 2009; Bedford *et al.,* 2009). Arginine is methylated at Arg2 (R2), Arg8 (R8), Arg17 (R17), Arg26 (R26) of histone H3, and Arg3 (R3) of histone H4 (Fig. 2.13) (Auclair and Richard, 2013).

Chromatin immonoprecipitatin result using H3K9me2 and H3K27me2 specific antibodies indicated histone modification in CaLCV IR region during the infection process in *N. benthamiana* and *A. thaliana* (Raja *et al.,* 2008). However, these two modifications are signature of repression of gene expression which indicated viral genome exploited the host PTM mediated gene regulation.

2.7.2.4.2.4 Histone ubiquitination

Histone H3, H2A and H2B are known to monoubiquitinated at specific residues. Histone H2A gets monoubiquitinated at K119 in yeast. Whereas the most preferred site of monoubiquitination of H2B is K120 in human and K123 in yeast (Fig. 2.13) (Schneider *et al.,* 2005; Shahbazian *et al.,* 2005). Although, H3 ubiquitination has been noticed but site of ubiquitination is still not clear. Ubiquitin is small molecule of ~8.0 kDa. Histone monoubiquitination takes place by formation of an isoopeptide bond between the carboxy-terminal glycine of ubiquitin and the ε-group of a lysine on different histones. This modification is nonspecifically involved in ubiquitination of several proteins. E2 conjugating enzymes are required for the ubiquitination of different substrate where as E3 ligase provides specificity to the reaction. In yeast, Rad6 (E2 conjugating enzyme) in cooperation with BreI (E3 ligase) forms the core group of proteins required for H2B monoubiquitination. Monoubiquitination of H2B recruits COMPASS/Set1 and other histone methylation factors and induces trimethylation of H3K4 (H3K4me3). H3K4me3 facilitate efficient interaction of RNA Polymerase II and other transcription factor which cooperatively activate the expression of gene (Fig. 2.14) (Weake and Workman, 2008).

In plant, UBC1/UBC2 functions as E2 conjugating enzyme with HUB1/HUB2 E3 Ligase enzyme. Polycomb group RING finger protein (Ring1b) functions as E3-ligase for H2A monoubiquitination. One of the studies in plants revealed HUB1 or HUB2 in coordination with UBC1 or UBC2 form monoubiquitination machinery which promote H3K4 trimethylation and regulate the flowering time in *Arabidopsis* (Fig. 2.15) (Cao *et al.,* 2008).

Interestingly, in plant HUB1 mutants showed increased susceptibility to *Botrytis cinerea* and *Alternaria brassicicola*. Interestingly, HUB1 interacts with MED21 mediator complex which is known to regulate RNA pol II activity. MED21 mutant line also indicated susceptibility to *B. cinerea* and and *A. brassicicola*. These results indicated role of HUB1 mediated gene expression involved in the defense against *B. cinerea* and *A.*

brassicicola (Dhawan *et al.,* 2009). Recent study has revealed the role of monoubiquitination of H2B in rapid modulation of many gene expressions during the photomorphogenesis (Bourbousse *et al.,* 2012).

Recent report indicated that adenovirus infection induced production of cellular type I interferon which activated a set of defense related gene. Activation of these defense related genes were correlated with enhanced monoubiquitination of H2B. Global increase in ubH2B was confirmed by ubH2B specific antibody and western blot analysis. More interestingly for the establishment of successful infection, Adenovirus E1A protein was interacting with hBreI complex and inhibited the monoubiquitination (Fonseca *et al.,* 2012).

Interestingly, a recent study suggested the role of monoubiquitinated H2B in DNA replication. In budding yeast, higher deposition of monoubiquitinated H2B was observed around origin of replication and its level was maintained in daughter strand during DNA replication. Surprisingly, in the absence of monoubiquitination of H2B, prereplication complex is formed but replication fork progression was slowed down. Collectively, this result indicated that H2B monoubiquitination is essential for the formation and stability of prereplication assembly and also needed for efficient progression of replication fork (Trujillo and Osley, 2012).

2.7.2.4.3 Cross talk between histone modifications and gene activation

Histones are known to be modified at several specific residues. Histone modification at one residue can influence the modification of other residues either of the same histone or different histone (Lee *et al.,* 2007). Many research studies have revealed monoubiquitination of H2B promote the methylation of H3K4. The cross talk between histones modifications have been extensively study in yeast. In yeast, COMPASS mediated H3K4 methylation and DotI mediated H3K79 methylation were dependent on Rad6/BreI catalyzed H2B monoubiquitination. COMPASS need subunit Cps35 for H3K4 methylation and interaction between COMPASS and Cps35 was promoted by

monoubiquitinated H2B. In the absence of monoubiquitinated H2B, Cps35 cannot interact with COMPASS and H3K4 methylation is disturbed which in turn affect the transcription. However, COMPASS can interact with RNA Polymerase II via paf1 complex and naturally present H3K4Me1. But, ubiquitination promote the recruitment of Cps35 which induces H3K4 methylation and facilitates the strong interaction between COMPASS and RNA Polymerase II (Fig. 2.14). All this action in coordination accelerates the transcription rate and efficiency (Lee *et al*., 2007).

Monoubiquitinated H2B facilities H3K4Me3 methylation in animal but it seems a conserved mechanism in plants also. Western blot analysis and Chromatin immunoprecipitation results indicated, in *Arabidopsis* hub1, hub2, orubc1, ubc2 plants lacked H2B monoubiquitination which, however, does not affect H3K4Me2 level but significantly reduced the H3K4Me3 deposition around chromatin of FLC genes (Cao *et al*., 2008).

2.8 Approaches used to identify resistant factor against geminivirus

One of the traditional methods to explore geminivirus resistant factor(s) was use of conventional plant breeding technique. After many generation analysis and tracking of specific markers, Ty1 gene was identified as resistant gene and found associated with all resistant *S. lycopersicum* plants against *Tomato yellow leaf curl virus* (TYLCV). But still mechanism of resistant is largely unknown. However, studies revealed that TYLCV resistance genes Ty-1and Ty-3 are allelic and code for DFDGD-Class RNA–dependent RNA polymerases belonging to the RDRc type. But RDRγ mediated resistance mechanism in tomato against TYLCV is still not clear (Verlaan *et al.,* 2013).

Degenerate primer of NBS-LRR was used to amplify R gene from common bean (*Phaseolus vulgaris* var. Othello) infected with *Bean dwarf mosaic virus*. Isolated R gene (RT4-4) used to develop transgenic *N.benthamiana* plants which were challenged with different viruses for functional analysis of RT4-4.RT4-4 plants did not confer resistant

against BDMV but able to provide successful defense against cucumber Mosaic Virus (CMV) (Seo *et al*., 2006).

Rapid amplification of cdna ends (RACE) experiment was performed to clone CYR1 that co-segregates with MYMIV (*Mungbean yellow mosaic virus*) resistant populations of *V. Mungo*. However, CRY1 expression was observed enhanced in MYMIV and *in silico* study also indicated interaction between CRY1 and coat protein of MYMIV but molecular mechanism of CYR1 mediated resistant could not be elucidated (Pal *et al*., 2007).

The 2IRGFP construct derived from *Tomato yellow leaf curl Sardinia virus* (TYLCSV) in combination with virus induced gene silencing and reverse genetic approach was successfully used to screen eighteen genes potentially involved in resistant mechanism. Out of 18, seven was analyzed and known to involve in antivirus defense mechanism. Half of the genes were known to involve in post translational modification. This study generated sufficient information to identify probable candidates involved in host plant resistance (Veerlan *et al*., 2013).

A comparative study of cDNA libraries from lines resistant (R) and susceptible (S) TYLCV infected plants indicated expression of 70 genes in resistant plant. Further analysis revealed that LeHT1 (hexose transporter 1) played crucial role in defense against TYLCV. LeHT1 silenced plants showed elevated level of TYLCV DNA accumulation and exhibited necrotic spots following infection with TYLCV. Similarly, necrotic spots were also observed when LeHT1 silenced plants were infected with *Bean dwarf mosaic virus*, *Cucumber mosaic virus* and TMV. These results suggested role of LeHT1 in providing resistance against TYLCV (Eybishtz *et al.,* 2010).

Recently, reduced accumulation of viral DNA was observed in tolerant cultivar H-88-78-1 infected with *Tomato leaf curl New Delhi virus* (ToLCNDV). Further analysis showed elevated accumulation of TYLCV specific siRNA in tolerant cultivar. To identify the host resistance factor in tolerant cultivar, suppression subtractive hybridization (SSH)

was carried out between mock and infected tolerant cultivar. SSH result indicated differential expression of 106 genes. Out of these, 34 were showed upregulation and 8 genes exhibited >4 folds upregulation. Therefore, cumulative accumulation of defense related transcripts and virus specific siRNA correlated with tolerance mechanism of tomato against TYLCV (Sahu *et al.,* 2010).

3.1 Plant growth condition

Seeds of chilli varieties (Punjab Lal, BS35 and Kalyanpur Chanchal) were obtained from Indian Institute of Vegetable Research (IIVR), Varanasi and seeds of *Nicotiana benthamiana* were obtained from Central Tobacco Research Institute (CTRI), Rajamundry, Andhra Pradesh. Chilli and *N. benthamiana* plants were grown under controlled conditions of 16 h light and 8 h dark period at 25°C ± 3°C with relative humidity of 60%. Seedlings were transferred at two leaves stage in new pots and plants were maintained under same conditions as mentioned above in an insect proof glass house.

3.2 Construction of highly efficient infectious clone of ChiLCV

Monomeric and tandem repeat clones of ChiLCV DNA A and associated betasatellite (DNA β) were available in our laboratory (Chattopadhyay *et al.,* 2008). In that study, a partial clone of DNA β was constructed at *Kpn* I and *BamHI* site followed by full-length insertion of DNA β (1.3 kb) in the binary vector, pCAMBIA2300 to obtain pTBeta. Tandem repeat of DNA β was confirmed by either restriction digestion or PCR using βC1 specific primer. In order to create a highly efficient infectious construct, full-length DNA A (2.7 kb) was inserted at *Sal* I site in pTBeta, containing tandem dimer of DNA β. To complete the tetramer construction, 2.7 kb DNA A was released from the tandem repeat construct of ChiLCV A (pTChA) by *Cla* I digestion which was subsequently inserted at *Cla I* site in the pCAMBIA2300 vector containing tandem dimers of both DNA β and DNA A, hereafter called as tetramer.

3.3 Ligation mixture for cloning of DNA

The volume of vector and insert were calculated according to their band intensity on the gel and using spectrophotometer. Vector and insert DNA were taken in a ratio of 1:3 and ligated in 1X T4 DNA ligase buffer with 1.0 µl T4 DNA ligase (EL0011, Thermo scientific, USA) in a final reaction volume of 20 µl. Ligation mixture was incubated at 16°C for 16 h as per manufacturer's instructions. A negative control/background reaction

was also set with vector, T4 DNA ligase and 1X T4 DNA ligase buffer. The ligation mixture was either used immediately for transformation or stored at -20° till transformation.

Table 3.1: Reaction components used for ligation.

Components	Amount
Vector	1.0 µl
Insert	3.0 µl
Ligase buffer (10x)	1.5 µl
ATP (25mM)	1.0 µl
T_4 DNA Ligase (10u/µl)	1.0 µl
Sterile distilled water (SDW)	7.5 µl
Total volume	15.0µl

3.4 Preparation of competent *E. coli* cells

The competent cells of *E. coli* strains DH5α was prepared by $CaCl_2$ method (Mendel and Higa, 1970). For preparing competent cells, a single colony of *E. coli* strain DH5α was inoculated into 3.0 ml of Luria Bartani (LB) media for primary culture. After 12-16 h, 1.0 ml of this primary culture was inoculated into 50 ml of LB media and allowed to grow at 37^0C, 220 rpm till the optical density of the culture OD600 reached a value of 0.35. The cells were chilled on ice for 15-20 min and centrifuged in SS-34 tubes at 6000 rpm for 5 min at 4^0C. The supernatant was discarded and cell were resuspended in 30 ml of ice cold sterile solution (6.0ml of 0.1M $CaCl_2$ + 24ml of 0.1M $MgCl_2$), mixed thoroughly and again incubated on ice for 30 min. After incubation, cells were centrifuged at 6000 rpm for 5 min at 4^0C. Supernatant was discarded and the cells were gently resuspended in 225µl sterile glycerol (100%) and 1275µl of 100mM $CaCl_2$. The cells were then aliquoted

into prechilled microcentrifuge tubes (MCT) and immediately stored at -80^{0}C till further use.

3.5 Transformation of *Eschericia coli*

Transformation of *E. coli* competent cells was performed according to Hanahan *et al.* (1991). Stored competent *E. coli* cells were taken out and kept on ice for thawing. The ligation mixture or appropriate concentration of plasmid DNA was added into competent cells, mixed gently and incubated on ice for 30 min. The cells were incubated at 42^{0}C for 2 min in a dry bath and immediately transferred on ice for 5 min. One ml of LB media was added to competent cells and incubated at 37^{0}C for 1h with constant shaking at 220 rpm. Cells were centrifuged at 6000 rpm for 5 min at room temperature and entire supernatant was discarded but 100µl. The cells were gently resuspended in the remaining LB medium and spread on LB agar plate containing the appropriate antibiotics. The plates were incubated at 37^{0}C overnight until colony appeared.

3.6 Plasmid DNA purification

Isolation of plasmid DNA by alkaline lysis method (minipreparation) was performed according to (Birnboim and Dolly, 1979). Overnight grown culture of *E. coli* (1.5 ml) was centrifuged at 13500 rpm, for 5 min and resuspended the pellet in 100µL of alkaline lysis solution I (50mM glucose, 25mM Tris-Cl pH 8, 10mM EDTA pH 8) by vigorous vortexing to dissolve the pellet and incubated at room temperature for 5 min. Freshly prepared alkaline lysis solution II (0.2N NaOH, 1% SDS) 200µL was added to each tube and mixed gently by inverting the tubes. One hundred and fifty microliter of prechilled akaline lysis solution III (5M potassium acetate) was added, and tubes were inverted gently to mix the solution followed by incubation on ice for 10 min. Tubes were centrifuged at 13500 rpm, 4°C for 15 min and supernatant was taken in fresh MCT. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently by inverting the tubes. The tubes were centrifuged at 13,000 rpm for 10 min at room temperature. The upper aqueous layer containing DNA was collected in fresh tubes and

0.8 volume of cold isopropanol was added in each tube. The tubes were incubated at -20°C for 20 min followed by centrifugation at 13500 rpm, 4°C for 25 min. Supernatant was discarded and the pellet was washed with 0.5 ml of 70% ethanol by centrifugation at 13500 rpm, 4°C for 10 min. The pellet was dried completely to remove any traces of ethanol and resuspended into 30µL of sterile distilled water. The quality of plasmid was analyzed on agarose gel electrophoresis and the concentration was measured by spectrophotometer.

3.7 Polymerase Chain Reaction (PCR)

Polymerase chain reaction for amplification and conformation of DNA A and DNA β was carried out in 25 µl of reaction mixture containing 1X Taq buffer, 0.2mM dNTPs, 2mM $MgCl_2$, 100 nM of forward and reverse primer each and 1U of Taq polymerase (Biotool, Spain). For the confirmation of clones gene specific primers were used on Eppendorf gradient Cycler (Eppendorf, Germany).

Table 3.2: Composition of PCR reaction mixture.

	Components	Stock conc.	Amount used	Working conc.
1	Taq Buffer	10X	2.5 µl	1X
2	dNTP	10mM	0.5 µl	0.2mM
3	$MgCl_2$	25 mM	0.2 µl	2mM
4	Forward primer	10µM	0.25µl	100nM
5	Reverse primer	10µM	0.25 µl	100nM
6	Taq Polymerase	5u/µl	0.2 µl	1U
7	Template DNA	-	0.5 µl	50 ng
8	SDW	-	20.6 µl	-
	Total volume		25µl	

Table 3.3: Programme used for PCR amplification.

	Programme	Temperature	Duration	
Step 1	Initial denaturation	94°C	5 min	
Step 2	Denataturation	94°C	30 sec	30 cycles
	Annealing	62°C	30 sec	
	Extension	72°C	1 min	
Step 3	Final extension	72°C	10 min	

3.8 Preparation of competent *Agrobacterium* cells

Agrobacterium tumefaciens strain EHA105 was used to prepare competent *Agrobacterium* cells. A single colony of *Agrobacterium* from fresh LB agar plate was inoculated into 3 ml of LB media [LB medium+1% glucose+ rifampicin (30 µg/ml)] and grown overnight at 28°C with vigorous shaking at 220rpm. Next day, 2.0 ml of this culture was used to inoculate 50 ml LB medium [LB medium+1% glucose+ rifampicin (30 mg/l)]. The culture was incubated at 28°C with vigorous shaking at 220rpm till the optical density (OD) at 600nm reaches 0.5. The culture was taken out and chilled for 5 min on ice and cells were harvested by centrifugation at 4°C, at 7000 rpm for 5 min. The pellet was resuspended gently in 10ml of ice cold 0.15M NaCl and incubated on ice for 15-20 min. The cells were again centrifuged at 7000rpm for 5 min at 4°C. The pellet was resuspended in 1.0 ml of ice cold 20mM $CaCl_2$ and 100µl of competent cells were aliquoted into prechilled microcentrifuge tubes. These tubes were frozen in liquid N_2 for 5 min and immediately stored at -80°C.

3.9 Transformation of *Agrobacterium tumefaciens*

A. tumefaciens competent cells were transformed with the plasmid construct according to Hofgen and Willmitzer (1978). *Agrobacterium* cells were taken out and thawed on ice, 1µg of plasmid DNA construct was added into competent cells and incubated on ice for 30 min. After 30 min, the cells were frozen into liquid N_2 for 2 min and thawed in 37°C water bath for 2-3 min and immediately transferred on ice for 5 min. One ml of LB medium (LB media + 1% glucose+ 30 mg/l rifampicin) was added to the cells and cells

were incubated for 4-5 h at 28°C at 220 rpm. After incubation cells were harvested by centrifugation at 7000 rpm for 5 min, excess of media was removed and the pellet was resuspended in remaining 100µl media. Subsequently, cells were spread on selection media (LB agar, 1% glucose, 30mg/l rifampicin and 50 mg/l kanamycin) and incubated at 28°C for 36-48 h.

3.10 Plant inoculation

Agrobacterium tumefaciens EHA105 was transformed with infectious clone of ChiLCV by freez and thaw method. *Agrobacterium* colony containing tetramer was confirmed with PCR and restriction digestion. Confirmed single colony was selected and culture was grown in LB (pH 7.0) containing rifampicin (30 µg/ml) and kanamycin (50 µg/ml) for 36 h at 28°C. Culture was centrifuged at 5000 rpm for 10 min and pellet was dissolved in sterilized distilled water. *Capsicum annuum* and *Nicotiana benthamiana* plants were pierced at four leaf stage around petiole and nodal region (Chattopadhyay *et al.,* 2008). *Agrobacterium* suspension at a desired concentration (OD600 = 0.8) were applied at wounds region and inoculated plants were kept in glass house under controlled conditions of 16 h light and 8 h dark period at 25°C $\pm$ 3°C.

3.11 Isolation of total genomic DNA from plant

Total genomic DNA from leaf tissue of either transgenic or non-transgenic plants was isolated according to the method described essentially by Dellaporta et al (1983) with fewer modifications. For small level preparation, DNA isolation was carried out into 1.5 ml microcentrifuge tube with the help of micropestles, while for large scale DNA preparation isolation was carried out using mortar and pestles. Leaf samples were dipped in liquid nitrogen and were ground with the help of micropestles till it becomes a fine powdery mass, followed by addition of 750 µl of extraction buffer. The samples were homogenized thoroughly and then stored in ice until proceeded further. One hundred microliters of SDS (10%) was added to the tube and mixed properly by inverting the tube. Samples were incubated at 65°C for 20 min with regular mixing at every 3 min

interval and then allowed to cool at room temperature (25±1^{0}C). Then, 250 µl potassium acetate 5M pH (5.8) was added to the tubes and mixed properly by inverting the tubes several times, followed by incubation on ice for 15 min. Tubes were centrifuged for 10 min at 13000 rpm at 4°C and supernatant was collected in fresh microcentrifuge tubes. Equal volume of phenol: chloroform: isoamyl alcohal (25:24:1) was added to the tubes and mixed well by inverting several time. Samples were again centrifuged at 13000 rpm for 10 min at room temperature and upper aqueous layer containing DNA was collected in fresh microcentrifuge tubes. Isopropanol (0.8 volume) was added to the tubes and samples were incubated at -20°C for 30 min to overnight. Samples were centrifuged at 13000 rpm for 30 min at 4°C to pelletize the DNA. Supernatant was decanted and the pellet was washed twice with 500 µl of 70% ethanol. Finally, the pellet was air dried and dissolved in 200 µl of autoclaved sterile distilled water. DNA was quantified using nanodrop spectrophotometer (ND1000, Thermo Scientific, USA). The quality and integrity of DNA preparations were analyzed on 0.8% agarose gel.

3.12 Southern hybridization

Southern hybridization was performed for viral DNA accumulation in virus infected plants following standard procedure (Southern, 1975). For southern blot analysis, 8.0 µg of total DNA, isolated from virus inoculated plants to detect viral DNA accumulation, was electrophoresed in agarose gel (0.8%). Gel was treated with 0.25M HCl for depurination for 10 min followed by denaturation solution (0.5M NaOH and 1.5M NaCl) for 45 min. Denatured gel was treated with neutralization solution containing 0.5M Tris-HCl and 1.5M NaCl twice for 30 min and 15 min, respectively. DNA was transferred onto the nylon membrane (MDI, India) using 10X SSC (3.0 M NaCl and 0.3M sodium citrate) by capillary method and cross linked onto positively charged nylon membrane following 1 min exposure to ultraviolet rays. Hybridization was carried out using radio labelled (αP^{32}-dCTP) specific probes in hybridization oven (Amersham, US) at 60°C. Blots were washed with washing solution (2.0X SSC and 0.1% SDS) for 15 min at room temperature and at 60°C for 5 min to remove excess of probes to avoid nonspecific

signals. The blots were kept in imaging plates for overnight and scanned using phosphoimager (Fujifilm, Japan). The signals were quantified by using the image analysis software (Quantity One, Bio-Rad).

3.13 Isolation of total RNA

Isolation of RNA from plant tissues was performed using trizol (Sigma, USA) and following manufacturer's protocol. For small level RNA preparation, tissue homogenization was carried out in 1.5 ml MCT with the help of micropestles and for large scale preparation homogenization was carried out in mortar and pestles. Young leaf samples were homogenized in liquid N_2 and homogenate were reuspended in TRI reagent (Sigma, USA) followed by addition of 200 µl of chloroform. Samples were incubated at room temperature for 10-15 min followed by centrifugation at 12000g at 4 °C for 15 min. The upper layer was transferred into fresh DEPC treated MCT and 0.8 volume of pre-cooled isopropanol was added and incubated on ice for 10 min. Samples were centrifuged at 12000g for 10 min at 4°C to precipitate RNA. The pellet was washed with 70% ethanol to remove salts by centrifugation at 10000g for 10 min at 4°C. The washing step was repeated three times. Finally, the pellet was air dried and dissolved in DEPC treated autoclaved water incubated by incubating the RNA sample at 65°C for 15 min.

3.14 Isolation of total mRNA

Total mRNA was isolated from total RNA using mRNA isolation kit (Roche, Basal) following manufacturer's protocol. Total RNA was diluted with 500 µl of lysis buffer followed by incubation at 65°C for 2 min. Three microliter biotin labeled oligo$(dT)_{20}$ was added to each sample. This mixture was then added in the MCT containing streptavidin coated magnetic particle (SMP) and incubated for 5 min at 37°C. The SMPs were separated from the liquid sample by magnetic particle separator. Five hundred microliter of wash buffer was added to resuspend the SMPs. Subsequently, the SMPs were separated from wash buffer using magnetic particle separator. The mRNA was isolated by resuspending the SMPs in distilled water.

3.15 Northern hybridization

Total RNA (10 µg) was isolated from infected and mock inoculated plants of chilli varieties (Punjab Lal and Kashi Anmol), followed by electrophoresis in 1.2% formaldehyde denaturing gel. The RNA was transferred onto nylon membrane (Hybond N+ Amersham, USA) by capillary method. The membrane was carefully removed and cross linked using UVP cross linker (Amersham, USA). Histone H2B specific probe was used to detect transcripts of H2B in mock and infected plants. The probe was labelled with α-P^{32} labelled dCTP using random hexamers, dNTPs-dCTP, Klenow fragments and Klenow buffers (Sambrook and Russell, 2001). Hybridization was carried out at 55°C overnight in a hybridization chamber (Amersham, USA). The excess of probe was washed with wash buffer (2XSSC and 0.1% SDS) twice for 15 min each at room temperature. Image was developed on phosphorimager (Fujifilm, Japan).

3.16 Synthesis of cDNA

Total RNA was treated with DNase I (Thermo Scientific, USA) for 30 min at 37°C which was subsequently inactivated by adding 5mM EDTA and heating at 65°C.

Table 3.4: Reaction mixture used for cDNA synthesis.

	Components	Stock conc.	Volume used	Final conc.	Manufacturer
1	RT buffer	5X	4.0 µl	1X	with RT
2	dNTP	10mM	2.0 µl	1mM	Thermo Scientific
3	MgCl2	25mM	2.0 µl	2.5 mM	Thermo Scientific
4	RNase Out		0.5 µl		Thermo Scientific
5	Reverse transcriptase	200 u/ µl	1.0 µl	200 U	Thermo Scientific
6	Oligo$(dT)_{18}$	0.5 µg/ µl	1.0 µl	0.5 µl	Thermo Scientific
7	Total RNA	5 µg/ µl	5.0 µl	1.0 µg	-
8	SDW	-	4.0 µl	-	-
	Total Vol.		20.0 µl		

For the synthesis of cDNA, 1µg of DNaseI treated total RNA was used with oligo(dT)18 primer and MuMLV reverse transcriptase (MuMLV RT H^-, Thermo Scientific, USA). Firstly, oligo$(dT)_{18}$ primer and total RNA was heated at 72°C for 5 min followed by snap chilling and addition of remaining component of the reaction mixture. Reaction mixture was incubated at 42°C for 1h and RT was inactivated at 72°C.

3.17 Construction of SSH cDNA library

The subtracted cDNA library was constructed by subtractive hybridization using the PCR-Select Subtractive Hybridization Kit (Clontech, USA). One µg of total $polyA^+$ mRNA was used for the first strand cDNA synthesis using first strand synthesis primer and reverse transcriptase. Samples were incubated at 42°C for 1.5 h. Samples were snap chilled to terminate the reaction. For the second strand synthesis, reaction mixture was added in the sample and incubated for 2 h at 16 °C followed by addition of T4 DNA polymerase. Reaction was terminated by addition of EDTA and glycogen mix and ds cDNA was purified by phenol/chloroform method. This dsDNA was digested with *Rsa* I which was further used for the adaptor ligation (Adopter 1 and adopter 2R). At first, hybridization adaptor 1 and adaptor 2R were ligated to tester (from infected plant). Samples were mixed with excess of *Rsa* I digested deriver (mock) cDNA. This sample was incubated at 98°C for 2 min followed by incubation at 68°C for 8 h. Second hybridization was performed by adding freshly denatured *Rsa* I digested cDNA obtained from the first hybridization sample. Second hybridization reaction mixture was incubated at 68°C for overnight. To dilute the sample, 200µl of dilution buffer was added in second hybridization sample. Diluted sample was used for the first PCR cycle which suppresses the unwanted and nonspecific amplification. Second PCR was performed to enrich SSH ESTs which were subsequently cloned in pGMT easy vector (Fig. 3.1 & 3.2) (Promega, USA).

3.18 Agarose gel mobility and PCR confirmation of SSH ESTs clones

Approximately 1µg of the plasmid DNA was loaded into 8% agarose gel which was run at 60 V for 2 h. Subsequent confirmation of the clone was done by PCR using M13 forward and reverse primer in 25 µl of reaction mixture. PCR was performed in eppendorf gradient cycler (Eppendorf, Germany) using the following programme-initial denaturation 94°C for 5 min followed by 30 cycles of (denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extention at 72°C 1 min) and final extension at 72°C for 10 min. PCR amplification was confirmed by gel electrophoresis analysis.

3.19 Sequencing of SSH ESTs clones

The pGMT-easy clones were isolated and purified by PEG method. Plasmid concentration was estimated on nanodrop (ND1000, Thermo scientific, USA) and also by comparative analysis of gel electrophoresis using 1µg of λ DNA as control. Approximately, 20-30 ng of purified DNA was sent for sequencing using commercially available sequencing facilities.

3.20 Data analysis and annotation of SSH ESTs

Vector sequences were removed from cDNA sequences of subtracted library using vecscreen programme on NCBI site. Analysis of cDNA sequence similarity to database sequences was conducted by comparison with non-redundant protein and nucleotide databases using BLASTX and BLASTN searches provided through the NCBI database. In addition, *Arabidopsis* database (TAIR) was also used for proper annotation with the already existing information. ESTs with significant database matches (E-value of $\leq$ 1e -5) were further classified into functional categories.

3.21 Reverse northern blotting

After checking of redundancy, 231 cDNA non-redundant clones were identified. These unique clones were further used for reverse northern blotting for further validation of

subtracted library. Subtracted cDNA library clones were amplified in a 96-well PCR plate using M13 forward and reverse primers in a 25 µl reaction following PCR programme - initial denaturation 94°C of 4 min, followed by 30 cycles of (denaturation at 94 °C for 30sec, annealing at 57°C for 30 sec and extension at 72°C for 1 min) and final extension at 72°C for 10 min. Both, the quality and quantity of the PCR products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. Purified 300 ng of PCR products were denatured by adding 0.6M sodium hydroxide and spotted on to Hybond N^+ membrane (Amersham Bioscience, UK) using dot blot apparatus (Bio Rad, USA). Tubulin was spotted as an internal control to treated samples. A PCR product of the neomycin II (NPTII) gene from the vector pCAMBIA2300 using primer sequences (5′-TTTTCTCCCAATCAGGCTTG-3 ′ and 5′-TCAGGCTCTTTCACTCCATC-3′) was also spotted as a phosphotransferase negative control to subtract the background noise. The membranes were neutralized with neutralization buffer (0.5 M Tris-Cl, pH 7.4, .5 M NaCl) for 3 min, washed with 2% standard saline citrate (SSC) and cross-linked using UV cross-linker. Total RNA (10 µg) was converted into radioactive cDNA probe using superscript III cDNA synthesis kit following manufacturer protocol (Invitrogen, USA). Hybridization was carried out at 60°C for 16 h. At first, washing was performed at room temperature for 15 min with 2X SSC & 0.2 %SDS followed by second washing with 1X SSC & 0.5 % SDS at 65 °C for 15 min.

3.22 Expression analysis by Self organization tree algorithm (SOTA)

The radioactive intensity of each spot was quantified as volume value using quantity one software (BIO RAD, USA). The local background value was subtracted resulting in the subtracted volume values which were normalized with internal control tubulin. Expression profiles of the ChiLCV infected and mock genes were analyzed by the hierarchical SOTA (Self-organizing tree algorithm) cluster on the log-transformed fold induction expression values across four time points by using Multi experiment viewer (MEV) software (The Institute for Genome Research; http: //rana.lbl.gov/EisenSoft ware.htm).

3.23 Primer designing

For primer designing, AC1 and βC1 sequences were available in the laboratory. For the host proteins, ESTs sequences generated from chilli var. Punjab Lal were considered for real time quantitative PCR (qRT-PCR). All primers were designed using primer express software (Applied Biosystem, USA) (Table 3.5).

Table 3.5: Primers used for qRT-PCR experiments.

No	OligoName	5'<-----Sequence----->3'	Length
1	CaThionin-FP	ACTAATGACCCTCAATGCAAAACA	24
2	CaThionin-RP	CACATCTATTCATGCATACGCACTT	25
3	CaNBS-FP	CATAACGGAGGAAAGCACGATAG	23
4	CaNBS-RP	GTTTTCACAGTCGCCTGCATT	21
5	CaLTP-FP	CGGCCGAGGTCAACTACGT	19
6	CaLTP-RP	GACGATCAGGAGTTGTGGTAGCT	23
7	CaATP/ADP–FP	TGGGCATTCACACATCTTCT	20
8	CaATP/ADP-RP	AATGACCGGATTTCCAGTTT	21
9	CaH4-FP	GGGTGGTGTGAAGCGTATCTC	21
10	CaH4-RP	AGGGCATAAACAACATCCATAGC	23
11	CaH2B-FP	AAAAAACCGGCAGCTGAAAA	20
12	CaH2B-RP	AGTCTCTACCGCCTTCTTCGATCT	24
17	CaTopoII-FP	TCCTTCCTTGGGCAGTCTAGAG	22
18	CaTopoII-RP	CACTGATAACATATGTCGCCTTCTTC	26
21	CanATMak3-FP	CATGGTGATGATGGAATCTGGTT	23
22	CanATMak3-RP	TCAGCCGGAAAGCATCTACA	20
23	CaPPO-FP	ACGTGGACAAGACTGTGAATGC	22
24	CaPPO-RP	CGATGTCCTCCAGCAGTTCAG	21
27	CaRibisCO-FP	TCCTCAGGAAGACATTGTCAAGATT	25
28	CaRibisCO-RP	TCAGTGCACCGAAAAAGTCAA	21
29	CaAsPer-FP	TCTCCTCTCCGACCCTGCTT	20
30	CaAsPer-RP	CAGTCCCAACGCTTCCAATC	20
31	CaRiboL37a-FP	CTGTGAGTTCTGCGGCAAGTA	21
32	CaRiboL37a-RP	AATACAGCTCTCTAAATACGAAACCA	26
33	CaUbiq-FP	GCCACTCAAAAGATAGCACTCATA	24
34	CaUbiq-RP	TGTTTTCATTCCTAAGTGCAGTG	25
35	caRiboL19-FP	GACGAATTGTAAGGTGGGTTGAC	23
36	caRiboL19-RP	AAAGGGAAGGGATAAAACATTCTAAAC	27

37	CaSKP1-FP	AGGGTTTGCTATTTTTCTCTCTCTACA	27
38	CaSKP1-RP	CGCAATCATCCTCGATCATG	20
45	CaRan-FP	TGCACAAGCACAGCATGAAA	20
46	CaRan-RP	GAAACTGATCGGCACTGAGCTA	22
49	CaHypo-FP	CCACATAAAATCAACAAACAAACCA	25
50	CaHypo-RP	TGCTCACAATTCTCCTGTTTTATCA	25
51	CaUKI-FP	TGGGCAGTAGAATTCACCGATA	22
52	CaUKI-RP	TGCCACTTCCCACGCTTT	18
53	CaUKII-FP	ATTCGCCGGAGGCTAAGC	18
54	CaUKII-RP	CGGCGGACATTCGTTCAG	18
57	CaUKIII-FP	CCGGGCAGGTGTCAAAGTT	19
58	CaUKIII-RP	TTACACAATATGCTACAGCCAAACC	25
59	CaSP1-FP	GCCAAAATCAACCACGACAA	20
60	CaSp1-RP	TCTCGAAGCGGTGCTCTGA	19
61	CaTubilin-FP	ACAGGTGAAGGAATGGACGAA	21
62	CaTubulin-RP	CTCGTAATATTCTCCCTCCTCATCA	25
63	CaActin-FP	ATCCCTCCACCTCTTCACTCTCT	23
64	CaActin-RP	CCAGCAAATCCAGCCTTAACC	21

3.24 Quantitative real time PCR (qRT-PCR)

The qRT-PCR was carried out using gene specific primers .The PCR reactions were carried out in 96 well plates in 20µl of reaction using SYBER green mix (Applied Biosystem, USA) under following PCR conditions- Initial denaturation at 94 °C for 10 min followed by 40 cycles of (denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extention at 72°C for 30 sec) and final extension at 72°C for 10 min. The experiments were repeated at least three times independently, and the data were averaged. The ct value of the genes was normalized with the ct value of the actin (considered as internal control) and for comparison of expression analysis ΔΔCT value was used to draw graph using PRIZM software.

3.25 Cloning of histone H2B gene from *Capsicum annuum* var. Punjab Lal and *N. benthamiana*

For amplification of H2B, primer was designed from *Capsicum annuum* var. Punjab Lal sequence generated as a part of this study. cDNA from Punjab Lal and *N. benthamiana* was used as template for amplification with *Pfu* fusion (New England Biolab, UK) enzyme. Blunt end product of the PCR was electrophoresed on 1% agarose gel. Amplicon corresponding to a band of 480 bp was excised from the gel, DNA was isolated and ligated in blunt end cloning vector pJET1.2 (K1231, Thermo Scientific, USA).

Table 3.6: PCR composition used for H2B amplification.

	Components	Stock conc.	Volume used	Final conc.	Manufacturer
1	Pfu buffer	5X	4.0 µl	1X	NEB
2	dNTP	10mM	0.5 µl	1mM	Fermentas
4	Forward Primer	10 µM	0.5 µl	250 nM	SIGMA
5	Reverse Primer	10µM	0.5 µl	250 nM	SIGMA
6	Pfu polymerase	0.5 µg/ µl	0.2 µl	0.5 µl	NEB
7	cDNA	-	2.0 µl	-	-
8	SDW	-	12.3µl	-	-
	Total volume		20.0 µl		

Table 3.7: PCR programme used for H2B amplification.

	Programme	Temperature	Duration	
Step 1	Initial denaturation	98 °C	1 min	
Step 2	Denaturation	98 °C	20 sec	30 cycles
	Annealing	57 °C	30 sec	
	Extension	72 °C	30 sec	
Step 3	Final Extension	72 °C	10 min	

Clone was confirmed by PCR and restriction digestion. For the amplification and confirmation of H2B gene following reaction component and programme was used.

3.26 Generation of protein interactome database

Protein interactome network was developed on STRING (www.string-db.org) using *Arabidopsis* database information

Table 3.8: Primers used for cloning of H2B and ChiLCV ORFs.

ORFs	5'<-----Sequence----->3'	Length
H2B FP	GAATTCATGGCACCAAAGGCAGCAGC	20
H2B RP	GGATCCCTAAGAGCTAGTAAACTTGGTAACTGC	27
ChiLCV-AC1 FP	GGATCCTAATGCCTAGGGCTGGGAGA	26
ChiLCV-AC1 RP	GAGCTCTCAACGCGTCGACGCCTGGTCC	28
ChiLCV-AC2 FP	GGATCCTAATGCGACCTTCATCACCCT	27
ChiLCV-AC2-RP	GAGCTCCTAAATACCCTTAAGAAATGACC	29
ChiLCV-AC3-FP	CATATGGATTCACGCACAGGG	21
ChiLCV-AC3-RP	GGCTCGAGTTAATAAATATTATATTTTATTG	31
ChiLCV-AC4 FP	GGATCCTAATGAGAATAGGGAACCTCATC	29
ChiLCV-AC4 RP	GAGCTCTTAAAATATTGAGGGCCGAAGAC	29
ChiLCV-AV1 FP	CATATGTCGAAGCGACCAGCAG	22
ChiLCV-AV1 RP	CTCGAGTCAGTTGGTTACAGAGTCGTAG	28
ChiLCV-AV2 FP	CATATGTGGGATCCATTAGTAAACGAG	27
ChiLCV-AV2 RP	GAGCTCTCACATCCCCTCGGCACATCTG	28
ChiLCVβC1-FP	GGATCCATGTTTCACCTCACAAAAT	25
ChiLCVβC1-RP	GGGTCGACTCATATATGTGCATTTATACC	29

3.27 DNA elution from agarose gel

For extraction of DNA from agarose gel, standard gel elution procedure was followed as essentially described in Gel extraction kit (Spin Jet, Fermentas, USA). Equal volume of dissolving buffer (weight of gel slice) was added in the gel slice and incubated at 55°C for 10 min with mixing in between. One fourth volume of isopropenol was added in the melted gel and kept on ice for 10 min. Sample was passed through column and spun at 13000 rpm for 1 min. Wash buffer (400 µl) was added in the center of the column and

spin at 13000 rpm for 1min. Column was air dried for 2 minute to evaporate the wash buffer completely. DNA was eluted by adding 25 µl of sterile distilled water.

3.28 Multiple Alignment and phylogenic tree construction of H2B

H2B sequences of several plants species was selected from gene bank (NCBI) in FASTA format. Multiple alignments were performed on ClustalW (http://www.ebi.ac.uk). Phylogenetic tree was also constructed on the same site using same sequences to understand their genetic relatedness.

3.29 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed following Saleh, *et al.* (2008) with some modifications. Four gram of leaves were crossed linked in cross linked buffer containing 0.4 M sucrose, 10 mM Tris–HCl (pH 8), 1 mM PMSF, 1 mM EDTA and 1% formaldehyde by applying vaccum for 10-15 min. Cross linking was stopped by addition of 2.5 ml of 2 M Glycine. After removing the water, leaves were homogenized in nuclei isolation buffer (0.25 M sucrose, 15 mM PIPES pH 6.8, 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 0.9% TritonX-100, 1 mM PMSF and 1:100 dilution of plant protease inhibitor. Homogenate was filtered through 4 layers of miracloth and centrifuged at 12000g for 20 min. White pellet of nuclei was washed two times with nuclei isolation buffer. Finally, white nuclei pellet was dissolved in 2 ml of nuclei lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.1% sodium deoxycholate and 1% Triton X-100), followed by centrifugation at 13000g for 20 min. Supernatant was sonicated five times at power 6 for 15 sec on and 1 min off. The sonicated sample was centrifuged at 13000g for 10 min at 4°C. Two hundred microliter of sonicated sample was taken and diluted five times. Before proceeding for the precipitation, the sonicated samples were precleared with 50µl of salmon sperm DNA/protein A agarose beads for 1h at 4°C. The sample was centrifuged at 3500g for 2-4 min at 4°C. Supernatant was taken and appropriate antibody was added and incubated overnight at 4°C with gentle rotation. Seventy five microliter of salmon sperm DNA/protein A agarose beads added in the

samples and incubated at 4°C with gentle rotation for 2h. The samples were centrifuged at 3500g for 2-4 minute at 4°C. The pellets were washed with following buffer in same sequence - low salt (150 mM NaCl, 20 mM Tris–HCl pH 8, 0.2%SDS, 0.5% Triton X-100 and 2 mM EDTA), high salt (500 mM NaCl, 20 mM Tris–HCl pH 8, 0.2%SDS, 0.5% Triton X-100 and 2 mM EDTA), LiCl (0.25 M LiCl, 1% sodium deoxycholate, 10 mM Tris–HCl pH 8, 1% NP-40 and 1 mM EDTA) and TE buffer (1 mM EDTA and 10 mM Tris–HCl pH 8.). For elution, 250ml of freshly prepared elution buffer was added in the sample and incubated for 15 min at room temperature. The sample was centrifuged at 3500g for 2-4 min at 4°C followed by addition of 20µl of 5M NaCl in the supernatant and incubated at 65°C for 4h to reverse the cross linking. For removal of histone proteins, following components [sample 10ml of 0.5 M EDTA, 20 µl of 1 M Tris–HCl pH 6.5, 1 µl proteinase K (20 $mgml^{-1}$)] were added and incubated for 2h at 45°C. Finally, chromatin was purified by phenol-chloroform method and dissolved in 50 µl of TE buffer.

3.30 Chromatin immunoprecipitation and polymerase chain reaction (ChIP-PCR)

PCR was carried out with chromatin as template using ChiLCV A promoter specific primer. To check the post translational modification of ORF region, AC2/AC3 specific primers were used. Following PCR reagents and programme are used-

Table 3.9: PCR composition used for ChIP assay.

	Components	Stock conc.	Amount used	Working conc.
1	Taq Buffer	10X	2.5 µl	1X
2	dNTP	10mM	0.5 µl	0.2mM
3	$MgCl_2$	25 mM	0.2 µl	2mM
4	Forward primer	10µM	0.25µl	100nM
5	Reverse primer	10µM	0.25 µl	100nM
6	Taq Polymerase	5u/µl	0.2 µl	1U
7	Template	-	2 .0µl	-
8	SDW	-	19.1 µl	-
	Total volume		25 µl	

Table 3.10: PCR programme used for ChIP assay.

	Programme	Temperature	Duration	
Step 1	Initial denaturation	94°C	5 min	
Step 2	Denaturation	94°C	30 sec	30 cycles
	Annealing	60°C	30 sec	
	Extension	72°C	30 sec	
Step 3	Final Extension	72°C	10 min	

3.31 Total histone extraction

Total histone was isolated from chilli var. Punjab Lal and *N. benthamiana* plants following standard protocol with some essential modifications to suit the experiments. Nuclei were isolated in nuclei isolation buffer containing 20 mM Tris-HCl (pH 7.5), 10 mM KCl, 10 mM MgCl2, 6% sucrose, 0.6% Triton X-100, 0.05% β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1:100 dilution of protease inhibitor. Homogenate was filtered through 4 layer of miracloth. Sample was centrifuged at 2800 rpm for 20 min at 4°C. Nuclei pellet was resuspended in nuclei lysis buffer containing 10 mM Tris HCl pH 7.5, 2 mM EDTA, 0.4 M HCl, 0.05% β-mercaptoethanol, 1 mM PMSF, 0.6% Triton X-100 and 1:100 dilution of protease inhibitor. Sample was incubated on ice for 4h with gentle rotation followed by centrifugation at 12000g for 20 min at 4°C. Supernatant was taken and histone was precipitated with 32% tri-chloroacetic acid. Histone pellet was washed twice with acetone, air dried and stored at -80°C till further use.

3.32 Detection of plant histones by western blotting

Histone pellet was dissolved in urea buffer and 10 µg of total histone was loaded onto 15% SDS polyacrylamide gel. Total histone was transferred on PVDF membrane which was subsequently processed for western blotting. Membrane was blocked in 5% skimmed milk for 2 h followed by washing with PBST (1X PBS and 0.05% Tween20). Membrane was incubated in primary antibody (H2B with 1:5000 dilution and H3K4 with 1:5000

dilution) for 2-3h. After three washings, membrane was incubated with anti rabbit IgG secondary antibody labeled with horseradish peroxidase (1:10000 dilution). Band was detected by adding H_2O_2 and DAB (3,3′-diaminobenzidine). For western blotting, antibodies to H2B (AbH2B) and H3K4 (AbH3K4) were used (Abcam, USA).

3.33 Cloning of H2B ORF in yeast expression vector pGBKT7

CaH2B and NbH2B were excised from respective clones of pJET1.2 vector by digestion with *Eco* RI/ *Bam* HI. Similarly, pGBKT7 was digested with restriction enzymes, *Bam*HI and *Eco* RI. H2B fragment released from pJET1.2 was eluted and ligated at *bam* HI and *Eco* RI site in pGBKT7. Clones were confirmed by restriction digestion using *Sal* I and *Eco* RI enzymes. PCR confirmation of the clones were carried out using H2B specific primers and following the PCR programme used for H2B as mentioned in section 2.26.

3.34 Cloning of ChiLCV ORFs in yeast expression vector pGADT7

ChiLCV ORFs (AC1, AC2, AC3, AC4, AV1, AV2 and βC1) were PCR amplified using ORF specific primers and following standard PCR programme. PCR products were cloned in pJET1.2 vector and clones were confirmed by digestion with appropriate restriction enzymes (AC1, AC2, AC3 and AC4 at *Bam* HI/*Sac* I, AV1 at *Nde* I/*Xho* I, AV2 at *Nde* I/*SaI* I). Viral ORFs were released from pJET1.2 vector and cloned in pGADT7 between *Sal* I and *Bam* HI restriction sites. Viral ORFs in pGADT7 were confirmed by restriction digestion and PCR using viral specific primers and following the same programme used for viral ORF amplification.

3.35 Yeast two hybrid assay

A single colony of yeast strain *Saccharomyces cerevisae* HA109 was grown in 50 ml YPD media containing yeast extract (20gm/l), peptone (10gm/l), and 40ml/l of dextrose (50%), pH 5.8 and kept on rotation at 30°C till OD reached >2.0. Primary culture was added in 300 ml of YPD media and kept for 3 h till OD600 reached 0.5-0.8. Yeast cells were pelletized at 1500g for 5 min and washed with sterile distilled water. To prepare

competent cells, 1.5 ml of 1X Tris-lithium acetate buffer was added to resuspend the pellet. Approximately, 100µl of competent yeast cells were used for transformation. In tube containing competent cells, 5µg denatured herring sperm DNA, 200 ng of respective pGBKT7 and pGADT7 clone each were added. PEG solution containing 1X tris buffer, 1x lithium acetate and 50% PEG was also added in the tube. Samples were gently vortexed and kept on rotation at 200 rpm at 30°C for 30 min. DMSO is added in all samples and kept at 42°C for 15 min followed by 10 min snap chilled. Samples were centrifuged at 1000g for 5-8 sec. Pellet was dissolved in 100µl of 1X TE buffer. Plating was carried out on YPAD plates lacking leucine and tryptophan (2DO). Colonies were appeared after 4-5 days which were streaked on 3 DO plate (-leu-his-trp) 5mM 3AT (3-amino-1,2,4-triazole) and subsequently on 4 DO plate (-ade-leu-his-trp).

4.1 Development of highly efficient infectious clone of ChiLCV

In the present study, a highly efficient infectious clone of ChiLCV was generated to ensure the delivery of both viral genome (DNA A) and betasatellite (DNA β) in a particular cell. Previous report suggested that infectivity of ChiLCV was increased in the presence of betasatellite (Chattopadhyay *et al.,* 2008). DNA A is the major genomic component which encodes for proteins required for replication, transcription and movement of viral genome. However, during co-infection DNA β functions as pathogenicity determinant and is involved in suppression of host defense machinery.

Conventionally, *Agrobacterium* cells harbouring tandem repeat constructs of DNA A are mixed in equimolar concentration with cells containing tandem repeat constructs of DNA β and this resultant mixture is then used as inoculum. Nevertheless, this method does not necessarily ensure entry of both the types of DNA molecules in any particular cell. As a consequence, some cells may receive either only DNA A or DNA β which indicates that the probability of receiving both DNA in a particular cell is low. Hence, achieving 100% infectivity seems not possible through traditional strategy used for agroinoculation, especially for a crop like chilli. Initially, this target was set up in order to understand the molecular basis of resistance in chillies. Therefore, a strategy was developed to construct an infectious clone of ChiLCV which could contain tandem repeat constructs of both DNA A and DNA β in a binary vector to ensure entry of both viral components in any given cell (Fig. 4.1). Accordingly, the tandem repeat construct of ChiLCV DNA A and DNA β were generated in binary vector, pCAMBIA2300. Construct was confirmed by PCR using primers specific to either AC1 (for DNA A) or βC1 (for DNA β) and by appropriate restriction digestions (Fig. 4.2). Binary vector harboring infectious construct (known as tetramer) was mobilized into *Agrobacterium tumefaciens* EHA105 strain. *A. tumefaciens* carrying tetramer clone was used to infect model plant *N. benthamiana* for assessing the infectivity of this newly generated construct of ChiLCV.

4.2 Infectivity analysis of highly efficient clone of ChiLCV

Model plant *N. benthamiana* was inoculated with *Agrobacterium* containing tandem repeat construct of either ChiLCV DNA A alone or mixed inoculum of ChiLCV DNA A & DNA β or with the newly generated tetramer clone. *N. benthamiana* was selected for initial infectivity analysis owing to its susceptibility against plant viruses. Symptom development was observed and recorded regularly. Symptom severity was assessed using 0-5 scale (Chattopadhyay *et al.,* 2008). Plants infected with only ChiLCV DNA A did not exhibit symptom at early stage of infection i.e. till 14 dpi which later on manifested symptoms like mild leaf curling (Fig. 4.3B & G). Whereas only one out of five plants (20%) infected with partial tandem repeat of DNA A and DNA β had developed severe symptom (Fig. 4.3C). In this case, the earliest symptom was noticed at 10 dpi which later on attained maximum severity scale of 5 at 24 dpi. Surprisingly, all of the five plants infected with tetramer showed severe symptom and incubation period was reduced to 7 dpi (Fig. 4.3D & G). Tetramer inoculated plants exhibited severe symptom like leaf curling, reduction in leaf size and stunting at later stage of infection (21 dpi) (Fig. 4.3F).

4.3 Comparative assessment of viral DNA accumulation

For detection and comparative analysis of viral DNA accumulation among plants, the sensitivity and specificity of PCR using ChiLCV specific primer were utilized. Total DNA isolated from infected *N. benthamiana* plants were used for PCR analysis. Results indicated low level of viral DNA accumulation in *N. benthamiana* plants infected with DNA A alone (Fig. 4.4A & B). In the case of DNA A and DNA β infected plants, one out of five plants showed higher accumulation of viral DNA. Remaining plants indicated presence of viral DNA, albeit the virus titer was significantly low (Fig. 4.4A & B). Surprisingly, all of the tetramer inoculated plants demonstrated presence of high level of viral DNA accumulation (Fig. 4.4A & B). Actin was considered as internal control. PCR

result could be correlated with the visible phenotype of infected plants and viral DNA accumulation.

Southern hybridization was carried out to validate the results obtained from PCR experiments and to further evaluate the efficiency of tetramer. Total DNA from infected *N. benthamiana* plants were transferred onto nylon membrane and hybridized with ChiLCV specific probe following standard protocol. No detectable signal could be seen from the plants infected with ChiLCV DNA A alone (Fig 4.5 A & D). Notably, only one plant indicated presence of higher level of viral DNA accumulation, out of five DNA A and DNA β inoculated plants (Fig. 4.5 B & E). In concurrent with the PCR result, all tetramer infected plants showed considerably high level of viral DNA accumulation (Fig. 4.5C & F). Since, cent per cent infectivity could be achieved with only tetramer construct, therefore, this highly efficient infectious construct was selected for further experiments.

4.4 Detection of early and late gene of ChiLCV

Despite, the proof of enhanced infectivity and viral DNA accumulation was generated, it was imperative to check expression of early and late genes of ChiLCV for further study. AC1 encodes for Rep protein which is indispensible for replication, is expressed at early stage of infection and AV2 encoded movement protein is expressed during the later phase of viral pathogenesis. These two viral genes were selected for studying relative level of early and late gene expression. For this, total RNA was isolated from tetramer infected plants and transcript level was detected by RT-PCR (reverse transcriptase PCR). AC1 and AV2 specific primers were used for the detection of AC1 and AV2 transcripts, respectively. In all three tetramer infected plants, AC1 and AV2 transcripts could be detected successfully (Fig. 4.6A, B & C). Level of AC1 was found to be relatively higher than AV2 transcript at 21 dpi.

4.5 Screening of chilli varieties

ChiLCV is known to infect several varieties of chillies and has been reported from different parts of India. However, some of the chilli varieties are known to be field resistant against ChiLCV (Kumar *et al.,* 2006). These resistant varieties can act as potential source for identification of resistance factor and to develop antiviral strategies against ChiLCV. Highly efficient infectious construct of ChiLCV, tetramer was used to screen field resistant varieties like BS35, Kalyanpur Chanchal and Punjab Lal (Balbir and Daljit, 2009; Kumar *et al.,* 2006). Approximately, 15 plants of each variety were inoculated with tetramer and the experiment was repeated twice. Symptom development was observed regularly till 28 dpi. However, no significant symptom of ChiLCD was observed in any of the resistant variety tested here. Therefore, PCR was employed to detect viral DNA accumulation at different time interval (7, 14, 21, 28 dpi). Total DNA from inoculated plants was isolated and detection of the viral genomic components was carried out using either AC1 specific primer for DNA A or βC1 specific primer for DNA β. PCR results indicated accumulation of significant amount of DNA A in all resistant varieties at 7 dpi and 14 dpi which showed sharp decline from 21 dpi onwards (Fig. 4.7A & C). Similar pattern was observed when βC1specific primer was used for detection of the satellite molecule (Fig. 4.7 B & D). It is relevant to mention here that one of the resistant varieties, Punjab Lal has been widely used by the plant breeders for developing resistance against ChiLCV for decades and is also an obvious choice of the farmers (Balbir and Daljit, 2009). Therefore, for identification of resistance factor, chilli var. Punjab Lal was considered for further experiments.

4.6 Identification of differentially expressed genes in resistant chilli var. Punjab Lal

To indentify the resistant factor(s) in chilli var. Punjab Lal, sensitivity and specificity of suppression subtractive hybridization (SSH) and PCR were exploited. SSH is a very efficient and sensitive technique which has been used successfully to identify specific genes involved in biotic and abiotic stress. SSH technique has generated a lot of

information which has further helped in functional characterization and elucidation of molecular mechanism of the candidate genes (Long *et al*., 2006, Pimentel *et al*., 2010).

The principle behind this technique is logically simple. Following exposure to either biotic or abiotic stress, host either enhances expression of some genes or initiates expression of a set of new genes to cope up with the situation. Transcripts from stressed plant and mock plant were isolated and hybridized under stringent condition. Similar transcripts were subtracted during the hybridization process where as upregulated transcripts and newly expressed genes were sorted out and cloned for further study.

The same principle was followed to identify the candidate resistance factor(s) from resistant chilli variety. It was anticipated that after infection with tetramer, resistant plant activated defense responses and initiated downstream signaling processes to inhibit the virus from multiplication and establishment of infection. Defense mechanism is complex phenomenon and may be governed by single or multiple genes. However, studies carried out to understand defense mechanism have revealed that specific pathogen either induces the expression of a specific gene or an array of defense related genes. Due to lack of any previous information regarding the defense mechanism operating in chilli against ChiLCV, a global level transcript study in infected and mock plants were considered to be a prerequisite and generation of SSH library was the first step towards this expedition..Chilli var. Punjab Lal was agroinoculated with tetramer and kept under controlled environmental conditions. Since, previous experiments indicated drastic reduction of virus accumulation in Punjab Lal at 21 dpi (discussed under section 4.5), it was hypothesized that this reduction in viral DNA could probably be due to the activation of host defense signaling at that particular time point of infection. Therefore, SSH was carried out at 21 dpi between mock and tetramer inoculated chilli var. Punjab Lal plants.

Table 4.1: ChiLCV induced differentially upregulated selected ESTs identified in resistant chilli variety Punjab Lal

EST	Annotation	Clone No	Organism	E-value	Fold upregulation
	Defence Related Proteins				
SC9	Thionin like protein	A11P1	*C. annuum*	3.00E-17	5.22
SC141	NBS-LRR	C4P3	*P. trichocarpa*	3E-14	8.13
	Transport				
SC149	Lipid transfer protein precursor	E1P3	*N. glauca*	1.00E-12	2.27
SC162	ADP,ATP carrier protein, putative	G10P3	*R. communis*	1.00E-69	2.76
	DNA organization replication/Transcription				
SC15	Histone H4 mRNA	D1P2	*C. annuum*	0.0	6.63
SC165	DNA topoisomerase II mRNA	H6P3	*N. tabacum*	1.00E-46	2.14
SC28	Histone H2B protein	C11P1	*A. thaliana*	1.00E-22	7.71
	Stress Response				
SC82	Stress-related protein 1 (SRP1	B9P2	*C. annuum*	6.00E-47	1.19
SC83	Lipid transfer protein	B11P2	*N. attenuata*	3.00E-29	5.02
	Signalling				
SC70	n-acetyltransferase mak3	H10P1	*R. communis*	3.00E-29	1.73
	Metabolism				
SC114	Polyphenol oxidase	G3P2	*N. tabacum*	3.00E-24	7.44
SC138	Xyloglucan endotransglycosylase /hydrolase 16	C1P3	*L. esculentum*	1.00E-32	2.77
SC143	Ribulose bisphosphate carboxylase activase	C8P3 D4P3	*N. tabacum* *C. annuum*	7.00E-20	2.88
SC147	Cytosolic ascorbate peroxidase			4.00E-86	7.36
	Protein metabolism				
SC39	60S ribosomal protein L37a	D12P1	*C. chinense*	1.00E-24	5.45
SC123	Ubiquitin-conjugating protein	F9P4	*C. annuum*	6.00E-04	2.50
SC129	Ribosomal protein L19	A10P3	*C. annuum*	1.00E-114	9.79
SC151	Skp1	E4P3	*C. annuum*	1.00E-20	2.00
SC202	Cyclophilin	C5P5	*C annuum*	4.00E-50	4.36
	Signalling				
SC145	Putative Ran protein	C12P3	*C. chinense*	2.00E-10	3.13
	Membrane protein				
SC214	Atg1 (Arabidopsis Transmembrane Protein G1p-Related 1	E8P5	*A. thaliana*	1.00E-23	3.14
	Unknown				
SC127	Conserved hypothetical protein	A3P3	*R. communis*	3.00E-50	7.01
SC160	Predicted protein(UK-i)	G5P3	*P. trichocarpa*	100E-.37	4.81 3.73
SC164	Unnamed protein product(Uk-ii)	H3P3	*V. vinifera*	400E-05	2.52
SC223	Unknown(UK-iii)	F7P2	-	-	

Accordingly, total RNA from tetramer and mock plants was isolated at 21 dpi and was used for the isolation of mRNA. Recommended amount of mRNA was taken for SSH experiments following the manufacturer's instructions. Subsequently, SSH was performed and quality of cDNA was assessed by *Rsa* I digestion (Fig. 4.8A). Comparative analysis of subtracted and non-subtracted SSH products was also performed (Fig. 4.8B). PCR products generated following SSH, were cloned in the pGEMT-Easy vector. SSH ESTs clones were confirmed by gel mobility and PCR using M13 specific primers (Fig. 4.9A & B). Notably, significant difference in mobility of the clones could be seen which were further confirmed by amplification of variable size of PCR product.

4.7 Sequencing and annotation of differentially expressed ESTs

Approximately, 480 EST clones were screened and sequenced. From the raw data, vector sequences were removed using vecscreen software (www.ncbi.nlm.nih.gov/VecScreen). Remaining sequences were searched for homology using NCBI and TAIR database. We carried out nucleotide to nucleotide BLAST (BLASTN) and nucleotide 6-frame translation-protein BLAST (BLASTX) were carring out on both these sites. Observations were recorded regarding parameters like the accession no, e-value and predictive function of each EST. After homology search, the transcripts were categorized on the basis of their putative function and their percentage were represented through piChart (Appendix 1).

4.8 Identification and classification of ChiLCV responsive genes in resistant variety

Randomly selected 480 colonies were screened, sequenced and annotated on NCBI and *Arabidopsis* (TAIR) database. The blast results indicated that 17% of the ESTs shared homology with genes involved in chromatin organization, transcription and translation whereas 20% ESTs showed homology with the genes involved directly or indirectly in metabolism process. Apart from this, other transcripts also showed homology with genes involved in different cellular and molecular processes of host. These results accounted for defense response (3%), growth and development (7%), transport (7%), stress response (3%), membrane protein (7%), signaling (3%) and protein degradation (20%). Due to

lack of complete characterization of several plant genomes, approximately 13% of genes did not share homology with any known characterized genes available on either NCBI or TAIR database (Fig.4.10). This global trasncriptome analysis in infected resistant chilli plants provided the first clue about the probable candidate gene(s) conferring natural resistance against ChiLCV.

4.9 Validation of SSH result by reverse northern

SSH clones were amplified by PCR using M13 forward and reverse primers. Approximately, 300 ng of PCR products were denatured in 0.6 N NaOH and subsequently, spotted on positively charged nylon membrane. Total mRNA from mock and infected plants was used for the preparation of radiolabelled probe using αP^{32}-dCTP. Nylon membrane was hybridized with radiolabelled cDNA and developed on the phosphorimager. Tubulin was taken as internal control where as neomycin phosphotransferase II (NPTII) was considered as negative control. Each spot was quantified using the quantity one software (BioRad) and the values were normalized with actin. Reverse northern result indicated the variation in the intensity of spots between virus and mock inoculated samples (Fig. 4.11). Quantification values of reverse northern indicated many fold upregulation of several genes (Table 4.1). No signal was observed in NPTII spot which indicated the quality and efficiency of the experiment.

Reverse northern result indicated alteration in the gene expression of different functional category of genes. Basal defense related genes viz., thionin like protein showed 5.22 fold upregulation whereas resistant gene NBS-LRR indicated 8.13 fold enhanced expression in resistant variety following ChiLCV infection. Some of the proteins involved in transport processes also showed elevated expression level. Precursor of lipid transfer proteins and ATP/ADP carrier proteins exhibited 2.27 and 2.76 fold upregulation, respectively. ChiLCV also induced the expression of genes involved in DNA organization, replication and transcription. For example, histone H4 and histone H2B level was enhanced upto 6.6 and 7.7 fold, respectively. Topoisomerase, the enzyme

actively involved in DNA replication was 2.1 fold upregulated. ChiLCV infection also caused minor increase in the expression of stress related protein (~1.2 fold). However, 2.27 fold elevated expression of lipid transfer protein was observed. N-acetyltransferase involved in several signal transduction pathway expression of which was altered in virus infected plant. Interestingly, n-acetyltransferase mak3 expression was only noticeably altered by 1.73 fold. Metabolism related genes also indicated differential upregulation following ChiLCV infection in resistant ChiLCV var. Punjab Lal. Polyphenol oxidase and xyloglucan endotransglycosylase / hydrolase expression were increased by 7.44 and 2.7 fold, respectively in coordination with the elevated expression level of Ribulose bisphosphate carboxylase activase (2.8 fold) and cytosolic ascorbate peroxidase (7.3 fold). Protein metabolism plays crucial role during plant-virus interaction. This notion was supported by enhanced transcript accumulation of ubiquitin (2.50 fold), ribosomal protein L37 (5.45 fold), ribosomal protein L19 (9.79 fold), SKP1 (S-phase kinase-associated protein 1) (2.0 fold) and cylophilin (4.3 fold). During infection, geminivirus genome is transported to nucleus with the help of several host proteins involved in nucleocytoplasmic trafficking. Alteration of gene expression of these proteins is expected to occur during pathogenesis. Ran is one of the karyophilic proteins involved in nucleocytoplasmic transport of cargos. Interestingly, 3.13 fold enhanced expression of Ran was observed in infected resistant chilli plant. Atg1 (*Arabidopsis* transmembrane Protein G1p-Related 1) is a membrane protein which was sorted out in SSH and its expression level was increased by 3.14 fold as analyzed by reverse northern assay. Reverse northern results suggested upregulation of several genes which did not share homology with any of the genes available on NCBI database and TAIR database. Hypothetical protein, predicted protein (Uk-I), unnamed protein product (Uk-II)and Unknown (Uk-III) exhibited 7.01 fold, 4.81 fold, 3.73 fold, and 2.52 fold enhanced expression respectively.

4.10 Global expression analysis of ESTs by self-organizing tree algorithm (SOTA)

Intensity of spots in reverse northern was measured and used to develop SOTA. Based on the similar expression profile, SOTA classified the transcripts and generated a hierarchical self-organizing tree. SOTA is a system of hierarchical cluster analysis for global expression data obtained from SSH hybridization. It is based on the standard statistical algorithms to analyze and arrange the genes according to similarity in pattern of gene expression. The output is represented graphically which conveys the clustering (Fig. 4.12). Colour combination was used to denote the upregulated and downregulated genes where green colour represented the low expression level and red colour indicated high level of expression. Initially, 11 groups were created on the basis of similar expression pattern. Colour pattern of the ESTs in group no 2 showed green colour in mock where as red color in infected samples which indicated maximum alteration in gene expression. Further 11 centroid images were also created using experimental viewer software (Fig. 4.13). Mean values were indicated in grey line whereas altered expression values were represented by pink lines. In centroid image group no 2, ESTs depicted similar expression pattern like SOTA, which was higher (above mean line) in virus inoculated sample and lower (below mean line) in mock inoculated samples. Therefore, candidate genes predominantly from group 2 corresponding to centroid image were selected to test their relative level following viral infection in both susceptible and resistant chilli variety.

4.11 Validation of reverse northern by qRT-PCR

A total of 22 genes were selected for the validation of reverse northern data by qRT-PCR using gene specific primers. Although, SSH was carried out between mock and ChiLCV inoculated plant of *C. annuum* var. Punjab Lal, comparative analysis of gene expression pattern in susceptible and resistant chilli varieties was logical requirement of the study. At the same time, it was necessary to check the level of these transcripts in a susceptible variety (Kashi Anmol) following ChiLCV inoculation. Samples were collected at 7, 14,

21 and 28 dpi from resistant and susceptible chilli plants. cDNA was prepared from 1µg of total RNA which was further diluted in the ratio of 1:10. Diluted cDNA (2 µl) was used for the qRT-PCR analysis and actin was taken as internal control. It is relevant to mention here that qRT-PCR result also indicated altered gene expression in resistant and susceptible chilli plants.

To validate the upregulation of NBS-LRR in reverse northern following ChiLCV infection, qRT-PCR was carried out to analyze the expression pattern at different time pont. At 7 dpi, NBS-LRR transcript accumulation in resistant variety was half of the level accumulated in susceptible variety (Fig.4.14A). But at 14 dpi, NBS-LRR accumulation level was enhanced 1.4 fold in resistant variety. Interestingly, result indicated ~4.3 fold higher accumulation of NBS-LRR transcripts in Punjab Lal at 21 dpi (Fig.4.14A). Similar result was also observed at 28 dpi where resistant variety indicated presence of approximately 1.4 fold higher accumulation of NBS-LRR transcript (Fig. 4.14 A). NBS-LRR conserved domain of plant resistant genes are known to be involved in the resistance mechanism against many pathogens including bacteria and viruses. They specifically recognize the pathogen and induce downstream defense signaling which eventually prevent the multiplication and spread of the pathogen. In majority of the cases, R gene mediated defense response involves hypersensitive reaction to confine the pathogen at the place of infection. Till date, no specific R gene has been identified and characterized against geminiviruses but some recent research work have indicated involvement of specific R gene in the mechanism of tolerance and resistance against geminiviruses (Sahu *et al.,* 2010; Pal *et al.,* 2007).

Polyphenol oxidase (PPO) expression pattern was found to be decreased with time of infection from 7 dpi to 28 dpi in susceptible chilli plant. At early stage of infection, PPO level was low (i.e. 14 and 2.5 fold less at 7 and 14 dpi, respectively) in resistant plant. But at later stage of infection, PPO expression was increased in resistant chilli plants. For example, at 21 dpi PPO transcripts were accumulated 1.5 fold higher in resistant plant than susceptible plants (Fig. 4.14B). Polyphenol expressions have been studied in many

plant species and its expression and activity have been demonstrated against many pathogens including fungi and bacteria. Its major role is to oxidize superoxide (O_2^-) to phenol. However, there is no evidence indicating role of PPO in imparting resistance against geminiviruses. Several reports indicated that PPO is involved in non-specific basal defense mechanism. Therefore, altered expression of PPO transcript during ChiLCV infection may be a part of the defense responses activated following virus infection.

In the present study, altered Ran expression level was observed in both susceptible and resistant chilli plants. At early stage, ChiLCV infection caused elevated level of accumulation of Ran transcript in susceptible chilli variety. Resistant variety showed ~7.5 fold lower expression at 7 dpi and 1.5 fold lower expressions at 14 dpi (Fig. 4.14C). However, qRT-PCR result of Ran expression at 21 dpi supported both the SSH and reverse northern result. At 21 dpi, resistant chilli plant contained 1.8 fold enhanced expression of Ran (Fig. 4.14C). Ran is well characterized karyophilic protein and is known to be involved in nucleocytoplasmic trafficking. Since, geminiviruses replicate in the host nucleus of the infected cell, therefore, they might exploit the host nucleocytoplasmic trafficking mechanism for the transport of viral genome and proteins.

Topoisomerase II expression was constantly higher at 7, 14 and 21 dpi in susceptible chilli var. Kashi Anmol (Fig.4.14D). However, topoisomerase II expression was increased in resistant variety at 14 dpi (8 fold in comparison to mock) and 21 dpi (5 fold in comparison to mock) but relative transcript level remained lower than susceptible variety throughout the course of experiments (Fig. 4.14D). Topoisomerase II expression level can be correlated with the susceptibility nature of the *C. annuum* var. Kashi Anmol. Topoisomerase II is one of the indispensable components of the DNA replication which is involved in DNA unwinding. Topoisomerase II has DNA cleavage properties which indicate its role in DNA recombination. Since, geminiviruses are DNA viruses which replicate through double stranded replicative form may require topoisomerase II for the

successful replication and production of new viral genome; alternatively it might play some unknown role during geminivirus infection.

Following ChiLCV infection, LTP expression was altered in both resistant and susceptible chilli plants but observed at enhanced level in Kashi Anmol at 7 dpi (4.8 fold) and 14 dpi (1.7 fold) (Fig. 4.14E). Nevertheless, at 21 dpi, resistant plant showed elevated level (4 fold) of LTP than susceptible one. Significant difference of transcript level was not observed at 28 dpi (Fig. 4.14E). LTP protein is a pathogenesis related protein which is known to be involved in defense response against bacterial, fungal and viral pathogens. *Cauliflower mosaic virus* infection in *Arabidopsis* and turnip (*Brassica rapa*) induced the expression of LTP. Although, LTP mediated mechanism of resistance is currently unknown but its enhanced expression following various pathogens indicates that probably LTP non-specifically participate in providing basal defense response in plants. The present study (by qRT-PCR analysis) showed elevated expression level of LTP in *C. annuum* var. Punjab Lal at 21 dpi which was in concurrent with results obtained from both the SSH and reverse northern. Elevated level of LTP following ChiLCV infection at 21 dpi could be correlated with reduced level of virus genome at 21 dpi in resistant var. Punjab Lal.

ChiLCV infection induced altered expression of tubulin in chilli plants. During the early stage of infection, higher expression of tubulin in susceptible chilli var. Kashi Anmol was observed i.e. 7.8 fold at 7 dpi and 2.8 fold at 14 dpi (Fig. 4.14F). At 21 dpi, resistant variety expressed 4.6 fold more in comparison to susceptible var. Kashi Anmol but at 28 dpi tubulin expression was similar in both the varieties (Fig. 4.14F). Tubulin is one of the major parts of the plant cytoskeleton. There are many reports which suggest the role of tubulin in virus movement for the establishment of successful disease in infected tissue. Geminiviruses also require nucleocytoplasmic, intracellular as well as intercellular movement of the virus genome and proteins and they exploit the cytoskeleton system of the plant to fulfill this purpose. Probably, this is the reason why ChiLCV infection induces higher expression of tubulin in the susceptible variety.

Ubiquitin expression was also altered during ChiLCV infection. Initially, ubiquitin transcript accumulation was detected higher at 7 dpi (4 fold) and 14 dpi (3 fold) in susceptible resistant variety (Fig. 4.14G). Surprisingly, ~5 fold elevated level of ubiquitin transcript was noticed in resistant variety in comparison to susceptible variety at 21 dpi. However ubiquitin expression level was decreased thereafter in resistant variety (Fig. 4.14G). Ubiquitination is one of the regulatory mechanisms of plant development, signaling, defense against pathogens etc. Role of ubiquitin in defense against plant viruses have been demonstrated recently.

Following ChiLCV infection, H4 transcript accumulation was significantly increased in susceptible variety as compared to resistant variety, for example, 11 fold at 7 dpi, 1.5 fold at 14 dpi and 2 fold at 21 dpi (Fig. 4.1H). Lower expression of histone H4 in resistant var. Punjab Lal may be correlated with the unfavorable condition for virus multiplication. Successful virus infection needs formation of minichromosome with coordinate organization with host histone proteins. H4 is one of the key constituents of nucleosomal assembly and chromatin organization and is known to be involved in gene regulation. Most of the trasncriptionally active genes are acetylated at several positions of histone H4. Active transcription is also required for expression of viral genes during infection.

Analysis of thionin expression by qRT-PCR in chilli verities revealed 10 fold increase in thionin expression in susceptible var. Kashi Anmol at 7 dpi which was further reduced at 14 dpi (2.3 fold). Interestingly, at 21 dpi thionin expression was drastically increased (44 fold) in resistant chilli var. Punjab Lal. This elevated level of thionin was also steadily maintained at 28 dpi in resistant plants (Fig. 4.1I). Thionin is produced at basal level in plants but known to be accumulated in high concentration following pathogen infection and considered as one of the PR (pathogenesis related) proteins involved in maintaining innate plant defense.

Elevated level of H2B expression was earlier observed by reverse northern analysis. H2B is one of the core components of nucleosomal assembly and is involved in chromatin

organization and maintenance. However, several researchers have suggested that H2B is also involved in gene regulation via post translational modification of histones in plant and animals. Post translational modification of H2B influences the modification of other histones which in turn regulates gene expression. Higher and constant level of H2B expression in resistant plants was noticed whereas in susceptible plant lower level of H2B transcript level was measured (Fig. 4.14J). Interestingly, at 28 dpi drastic reduction of H2B level was observed in susceptible variety. Formation of minichromosome using host histone proteins is a prerequisite for successful transcription of DNA viruses. Impaired post transcriptional modification of histones might inhibit virus multiplication.

ATP/ADP transporter facilitates transport of ATP and is generally expressed more in stem and root than in mature leaves. However, there is no direct or indirect evidence of involvement of ATP/ADP transporter in geminivirus infection or during any other plant virus pathogenesis. In the present study, ATP/ADP transporter expression was altered and found to be upregulated in resistant chilli plant by reverse northern blotting. Similar result was also obtained by qRT-PCR which indicated higher transcript accumulation in resistant plants. At early stage of infection, there was not much difference of ATP/ADP transcript accumulation in mock and virus infected plants at 7 dpi; however, ATP/ADP expression was increased at 14 and 21 dpi. When compared, ATP/ADP transcript accumulation was found to be steadily higher in resistant var. Punjab Lal throughout the period under study (Fig. 4.14K).

SKP1 expression was altered during ChiLCV infection in both resistant and susceptible chilli variety. At 7 dpi, SKP1 transcript accumulation was observed to be ~3 fold higher in susceptible variety which became equal at 14 dpi like the resistant variety. Interestingly, at 21 dpi resistant plants showed 1.5 fold increase in SKP1 expression than the susceptible one followed by a steady decline at 28 dpi. SKP1 is one of the major constituents of the proteosomal system which is involved in development, signaling and defense system of the host. Role of SKP1 in geminivirus pathogenesis has also been reported (Fig. 4.14L). N-acetyltransferase (MAK3) is involved in flowering and

developmental processes in model plant, *A. thaliana*. Its function is well characterized in yeast where it acetylates N-terminal of several proteins. N-acetyltransferase acetylates N-terminal of *gag* coat protein of *Saccharomyces cerevisiae* L-A ds RNA virus of the yeast (Tercero et al., 1993). This acetylation is important for the assembly of virus particle. In ChiLCV infected resistant plants, increased level (7-21 dpi) of N-acetyltransferase was observed. In contrary, N-acetyltransferase expression declined steadily in in susceptible plants during the course of infection (Fig. 4.15M).

ChiLCV infection also caused alteration in expression level of cyclophilin in both the varieties. At 7 dpi, susceptible plant indicated ~4.5 fold increased expression of cyclophilin than resistant plants. But at later stage of infection (14, 21 and 28 dpi), cyclophilin expression was found to be at par in both resistant and susceptible chilli varieties (Fig. 4.15N). Thus, qRT-PCR data indicated similar expression pattern and equal accumulation of cyclophilin transcripts in both type of plants. Cyclophilin is prolyl isomerase which catalyzes cis–trans isomerization of the peptidyl–prolyl bonds in protein that determines the structure, function and localization of the candidate proteins. This result implicated probable involvement of cyclophilin for proper folding and organization of host and virus proteins.

ChiLCV infection also induced expression of ascorbate peroxidase (APX) in resistant chilli variety, upregulation of which was also confirmed by both SSH and reverses northern analysis. Further analysis of APX expression by qRT-PCR indicated reduced level of APX at 7 dpi (5 fold) and 14 dpi (4.8 fold). Nonetheless, elevated level of APX was observed at 21 dpi (2.2 fold) in resistant var. Punjab Lal (Fig. 4.15O). APX, a detoxifying enzyme is involved in the conversion of H_2O_2 into H_2O during plant defense response. Upregulation of APX has previously been reported against other plant viruses and bacteria infecting chilies.

The qRT-PCR result indicated higher level expression of RuBisCO in susceptible chilli var. Kashi Anmol throughout the course of experiment. Concomitantly, reduced level of

RuBisCO expression was observed in resistant plants (Fig. 4.15P). RuBisCO is the most abundant and key enzyme involved in energy production of the plants. Recent information revealed interaction between potyvirus P3 protein and RuBisCO which could be correlated with virus pathogenesis and symptom development. Geminiviruses may also target this enzyme during pathogenesis in order to create compatible cellular environment.

Transcript level of ribosomal protein L19 was enhanced following ChiLCV infection. Nonetheless, susceptible chilli variety indicated enhanced level 5 fold (at 7 dpi) and 2.13 fold (at 14 dpi) over the resistant variety. At 21 dpi, ribosomal protein L19 expression did not show significant difference between these varieties. However, elevated level (7.62 fold) of ribosomal protein L19 was observed at 28 dpi in susceptible variety than resistant one. In resistant plant, ribosomal protein L19 expression was comparatively less but it increased from 7 dpi to 21 dpi, followed by decrease at 28 dpi (Fig.4.15Q). Similar result was observed on expression of ribosomal protein L37. Ribosomal protein L37 expression in susceptible var. Kashi Anmol was higher at 7 dpi (~2.5 fold) and 14 dpi (~5 fold) but remain equal at 21 dpi. At 28 dpi, ribosomal protein L37 expression was elevated ~2.5 fold in comparison to resistant var. Punjab Lal (Fig. 4.15R). Ribosomal proteins play key role during ribosomal assembly and translation (Gamalinda *et al*., 2013). But their expression is known to alter during virus infection in plant and animals. In animal system, HIV infection induces expression of ribosomal protein L37. Recent study also revealed expression of ribosomal proteins during *Potato virus A* infection. Interestingly, in this study the altered expression of ribosomal protein L19 and L37 was observed in susceptible plants.

Owing to lack of complete genome sequences of chilli, some of the cloned ESTs did not share any homology with the sequences available on NCBI and TAIR database. One of the ESTs shared similarity with unknown hypothetical protein and showed significant differential expression during geminivirus infection. ChiLCV infection causes upregulation of expression of this gene. Interestingly expression of hypothetical protein

was constantly higher in susceptible variety in comparison to resistant plant. Comparative expression analysis by qRT-PCR suggested role of this hypothetical proteins as susceptible factor as level of this transcript was very low in resistant variety (Fig. 4.15S). Expression of unknown I was maintained at higher level in susceptible variety till 21 dpi, followed by a decrease at 28 dpi (Fig. 4.15T). Notably, unknown II transcript accumulation was observed comparatively higher in resistant variety throughout (Fig. 4.15U). However, unknown-III expression did not indicate any significant difference at any stage of infection except at 21 dpi when elevated expression (>7 fold) was observed in resistant plant (Fig. 4.15V).

4.12 Detection of H2B transcript in resistant and susceptible plant infected with ChiLCV

To validate reverse northern and qRT-PCR results indicating higher expression of H2B in resistant variety, study was conducted to test H2B level by northern blotting technique. For this, total RNA was isolated from infected resistant and susceptible verities of chilli at 7, 14, 21 and 28 dpi. H2B specific probe was used to detect the transcript by northern blotting. Northern blotting result indicated low expression of H2B in susceptible plant but high level of expression was observed in the case of resistant variety. This result was in concurrent with information generated through reverse northern blotting and qRT-PCR, highlighting role of H2B in natural conferring resistance in chilli (Fig. 4.16).

4.13 Multiple sequence alignment and phylogenetic analysis of H2B in plants

Histone H2B sequences of various plant species were obtained from NCBI database and multiple sequence alignment was performed using ClustalW software available within EMBL server. H2B sequences analyzed were deduced from wide range of organisms including dicotyledonous plants (*Capsicum, Nicotiana* etc), mosses (*Selaginella*) and human. Length of the H2B was found to differ among the organisms for example, 126 aa in the case of human and 154 aa in the case of *Arabidopsis lyrata.* ClustalW results indicated conservation of C-terminus end of the H2B but N-terminus was found to be

variable (Fig.4.17). However, some of the amino acid like lysine at several locations (K4, K9, K10, K14, K15) is conserved in N-terminus of H2B among majority of the plant species. Non-conserved N- terminus sequence of H2B of various plant species suggests plausible use of different gene expression mechanism in diverse plant species. However, C-terminal part of the H2B is highly conserved in almost all plant species.

Phylogenetic tree of H2B was generated using NCBI which indicated close relationship of *Capsicum annuum* H2B (generated as a part of the study) with members of the *Solanaceae* family especially with *Solanum tubersum* and *Solanum lycopersicum*. Phylogenetic distance between *N. benthamiana* and *C. annumm* was considerably longer, indicating their genetic divergence (Fig. 4.18). H2B protein sequences from *C. annuum* var. Punjab Lal and *N. benthamiana* were used to perform homology modeling using Phyre2.0 software. Homology modeling result indicated the structural conservation of H2B at C-terminal region with some noticeable differences at N terminal (Fig. 4.19).

4.14 Generation of interactome network of H2B on STRING

Persistent elevated level of histone H2B expression suggested its role in resistant mechanism of the chilli plant. H2B is involved in the assembly of nucleosome and organization of chromatin. To explore the role of H2B in plant system and to find out its putative interacting partners, the *Arabidopsis* database was used to generate an interacting protein network of H2B using STRING site (www.string-db.org). Since less information is available about the chilli genome database, therefore, the information of model plant *Arabidopsis* was exploited to draw a tentative interactome network of H2B. STRING generated an interacting network of H2B which indicated other histones (histone H3 and histone H4) are major interacting partners. STRING information also suggested putative interaction between H2B and histone monoubiquitination protein 1 (HUB1) (Fig. 4.20). HUB1 is homologue of BRE1 (E3) protein which is involved in the monoubiquitination of histone. Therefore, studying correlation between histone post-translational modification and monoubiquitination machinery was important to elucidate the

mechanism of natural resistance in chilli against ChiLCV. Evolutionary tree on STRING indicated the conservation of histones in broad range of organisms, however, for HUB1 lack of sufficient genetic information in diverse plant species remained the main bottleneck of this study (Fig. 4.21).

HUB1 was also known to interact with ubiquitin conjugating enzyme (UBC2) to form complete monoubiquitination machinery which is involved in the monoubiquitination of H2B. Protein interactome network of UBC2 indicated its interaction with HUB1 and HUB2 which in turn was also known to interact with H2B. This flow of interaction (UBC2>HUB1>H2B) suggested that monoubiquitination of H2B may be directly or indirectly correlated with regulation of virus gene and may be the cause of reduced level of viral DNA accumulation at 21 dpi.

4.15 Post translational modification of histone

Histone proteins viz, H3 and H4 are the major histones involved in nucleosome assembly and chromatin organization. Apart from their structural role, they are also known to regulate gene expression. Post-translational modification at specific amino acids of H3 and H4 is responsible for either activation or repression of promoter of a gene. These modifications are influenced by many other cellular factors and processes. One of the factors is H2B, post-translational modification of which can also affect the modification of H3 (Weake and Workman, 2008; Ying Cao *et al.,* 2008). However, no information on H2B mediated modification of H4 is currently known. Therefore, the present study focused on understanding the functional relationship between post-translational modification of H2B and H3 and regulation of viral gene expression.

4.16 Post translational modification of histones and viral gene expression

Geminiviruses replicate in the host nucleus via double stranded DNA replicative form (RF). This ds RF also make minichromosome through recruitment of histone proteins. Depending on the virus genome size, nucleosome number may vary between 11-12

(Pilartz and Jeske, 2003). Minichromosome of virus utilizes the host machinery to regulate the viral gene expression via post translational modification of histone proteins.

Histone H3 modifications play crucial role during gene expression as well as hetrochromatin formation. Mono-, di and tri-methylation of H3K9 and H3K27 are associated with repression of gene expression. Interestingly, H3K4 trimethylation (H3K4me3) is known to be responsible for activation of gene expression in eukaryotes. Recent studies have suggested that H3K4me3 is influenced by modification of other histones. Studies using animal model have indicated that monoubiquitination of histone H2B at K120 in human and K123 in yeast promote H3K4me3. This information was used to study regulation of ChiLCV gene expression in resistant chilli var. Punjab Lal and highly susceptible model plant, *N. benthamiana.* Therefore, to analyze the post translational modification of minichromosome of ChiLCV during gene expression chromatin immunoprecipitation assay (ChIP) was carried out using specific antibodies.

4.17 Analysis of trimethylation at H3K4 in chilli var. Punjab Lal and *N. benthamiana*

It is presumed that gene expression strategy of ChiLCV depends on the host machinery via post transcriptional modification of histones. Trimethylation of H3K4 (H3K4me3) can be considered as the major modification during the gene expression of ChiLCV. For the analysis of H3K4me3, ChIP assay was conducted using specific antibody against H3K4me3. Chromatin immunoprecipitation was performed at 21 dpi from harvested plant samples following standard protocol. Recommended amount of antibody (5 µg) was used for precipitation. Precipitated chromatin was used as template for the detection of H3K4me3 occurring at virus promoter. For PCR, ChiLCV CR region specific primers were used (Fig.4.22E). ChIP-PCR result indicated presence of amplified viral genome in the positive control input (sonicated chromatin) confirming the presence of viral DNA (Fig. 4.22 A & B). ChIP-PCR from the mock chilli plant did not yield amplification whereas in infected resistant chilli plant, amplification of faint band could be noticed

although barely perceptible (Fig. 4.22 A & B). In model plant *N. benthamiana,* ChIP-PCR showed amplification in input control whereas no amplification was observed in mock sample, as expected (Fig. 4.22 C & D). In the case of infected *N. benthamiana* plant, high intensity amplification of viral CR could be detected (Fig. 4.22 C & D)). This clearly showed involvement of H3K4me3 at the promoter region of ChiLCV.

ChIP-PCR result indicated the H3K4me3 at the promoter of ChiLCV DNA A in *N. benthamiana* which was impaired in chilli (Fig. 4.22). Further, attempt was also made to analyze H3K4me3 at ORF region of ChiLCV i.e. at the AC2/AC3 overlapping region (Fig. 4.23 D). Notably, similar result was observed as in the case of ChIP analysis of viral promoter (Fig. 4.23 A & B). In input control, amplification was observed in both infected chilli and *N. benthamiana* but in H3K4me3 ChIP sample, the desired amplicon could be observed only in the case of infected *N. benthamiana* unlike infected chilli var. Punjab Lal plants (Fig. 4.23 A, B & C). As expected, mock inoculated plants failed to indicate ChiLCV presence. In all of the above cases, no-antibody control was selected where the samples were processed in the same way with the treated samples (Fig. 4.23 A, B & C). PCR of these samples did not indicate viral presence thus ruling out contamination / error during the experimentation. Internal control, actin always showed steady basal level of expression (Fig. 4.23 A, B and C). In this case, desired size of amplicon was used in input, mock and infected plant of chilli and *N. benthamiana*. These results further reiterated experimental quality and precision.

4.18 Analysis of monoubiquitination of H2B in chilli var. Punjab Lal and *N. benthamiana*

Recent studies have suggested involvement of monoubiquitination of H2B in the regulation of H3K4me3. Results obtained from ChIP-PCR using H3K4me3 antibody indicated low amplification of viral DNA in resistant chilli variety. Nonetheless, high level of viral DNA in *N. benthamiana* indicated indispensable role of H3K4me3 in viral gene expression in a permissive host. Impaired H3K4me3 in chilli could be due to either

lack of or inefficient monoubiquitination of H2B. To ascertain the codependency of H3K4me3 on monoubiquitination of H2B, ChIP assay was carried out using monoubiquitinated H2B antibody. This antibody was not generated from the plant peptide rather it was specific for the human H2B monoubiquitinated at K120 position. It is relevant to mention here that this antibody can cross react with monoubiquitinated H2B of plants (based on manufacturer's recommendations). In the first set of ChIP assay, presence of viral DNA could not be confirmed in both chilli and *N. benthamiana*. Therefore, needful modifications carried out o standerized the ChIP protocol including use of increased amount of antibody and sonicated chromatin for ChIP. For this study, the primer set specific for amplification of ChiLCV CR region was used to detect the viral genome (Fig. 4.22 E). In the case of chilli, ChIP-PCR result indicated amplification of virus in input control but not in the mock sample (Fig. 4.24 A & B). Notably, reduced level of virus amplification was obtained in infected chilli resistant plant (Fig.4.24 A & B). As expected, no amplification was observed when no-antibody control was used. In the present study, actin was used as internal control amplification of which was detected and confirmed in chilli and *N. benthamiana*. Together, these results indicated that monoubiquitination of H2B is common mechanism of gene regulation of the host which can also be exploited by virus.

4.19 Quantitative analysis of post translational modification of histones in chilli var. Punjab Lal and *N. benthamiana*

The Global level of H2B monoubiquitination in mock and infected chilli and *N. benthamiana* was also analyzed in this study. Till date, no standard protocol is available for isolation of total histone from any solanaceous plants including chilli and *N benthamiana.* Therefore, the present study was undertaken to optimize an efficient protocol for analysis of global histone level in these plant species. Total histone was extracted from chilli and *N. benthamiana* plant by acid extraction method. Equal amount of total histone was electrophoresed on 15% SDS-PAGE (Fig. 4.25A) which was subsequently transferred on PVDF membrane. Presence of histone was detected using

H2B specific antibody. Approximately 17-18 Kda size of protein could be detected, as expected. Surprisingly, additional prominent bands of approximately 42 Kda were also noticed. Presumably, these bands could be modified H2B which were monoubiquitinated at least at 3 places. Since the molecular size of the ubiquitin is ~8 Kda and the size of the band is ~42 kDa. It indicates that three ubiquitin (8x3=24 Kda) are attached with H2B (~18 kda) giving rise a band of molecular weight ~42 kDa (Fig.4.25B).

Western blotting assay was carried out from the mock and virus inoculated samples collected at different time points viz., 7, 14 and 21 dpi. Results indicated constant level of H2B proteins in mock and infected plants of chilli and *N. benthamiana.* However, modified H2B level was altered during virus infection. In the case of chilli, low level of modified H2B was observed in mock whereas enhanced level was observed in infected plants (Fig. 4.26, Row-2). In contrary to chilli, higher level of modified H2B was detected in *N. benthamiana* mock plants which showed steady decline with the progression of ChiLCV infection (Fig. 2.26; Row-2).

Since monoubiquitination of H2B promotes H3K4me3, therefore attempt was also made to detect global level of H3K4me3 in chilli and *N. benthamiana* plants. Western blotting results indicated high level of modified H3K4me3 protein in mock plant of chilli (Fig 4.26, Row-1). But in infected plants low level of H3K4me3 was detected at 7 dpi, level of which was further increased later on at 14 and 21 dpi (Fig. 4.26, Row-1). Contrary to the observations made in chilli var. Punjab Lal, results in *N. benthamiana* indicated high level of H3K4me3 modification in mock plants. However, infected *N. benthamiana* plants showed persistent decline during the course of study i.e. till 21 dpi (Fig. 4.26, Row-1). Level of H3K4me3 in infected chilli and *N. benthamiana* plants could be correlated with monoubiquitinated H2B level in these plants.

4.20 Interaction study of ChiLCV proteins and Histone H2B

In order to elucidate the mechanism of resistance in *C. annuum* var. Punjab Lal and to identify the interacting viral factor, yeast two hybrid system assay (Y2H) was carried out

to study the interaction between host factor (*C. annuum* var. Punjab Lal and *N. benthamiana*) and viral proteins**.**

Table 4.2: Interaction among ChiLCV ORFs with CaH2B and NbH2B.

	Viral ORFs	CaH2B		NbH2B	
		3 DO (-Ade-Trp-Leu)	4DO (-His-Ade-Trp-Leu)	3 DO (-Ade-Trp-Leu)	4 DO (-His-Ade-Trp-Leu)
1	AC1	+	+	-	-
2	AC2	-	-	-	-
3	AC3	-	-	-	-
4	AC4	-	-	-	-
5	AV1	-	-	-	-
6	AV2	-	-	+	+
7	βC1	-	-	-	-

(+) interaction, (-) No interaction; Ade-Adenin, Trp-tryptophan, His- Histidin and Leu- Leucine.

H2B from *C. annuum* var. Punjab Lal (CaH2B) and *N. benthamiana* (NbH2B) were cloned in yeast expression vector (pGBKT7) where as viral proteins (AC1, AC2, AC3, AC4, AV1, AV2 and βC1) were cloned in pGADT7 vector (Fig.4.27). Both the plasmids were cotrasnfected in *S. cerevisae* strain HA109 by PEG mediated transformation and placed on two drop out medium lacking tryptophan and leucine (-Trp, -Leu). Several colonies appeared after 3-4 days incubation at 30 °C. A few colonies were selected and streaked on 3 drop out plate lacking histidine, tryptophan and leucine (-His,-Leu,-Trp) supplemented with appropriate amount of 3AT(3-Amino-1,2,4-triazole). Colonies from the plates cotransfected with empty vector pGBKT7 and PGADT7, empty pGBKT7 & and pGADT7 containing virus ORF empty pGADT7 vector and pGBKT7 containing host factor were taken as negative. For positive control, p53 and SV40 large T-antigen (Tag) combination was considered. Interestingly, yeast growth could be observed both in positive and CaH2B with AC1 combination (Fig 4.28A). Neither any other combinations nor the negative controls showed growth on 3DO plate supplemented with 5 mM 3-AT. Subsequently, the colonies were placed on 4 DO plate lacking histidine, tryptophan, leucine and adenine (-His,-Leu,-Trp, -Ade). It was observed that ChiLCV AC1 could interact with CaH2B on 4 DO plates too (Fig 4.28B). However, no interaction between NbH2B and AC1was observed (Fig4.28C). Surprisingly, NbH2B could interact with

AV2 even under stringent conditions like 3DO supplemented with 5 mM 3-AT and 4DO medium (Fig.4.29 A&B). Contrary to this, CaH2B and AV2 could not interact (Fig. 4.29 C).

Chilli is the one of the most economically important vegetable and spice crops in India. It is also used for different purposes and has several medical applications including promising source for cancer treatment .Although chilli is cultivated in almost every states in India, its production is severely affected by chilli-infecting begomoviruses. Chilli-infecting begomoviruses are considered as the most devastating pathogens of chillies in India and cause severe crop loss every year. Among the chilli-infecting begomoviruses, *Chilli leaf curl Multan virus* (ChiLCV) is the most predominant begomovirus species and deserves attention for control. However, a few chilli varieties have been identified as field resistant to ChiLCV which can serve as potential sources of resistance. Therefore, it is important to identify resistance factor in order to develop efficient antiviral strategy against ChiLCV. ChiLCV is a member of the genera *Begomovirus* belonging to the family *Geminiviridae* which is characterized by geminate shaped virions (Fig. 2.3D). All geminiviruses have single stranded circular genome (2.5-3 kb) and are known to be either monopartite or bipartite. Monopartite viruses are associated with small satellite DNAs of ~1.3 kb size (α and β) (Mansoor *et al.,* 1999; Hanley-Bowdoin, 2000; Briddon *et al.,* 2003; Jeske, 2009).

ChiLCV is a monopartite geminivirus with single stranded small circular genome (DNA A) of 2750 bp and is associated with betasatellite (DNA β) of 1361 bp (Chattopdhyay, *et al.,* 2008). Like other geminiviruses, ChiLCV replicates in the host nucleus via double stranded circular replicative form (RF) which form minichromosome like structure by utilizing host proteins (Gutierrez, 2000a). Further, the minichromosome can be transcribed bidirectionally to produce viral proteins.

Previous studies have suggested that ChiLCV DNA A in association with DNA β induced severe symptom in *N. benthamiana* plant (Chattopadhyay *et al.,* 2008). In the present study, a highly efficient infectious clone of ChiLCV consisting of tandem repeats of DNA A and DNA β both in a binary vector, pCAMBIA2300. The resultant clone has been termed as tetramer. Comparative assessment of the infectivity of tetramer was analyzed in susceptible model plant *N. benthamiana*. Infection of *N. benthamiana* with

ChiLCV DNA A alone exhibited less pronounced and delayed symptoms. Whereas plants infected with DNA A and DNA β demonstrated severe symptom which appeared earlier than plant inoculated with DNA A alone. However, only 20% of the infected plants exhibited typical symptoms characteristic to leaf curl disease. This could be due to conventional method of mixing equimolar concentration of viral genomic components before agroinoculation. In this case, at cellular level, majority of the cells might receive either DNA A or DNA β and very few cells might encounter both DNA A and DNA β together. Contrary to it, all tetramer infected plants showed severe leaf curling symptoms characteristic to the disease. Tetramer inoculation ensured entry of both the genomic components of ChiLCV in all infected cells. Further, assessment of infectivity was carried out by comparative analysis of viral DNA accumulation by PCR and southern blotting technique. ChiLCV DNA A infected plants could not accumulate detectable amount of viral DNA. However, PCR and southern blotting experiments revealed higher accumulation of viral genome in ChiLCV DNA A and DNA β infected plants. Similar result was also observed in tetramer infected plants. Nonetheless, in the case of DNA A and DNA β, only one out of five plants showed significant amount of viral DNA accumulation whereas all tetramer infected plants exhibited higher viral titer. In the later case, due to presence of both molecules, ChiLCV pathogenicity was enhanced. Previous reports have revealed that DNA β is a pathogenicity determinant which helps in virus movement and thereby, influences pathogenicity (Chattopadhyay *et al.,* 2008; Briddon *et al.,* 2003). In an infected cell, DNA A provides all the necessary proteins required for replication, transcription and transport (Lazarowitz , 1992; Hanley-Bowdoin *et al.,* 2000). Eventually, DNA A in coordination with DNA β caused severe chilli leaf curl disease symptoms and tetramer assured cent per cent infectivity.

The early and late gene expression of ChiLCV DNA A was confirmed by RT PCR using either AC1 or AV1 specific primers. Results demonstrated successful expression of virus ORFs viz., AC1 and AV1 from all tetramer infected plants. In general, geminivirus AC1 ORF expression is driven by left promoter which is known to be activated at early stage of infection and AV2 ORF expression is regulated by right promoter which is activated at

later stage of infection (Shivaprasad *et al.,* 2005). This result has also identified 21 dpi as the optimum time for analysis and detection of viral DNA accumulation as well as transcripts.

This highly efficient infectious clone (tetramer) was further used for the screening of resistant chilli varieties. For this, field resistant chilli varieties (Kalyanpur Chanchal, Punjab Lal) and one collection, BS35 were selected (Rai *et al*., 2013). These varieties are promising sources for the resistance factor to develop antiviral strategy. None of the resistant plants could manifest symptom of ChiLCD nor could viral DNA be detected by southern blotting assay. Furthermore, sensitive method like PCR was used to detect the viral genome accumulation in resistant chilli plants. PCR results revealed initial presence of AC1 and βC1 at 7 dpi which maintained up to 14 dpi. But viral genome accumulation declined drastically in all resistant plants from 21 dpi onwards. The results obtained here indicated that at early stage of infection ChiLCV could dominate and exploit the host machinery for replication, transcription and movement. At initial stages, infected cells receive only viral genome and coat proteins (CP). CP carries the viral genome into the nucleus where it forms minichromosome to facilitate transcription of early genes to produce viral proteins involved in early steps of pathogenesis i.e. replication and transcription. At this stage, host may not be able to detect the viral genome and proteins, probably due to their low concentration which is below the detectable threshold level of host defense machinery. With the progress of the infection, late genes derived viral proteins are involved in encapsidation and movement between cells. Gradual accumulation of viral factors can cross the threshold limit and as a consequence viral presence is sensed by host defense machinery which eventually gets activated around 21 dpi, as observed here. Therefore, cumulative effect of host defense responses could inhibit ChiLCV replication and spread as reflected by reduced viral DNA accumulation at 21 dpi in all resistant varieties.

Capsicum with 12 chromosomes has very large (2700 Mb) and complex genome organization (www.solgenomics.net). Lack of complete *Capsicum* genome information

and absence of previously characterized resistance factor(s) against ChiLCV, compelled to perform the global level trasncriptome analysis in infected resistant var. Punjab Lal.

Till date, many resistant factors have been characterized against several plant viruses and mechanisms of resistance have also been elucidated. One of the well characterized mechanisms is N gene mediated dominant resistant mechanism against *Tobacco mosaic virus*. A defense response involving hypersensitive response is activated following recognition of replicase protein of TMV by N gene product (Whitham *et al*., 1996; Marathe *et al.,* 2002). Similarly, Rx1 and Rx2 resistant genes from potato are also known to confer resistance against *Potato virus X* (Bendahmane *et al*., 1999; Bendahmane *et al*., 2000). Rx protein recognizes coat protein of PVX and initiates downstream defense signaling including localized cell death (HR). Sw5 recognizes movement protein of *Tomato spotted wilt virus* and confers resistance in tomato plant (Spassova *et al*., 2001). HRT (HR to *Turnip crinkle virus*) recognizes coat protein of *Turnip crinkle virus* and initiates defense response in *Arabidopsis thaliana*. Dominant resistance has been reported from a wide variety of plant species against a range of plant viruses. These resistance factors have been well characterized and their mechanism of action have been elucidated. In the present study, hypersensitive reaction, the signature of dominant resistance mechanism against plant viruses could not be seen when chilli resistant varieties were inoculated with ChiLCV. Therefore, the present observation suggests the previous reports which indicated that resistance against ChiLCV is not controlled by dominant resistant gene in chilli.

Although resistance through dominant gene is common in many plant species, recessive resistant locus have also been characterized in some plant species. In majority of these cases, recessive resistance is governed by either due to non-availability of host factor(s) or presence of mutated form which ultimately impede viral multiplication. PVR^1 and PVR^2 loci in pepper plant is well characterized recessive resistant gene which confers resistance against *Potato virus Y* and *Tobacco etch virus* in pepper plants (Kyle and Palloix, 1997; Kang *et al*., 2005). Recent studies on eukaryotic initiation factor 4E

(eIF4E) indicated its role in conferring recessive resistance against *Barley yellow mosaic virus* (BYMV) and *Melon necrotic spot virus* (Ashby *et al*., 2011). Potyviruses contain viral genome-linked protein (VPg) at their 5′ end of RNA genome. This VPg mimics the structural configuration of 5′ cap of eukaryotic mRNA and interacts with host translational machinery to produce viral proteins. In the presence of mutant copy of this essential gene (eIF4E), VPg can not interact with host translational machinery and thereby, viral translation is hindered. Thus, mutant copy of eIF4E confers recessive resistance against potyviruses (Ruffel *et al*., 2002).

Development of chilli leaf curl disease is a complex phenomenon which involves compatible interactions between host and virus proteins at several stages of pathogenesis. Viruses have the ability to exploit and recruit host machinery for their sustenance in a permissive host. But plants are also evolved with several basal and specific defense mechanisms.

SSH is a very sensitive and specific technique and have been widely used successfully for exploration of candidate genes involved specifically in plant defense (Xiong *et al.,* 2001; Sahu *et al.,* 2010; Acharyya *et al.,* 2011), stress (Aguilar-Hernández *et al.,* 2011; Deokar *et al.,* 2011) or development (PMbeguie-A-Mbeguie *et al.,* 2007; Pimentel *et al.,* 2010). One of the earlier results of SSH has led to identification of 34 distinct immediate early (IE) defense-related genes in rice against blast fungus, *Pyricularia grisea* (Xiong *et al.,* 2001). This information facilitated the further research for the elucidation of resistance mechanism in rice against this pathogen. Recently, in a tolerant cultivar of tomato, 106 non-redundant ESTs were obtained by SSH against *Tomato leaf curl New Delhi virus* (Sahu *et al.,* 2010). Further analysis indicated >2.5 upregulation of 34 genes including NBS-LRR gene. SSH was also used for identification of exploration of resistant factor in *Hibiscus cannabinus* (Mesta) plants infected with *Mesta yellow vein mosaic virus* (Acharyya *et al.,* 2011). Reverse library analysis suggested significant down regulation of 7 genes including that of defense related suppressor of G2 allele of *skp1* (SGT1) which was considered as modulator of resistance against the virus (Acharyaa *et al.,* 2011).

In the present study, SSH principle was employed to identify and elucidate the mechanism of natural resistance in chilli var. Punjab Lal. ChiLCV infection resulted in altered expression of different functional groups of genes in chilli var. Punjab Lal. Altered gene expression was monitored by either elevated level of gene expression or expression of a new set of gene(s). Presumably, altered level of gene expression in Punjab Lal is correlated with the mechanism of natural resistance against ChiLCV. Among the differentially expressed genes, transcripts level of which were either up regulated or newly expressed following ChiLCV infection, might act as the probable candidate resistance factor. Considering the above hypothesis SSH was carried out between mock and ChiLCV infected Punjab Lal plants at 21 dpi. Transcripts with similar expression level from mock and infected plants were hybridized and omitted from the next step of SSH. Differentially expressed transcripts did not hybridize due to lack of hybridizing counterpart in mock plants. Therefore, these transcripts were sorted out and subsequently PCR amplified using adaptor specific primer and cloned for further study.

All SSH clones were sequenced and after confirming redundancy 231 unique ESTs were annotated using NCBI and TAIR database. Homology search results revealed presence of different category of ESTs. Functional categorization of all clones of SSH indicated their similarity with ESTs involved in signal transduction, chromatin organization, gene regulation, defense mechanism, protein metabolism, plant growth and development, ribosome assembly etc. SSH result suggested alteration of array of gene expression which is functionally involved in different physiological or cellular processes. This finding suggests that ChiLCV infection globally affects the several cellular processes which may cross talk among each other. In a living organism, one signaling pathway is related to other either directly or indirectly. Therefore, alteration of any pathway usually affects the others. For example, alteration of histone gene expression following ChiLCV might affect the expression of several genes, which are regulated by histone modifications.

Following pathogen attack, plants express some genes which in coordination with other host gene products confer resistant against pathogen. Validation of SSH result was

carried out by reverse northern assay. Result supported the SSH information and indicated alteration of gene expression in Punjab Lal plant following ChiLCV infection. Quantitative and comparative analysis of spots on mock and infected samples revealed upregulation of some specific set of genes.

Reverse northern involved use of radiolablled cDNA prepared from transcripts from mock and infected Punjab Lal plants. Higher the transcripts level, greater will be the corresponding radiolabelled cDNA and therefore, intensity of the spots will also be enhanced. Reverse northern analysis is a reliable technique and has been previously used to validate SSH results (Sahu *et al.,* 2010; Acharyya *et al.,* 2011). Comparative quantification of intensity of spots between mock and infected samples revealed differential expression of genes in ChiLCV infected plants. Many genes like NBS-LRR, LTP, histone H4, topoisomeraseII, thionin, ascorbate peroxidase, polyphenol oxidase and stress related proteins were upregulated several folds. Reverse northern result indicated probable role of these genes in conferring natural resistance. Expression analysis of these genes were further analyzed at 7, 14, 21 and 28 dpi by qRT-PCR in Punjab Lal and was also compared with gene expression level in susceptible chilli variety Kashi Anmol. Genes which showed significant upregulation in reverse northern were selected for further analysis.

Reverse northern results indicated upregulation of NBS-LRR genes in Punjab Lal at 21 dpi. NBS LRR is the conserved domain known in resistant proteins. R gene confers resistance against bacteria, viruses, and insects. Following pathogen infection, expression of resistant gene is induced which recognizes elicitor produced by the pathogen and initiate downstream defense signaling to block the pathogenesis (Whitham *et al*., 1996). Till date, several R genes have been isolated and characterized against bacteria and viruses. N gene of tobacco is a member of the Toll-interleukin-1 receptor like-nucleotide-binding site-leucine rich repeat (TIR-NBS-LRR) class of R genes which recognizes replicase protein of TMV and induces hypersensitive response to inhibit the virus replication (Whitham *et al.,* 1996). Several other genes containing conserved NBS-LRR

domain have been identified from *Arabidopsis* (Gassmann *et al.,* 1999; Takahashi *et al.,* 2002), tomato (Lauge *et al.,* 1998; Sahu *et al.,* 2010), pepper (Wan *et al.,* 2012) and potato (Bendahmane *et al.,* 1999). In one of these studies, a candidate resistant gene CYR1 was observed segregating with resistant population of *Vigna mungo*. *In silico* analysis of gene sequence of CYR1 revealed presence of conserved NBS-LRR domain and docking study suggested potential interaction between CRY1 and CP of MYMV (Maiti *et al.,* 2012). However, experimental evidences (*in vivo* and *in vitro*) to support this hypothesis are presently lacking. One of the recent reports indicated upregulation of NBS-LRR gene in tomato cultivar tolerant against ToLCNDV (Sahu *et al.,* 2010). The present observation is also in concurrence with these results. Following ChiLCV infection, expression of NBS-LRR gene was upregulated >5 fold as compared to susceptible plant. NBS-LRR mediated defense mechanism against geminiviruses is largely unknown and need further experimental support to elucidate the role of NBS-LRR in providing resistance against ChiLCV.

In the present study, significant difference in transcript accumulation of Ran between resistant and susceptible plants was not observed. Ran encodes for 24 kDa karyophilic protein with GTPase activity. It is one of the important cellular factors involved in nucleocytoplasmic transport of the cargo. Geminivirus genome and majority of the geminivirus proteins (AC1, AC2, AC3, AV1, and βC1) are known to be localized in the nucleus. Geminivirus encoded proteins (AV1/NSP) are also involved in bidirectional transport of viral genome (Stanley and Gay, 1983; Noueiry *et al.,* 1994; Fondong, 2013). Mechanism of nucleocytoplasmic trafficking of viral protein is not known. Recent studies on nucleocytoplasmic transport of geminiviruses revealed that NSP of *Cabbage leaf curl virus* (CaLCV) interact with NSP interacting GTPase (NIG) protein (Carvalho *et al.,* 2008). NSP is known to be localized in the nucleus in absence of MP, which relocalized NSP in cytoplasm in a cooperate manner. NIG also relocalized NSP in cytoplasm when NIG and NSP are coexpressed (Carvalho *et al.,* 2008). Previously, interaction between CP of MYMV & TYLCV and importin-α, another karyophilic protein indispensible for nucleocytoplasmic trafficking have also been demonstrated (Guerra-Peraza *et al.,* 2005).

All of these previous results and altered expression of Ran in resistant and susceptible plants indicated that geminiviruses may exploit conserved nucleocytoplasmic trafficking of host for their protein transport.

Polyphenol oxidase (PPO) is a tetrameric copper containing 52-64 Kda protein (Van-Gelder *et al.,* 1997) which also possesses two sites for binding of oxygen containing aromatic compound (Mayer, 2006). PPO catalyses the *o*-hydroxylation of phenols produced during oxidative burst following pathogen attack (Raj *et al.,* 2006). Hence, it functions as scavengers which protect the cell damage from reactive oxygen species and reactive phenolic molecules. Pathogen attack induces the activation of defense machinery which also involves oxidative burst to prevent the pathogen multiplication and prohibit its spread (Fobert *et al.,* 2005; Torres *et al.,* 2005). But higher accumulation of reactive oxygen species can also cause cell damage and may interfere in several physiological and cellular processes (Jacobson *et al.,* 1996; Dangl and Jones, 2001). Many reports indicated that PPO is involved in maintaining the basal defense against fungi, bacteria and viruses (Poiatti *et al.,* 2009; Mayer and Harel, 1979; Constabel *et al.,* 1995; Thipyapong *et al.,* 1995). Elevated level of PPO transcripts in resistant chilli var. Punjab Lal can be correlated with activation of basal defense following ChiLCV infection.

Ubiquitin is a conserved protein with 76 amino acids and attached to a specific substrate via sequential process involving three enzymes E1, E2 and E3 (Hershko and Ciechanover, 1998). Ubiquitin proteosome system (UPS) is a regulatory system in plant and animal which controls degradation of misfolded/damaged proteins or proteins carrying specific destruction signal. Role of UPS has been characterized in several physiological and cellular processes including cell cycle, programmed cell death, sucrose and hormone signaling, plant development and plant defense (Kim *et al*., 2006; Jung and Grune, 2008, Vierstra, 2009). Ubiquitin ligase expression is altered during biotic and abiotic stress (Cho *et al.,* 2006). Incompatible strain of powdery mildew (*Plasmopara halstedii*) infection induced higher accumulation of ubiquitin transcript level in sunflower hypocotyls but no alteration in ubiquitin transcripts was observed when same plants were

infected with compatible strain (Mazeyrat *et al.,* 1999). Similar result was also observed when *Capsicum annuum* var. Bugang was infected with TMV incompatible strain (TMV-P0) and compatible strain (TMP-P1.2). TMV-0 infection enhanced the expression of RPN7 (regulatory particle non-ATPases 7) which was correlated with programmed cell death (Lee *et al*., 2006). Several reports have indicated that plant virus movement proteins are the prime target for degradation by host 26 proteosome machineries as observed for TMV, *Turnip yellow mosaic virus*, TGBp3 (Triple gene block proteins 3) of *Potato leaf roll virus* (Drugeon & Jupin, 2002; Vogel *et al.,* 2007; Ju *et al.,* 2008). This host defense response is currently being recognized as common mechanism to regulate virus spread. Recent reports have suggested interaction between *Tomato yellow leaf curl Sardinia virus* Spain isolate (TYLCSV) C2 protein with CSN (COP9 signalosome) complex of UPS system in *Arabidopsis* which modulates hormone signaling pathways involved in plant defense (Lozano-Duran *et al.,* 2011). C2 interaction with CSN severely impairs the jasmonic acid pathway which contributes to geminivirus defense. ChiLCV infection results in quantitatively higher accumulation of ubiquitin in resistant plant which can be correlated with activation of UPS against viral infection. Reduced level of virus DNA may also be correlated with low level of viral proteins which are targeted by UPS. Nevertheless, mechanism of UPS mediated ChiLCV/geminivirus protein degradation is not known. Further experiments and study are needed to decipher this ubiquitous mechanism.

Upon pathogen infection, plants activate complex set of defense responses involving array of genes having diverse role (Ascencio-lbanez *et al.,* 2008). Resistance of plants to pathogen depends on its capacity for fine tuning of gene expression and coordinating other biological processes. Cumulative effect of defense responses involves the cross talk between several signaling pathways (Koornneef and Pieterse, 2008; Genoud and Métraux, 1999) including transcription, translation, defense, metabolism, development etc. In the present study, upregulation of ATP/ADP transporter in resistant chilli plants was initially observed by northern blotting and was further validated by qRT-PCR. Comparative expression of ATP/ADP transporter was constantly higher throughout the

infection in resistant chilli variety. ATP/ADP transporter catalyzes the highly specific transport of ATP across a membrane in an exchange mode with ADP (Pebay-Peyroula *et. al.,* 2003). Less information is available indicating role of ATP/ADP transporter during plant pathogen interaction. One of the early studies indicated enhanced resistance to *Ervinia carotovora in* transgenic potato plants could be correlated with reduced level of ATP/ADP transporter (AATP1) (Conrath *et al.,* 2003). Further, it was demonstrated that AATP1 antisense leaves had potential to produce enhanced level of H_2O_2 and several defense related genes which collectively delayed the appearance of disease symptoms (Conrath *et al.,* 2003). On the basis of above information, it can be hypothesized that during plant pathogen interaction energy is required for both the partner. Activation of several defense and signaling machinery require efficient and constant supply of energy. Improper energy influx may lead to failure of specific machinery. Resistant chilli variety is naturally well equipped with constant higher expression of ATP/ADP transporter which might have contributed to the defense response against ChiLCV.

Comparative analysis of gene expression in resistant and susceptible chilli plants following ChiLCV infection also indicated upregulation of some genes in susceptible plant. Higher accumulation of ribosomal protein L19 and L37 transcripts was detected in susceptible plants. L19 represents as the large subunit of ribosome which contains two globular domains connected with an extended segment(Gamalinda *et al*., 2013). One of the domains of L19 is localized at the surface of the large subunit and is involved in the formation of inter subunit bridge between small and large subunit of ribosome (Yang *et al.,* 2011). In animal, L19 facilitates pathogenesis of the *Epstein-Barr virus* (Yang *et al.,* 2011). Ribosomal protein L37 is also one of the important constituents of ribosome and is required for efficient translation. Recent information suggested upregulation of ribosomal protein L37 in rat brain due to HIV-1 infection (Li *et al.,* 2013). However, direct mechanistic role of ribosomal proteins (L19 and L37) in viral pathogenesis is elusive and requires further studies. This study provides the first clue of plausible involvement of ribosomal proteins in plant virus pathogenesis.

Geminiviruses form minichromosome using host histone proteins and regulate viral gene expression through post translational modification of histone proteins. Recent studies on movement of BDMV revealed interaction of histone H3 with viral CP and MP. H3 was found to be localized in the cell cytoplasm and cell periphery along with MP (Zhou *et al.,* 2011). Recently, deep trasncriptome sequencing methods was employed for the analysis of trasncriptome in mock, symptomatic, and recovered pepper leaves following PepGMV infection. Results indicated differential expression of several genes including histone H4 (Gongora-Castillo *et al.,* 2012). In the present study, upregulation of histone H4 was noticed in the susceptible chilli plant. Histone H4 is known to occur as dimer with H3. Therefore enhanced accumulation of histone H4 can facilitate formation of minichromosome and movement complex for successful pathogenesis in a compatible plant species.

Microtubule interaction with viral MP is indispensable for intra- and intercellular movement of plant viruses. TMV MP is localized, along with the microtubule, in infected cells (Byko *et al*., 2000). TGB of potyviruses are also known to interact with microtubule during the virus movement (Wright *et al*., 2010). Therefore, enhanced transcript level of tubulin in susceptible chilli plant appears to be a prerequisite for efficient movement of ChiLCV which in turn govern pathogenesis.

Thionin, a small cys-rich highly basic protein having antimicrobial activity is known to be involved in eliciting plant defense against several bacteria and fungi (Epple *et al.,* 1997; Pelegrini *et al.,* 2005). Sequence analysis of EST sorted out in SSH of resistant var. Punjab Lal indicated the similarity with γ-thionin of *Capsicum annuum* and defensin of *Solanum lycopersicum.* The role of γ-thionin has been elucidated in providing defense against broad range of fungi (Terras *et al.,* 1995). Elevated level of PDF1.2 gene (which encodes for defensin) expression was observed in *N. benthamiana* plants infected with TMV (Mitter *et al.,* 1998). Therefore, enhanced expression of γ-thionin indicated possible role of this protein in imparting basal defense responses in resistant chilli plants.

Ascorbate peroxidase (APX) is major H_2O_2 scavenging enzyme found in the cytosol, chloroplast and mitochondria of higher plants and catalyzes the conversion of H_2O_2 into H_2O using ascorbate as electron donor (Singh *et al*., 2010; Caverzan *at al*., 2012). *Cladosporium fulvum* infection caused elevated level of APX activity in tomato. MYMIV resulted in enhanced accumulation of APX in Kenaf (*Hibiscus cannabinus* L.) and roselle (*Hibiscus subdariffa* L.) (Sarkar *et al*., 2010; Kundu *et al*., 2013). APX upregulation is indicative of pathogen attack which in turn activates plant defense response and produces reactive oxygen species including H_2O_2 production in order to limit the pathogen spread. For maintaining the ROS homeostasis, plants produce ascorbate peroxidase to scavenge extra H_2O_2.. Elevated level of APX in Punjab Lal can be linked with this notion.

Some EST sequences (hypothetical protein, unknown I, unknown II and unknown III) did not share homology with any known genes on NCBI and TAIR database. However, differential expression of these transcripts indicated their role during ChiLCV pathogenesis. Elevated level of hypothetical protein in susceptible chilli var. Kashi Anmol suggested its role in host susceptibility. Expression level of unknown I was slightly higher in susceptible variety and could support ChiLCV pathogenesis. In contrast, transcript level of unknown II and unknown III was higher in resistant variety Punjab Lal. Further research is necessary to study functions of these proteins in plant biology vis-a-vis geminivirus infection.

Interestingly, persistent and higher accumulation of histone H2B transcripts was observed in resistant chilli plants. Histone H2B is a constituent of nucleosome (Bevykin *et al.,* 1988; Hondele *et al.,* 2013) and is involved in eukaryotic gene regulation (Batta *et al.,* 2011). Geminivirus minichromosome mimics eukaryotic chromatin organization (Pilartz and Jeske, 1992; Pilartz and Jeske, 2003). Therefore, viral gene regulation could also be governed by principles of host gene expression strategies. Lower levels of ChiLCV DNA might be due to reduced levels of viral proteins probably owing to impaired transcription. Further, it is known that transcription is regulated by post translational modification of histones. At early stage of infection, ChiLCV genome accumulated at significant and

detectable level which was maintained up to 14 dpi, followed by a sharp decline. This drastic reduction in viral genome accumulation can be correlated with less availability of essential viral proteins required for replication. Lower levels of viral genome and higher accumulation of H2B transcript at 21 dpi indicated host dependence of ChiLCV replication via H2B mediated gene regulation. Therefore, further effort was made to elucidate histone H2B mediated defense mechanism in resistant chilli variety.

For this, the H2B gene was cloned from both chilli var. Punjab Lal and model plant *Nicotiana benthamiana.* Sequence analysis and multiple sequence alignment revealed C-terminal conservation of H2B whereas N-terminal was variable among different plant species. Phylogenetic tree indicated close relationship of H2B from chilli var. Punjab Lal with tomato and other solanaceous plants.

To predict the interacting partner(s) of H2B, *in silico* analysis was carried out on STRING using *Arabidopsis* interactome information. STRING result suggests direct interaction between histone proteins H3 & H4 with H2B. Physical interaction between these histones also indicated their coordinate function in gene regulation. Reports indicated that post translational modifications of one histone protein can affect modification of others. One of the candidate, HUB1 (histone monoubiquitination 1) protein has been identified as interacting partner of H2B. HUB1, a RING E3 ligase is involved in the monoubiquitination of histones H2A and H2B (Liu *et al.,* 2007; Cao *et al.,* 2008). Therefore, it was decided to explore the role of monoubiquitination of H2B on viral promoter activation. One of the early reports of HUB1 in plants indicated its role in plant growth and development (Liu *et al.,* 2007; Cao *et al.,* 2008) and defense (Dhawan *et al.,* 2009). HUB1 mutant exhibited abnormal leaf growth and colour, reduced biomass, reduced growth of primary leaf (Liu *et al.,* 2007; Himanen *et al.*, 2012). Detailed analysis of this mutant and kinematic study of root and leaf growth using flow cytometry suggested defects in cell cycle (Himanen *et al.*, 2012). Cell cycle duration was increased in young leaves of HUB1 mutant which caused early entry into the endocycles. Trasncriptome analysis of HUB1 revealed altered expression of the genes involved in the

regulation of G2-to-M phase transition of cell cycle (Himanen *et al.*, 2012). Together, these early reports have shed light on the regulation of gene expression by monoubiquitination of H2B in plants. Later on, some more experiments were conducted to correlate the function of monoubiquitinated H2B and gene expression. Loss-of-function allele of HUB1 in *Arabidopsis* plants showed enhanced susceptibility to *Alternaria brassicicola* and *Botrytis cinerea* while HUB1 over expressed lines conferred resistance to *B. cinerea* (Dhawan *et al.,* 2009). Detailed study revealed interaction between HUB1 and MED21, a subunit of the mediator complex that regulates RNA polymerase II in *Arabidopsis.* MED21 RNA interference line showed enhanced susceptibility to *B. cinerea* where as MED21 T-DNA insertion line was embryonic lethal indicating indispensable role of MED21 in plant growth and development (Dhawan *et al.,* 2009). Enhanced global level of monoubiquitinated H2B was observed upon activation of 177 genes during *Arabidopsis* photomorphogenesis (Bourbousse *et. al*., 2012). Recent study indicated deposition of monoubiquitinated H2B around origin of replication in budding yeast which was eventually maintained in daughter strand by the cooperatives action of monoubiquitination machinery (Kelly and Mary 2012). Interestingly, in the absence of monoubiquitination of H2B, although prereplication complex is formed and activated but replication fork progression was slowed down. This finding suggested the role on monoubiquitinated H2B for the assembly and stability of prereplication complex and efficient progression of replication forks (Kelly and Mary, 2012). This unique modification of H2B regulates the histone H3 trimethylation at lysine 4 (H3K4). H3K4 trimethylation is signature of gene activation in animal, plant and yeast. In the absence of H2B monoubiquitination, H3K4 trimethylation efficiency is reduced and gene expression is also negatively regulated. Role of post translational modification in the expression analysis of galactose inducible gene, GAL1 in *S. cerevisiae* revealed that monoubiquitination of H2B is indispensible for H3K4 methylation and recruitment of RNA polymerase II (Shukla *et al.,* 2006). GAL1 expression is significantly reduced in the absence of H2B monoubiquitination at lysine 123 (Shukla *et al.,* 2006).

Chromatin immunoprecipitation assay using specific antibody (H3K4me3) indicated post translational modification of histone. ChiP-PCR results obtained from highly susceptible *N. benthamiana* plant revealed accumulation of higher levels of viral genome which suggested proper and efficient involvement of post translational modification machinery in this host. Whereas in resistant chilli plant post translational modification of histones was impaired as confirmed by low accumulation of virus in ChiP-PCR result. ChIP-PCR was performed using ChiLCV promoter specific primers and the result suggested H3K4me3 modification in promoter region indicating activation of geminivirus promoter. In resistant plant, lesser H3K4me3 modification is the key reason for insignificant detection of virus using promoter specific primers which is incidentally related to reduce accumulation of ChiLCV genome.

Monoubiquitination of H2B in the promoter region of ChiLCV genome was confirmed in *N. benthamiana* which could be correlated with higher accumulation of viral genome. In contrary, reduced monoubiquitination of H2B around the ChiLCV promoter region was observed in resistant chilli plant which could be due to low deposition of H3K4me3. Thus, it can be postulated that impairment in monoubiquitination of H2B reduces the H3K4me3 which in turn negatively affect the ChiLCV gene expression. As a consequence, viral replication is also reduced.

Further attempt was also made to analyze alteration in global H2B ubiquitination and H3K4me3 modification in mock and infected plants of resistant chilli variety and *N. benthamiana*. Western blotting results indicated lower levels of modified H2B in mock resistant plant whereas in infected resistant plants higher accumulation of modified H2B was observed. Previous studies have suggested the role of ubiquitinated H2B in activation regulation of gene expression in plant and animal (Lee *et al*., 2007; Cao *et al*., 2008; Fonseca *et al.,* 2012). In the present study, constant amount of unmodified H2B (17-19 Kda) in mock & infected chilli plants and higher levels of modified H2B (~44 Kda) in infected chilli plants was observed. Larger mass of modified H2B is indicative of attachment of three ubiquitin molecules. Therefore, elevated levels of modified

(ubiquitinated) H2B can be correlated with activation of defense related genes following ChiLCV infection.

N. benthamiana mock plant indicated higher levels of modified H2B while ChiLCV infection resulted in constant decrease of modified H2B. This result can be correlated with reduced expression of genes involved in either regarding defense mechanism or defense signal transduction pathways.

Notably, higher levels of modified H3K4me3 were observed in mock resistant plant. Higher levels of H3K4me3 modification indicates that apart from regulation by monoubiquitination of H2B, H3K4me3 is also regulated by some unknown mechanism (Shilatifard, 2012). Nevertheless, during the early phase of infection (7–14 dpi), lower levels of H4K4me3 was observed in resistant chilli plants which can be correlated with reduced expression of genes involved in defense and higher accumulation of viral DNA during this period. At a later stage of infection cycle (21 dpi), H3K4me3 level is increased which is indicative of activation of defense related genes coupled with reduction of viral DNA. In *N. benthamiana* plants, H3K4me3 modification was higher similar to resistant plant but H3K4me3 modification evidenced steady decrease during the course of study which can be linked to repression of defense related genes.

ChIP assay indicated that inefficient post translational modification of virus minichromosome has resulted into low expression of viral transcripts and consequently virus proteins in resistant chilli var. Punjab Lal. To explore the reason for impaired post translational modification in resistant plants, yeast two hybrid assay (Y2H) was performed between virus protein and plant H2B (from chilli, CaH2B and *N benthamiana*, NbH2B).Y2H results have established interaction between CaH2B and ChiLCV AC1 ORF. Interestingly, AC1 failed to interact with NbH2B.

Rep (encoded by AC1) is indispensible for virus replication and known to posses DNA binding and unwinding activity (Chaudhury *et al.,* 2006; Pasumarthy *et al.,* 2010; Kittelmann *et al*., 2009; Nash *et al.,* 2011). In a previous study, geminivirus Rep was

shown to interaction with histone H3 in insect cells but the fate of this interaction was not elucidated (Kong *et al*., 2000). Rep essentially creates nick in the nonanucleotide seqeuence located in the stem loop structure of viral genome. During replication, Rep remains attached at the 5' end of the nicked genome or functions as helicase. Geminivirus Rep also interacts with several host factors necessary for replication like PCNA, RFC, E2F and cell cycle progression like RBR (Luque *et al*., 2002; Xie *et al*., 1995; Xie *et al.,* 1996, Ach *et al.,* 1997; Kong *et al.,* 2000; Arguello-Astorga *et al.,* 2004; Pavlov *et al.,* 2004). Interaction of Rep with CaH2B might hamper proper functioning of Rep and therefore, ChiLCV replication gets affected. Additionally, Rep can also interact with and mask CaH2B *in vivo* and make it inaccessible for monoubiquitination machinery (Fig. 5). Higher accumulation of H2B in Punjab LaL can bind and recruit majority of the Rep protein, thus creating dearth of this indispensible protein. Punjab Lal with altered H2B expression and varied N-terminal region of H2B does not afford a permissive cellular environment for ChiLCV replication and transcription.

Y2H assay revealed interaction between NbH2B with ChiLCV AV2 (movement protein, MP) in a permissive host. Notably, CaH2B did not interact with AV2. Recent reports have revealed that geminivirus proteins NSP and MP of BDMV could interact with H3 to form a complex for efficient movement of viral genome (Zhou *et al.,* 2011). Higher accumulation of viral genome and severe symptoms on ChiLCV infected *N. benthamiana* plants can be correlated with efficient intra- and intercellular transport of viral movement complex. Association of H2B with viral genome and interaction with MP creates a compact genome which facilitates smooth transport of movement complex through nuclear pore complex and plasmodesmata. Thus, formation of efficient transport of viral movement complex is also prerequisite for successful establishment of disease in a permissive host. *N. benthamiana* has evolved with appropriate strategy for efficient transport of viral genome unlike resistant chilli variety Punjab Lal, where ChiLCV failed to exploit host proteins for formation of competent movement complex.

Therefore, ChiLCV resistant variety Punjab Lal employs dual strategy to restrict viral infection. Firstly, from host point of view, natural inefficient post transcriptional machinery for histone modification has led to impaired virus gene expression. Secondly, CaH2B interacts and interferes with ChiLCV Rep mediated functions. In addition, ChiLCV AV2 fails to form a movement complex in this non-permissive host.

Chilli is an important multipurpose crop being used as vegetables, spices and for medicinal purposes. In India, chilli production is affected by many pathogens including viruses. *Chilli leaf curl Multan virus* (ChiLCV), a *Begomovirus* within the family *Geminiviridae* has emerged as the predominant species causing leaf curl disease in chillies. However, field resistant varieties against ChiLCV are available and are being used in resistance breeding programme for the genetic improvement of chillies in India. Therefore, the present study was aimed to elucidate mechanism of natural resistance mechanism in a chilli variety, Punjab Lal.

In the present study, a highly efficient infectious construct (tetramer) containing tandem repeats of viral genome (DNA A) and betasatellite (DNA β) was developed in a binary vector, pCAMBIA2300 in order to ascertain delivery of both the genomic components in every plant cell. Cent percent infectivity was achieved using this construct in model plant *Nicotiana benthamiana.*

Agroinoculation procedure of chilli varieties was standardized using the newly generated tetramer construct. Screening of field resistant varieties (Punjab Lal, Kalyanpur Chanchal and BS-35) was performed using this construct through *Agrobacterium tumefaciens* mediated delivery of viral DNA components. *C. annuum* var. Punjab Lal was selected for the further study based on the resistant response following ChiLCV (tetramer) inoculation and its durable field resistant nature for over two decades.

Extensive trasncriptome analysis of chilli var. Punjab Lal plants was carried out using suppression subtractive hybridization and reverse northern. A total of 231 non-redundant transcripts putatively involved in different cellular processes of plants were identified. Upregulation of the genes was further confirmed by qRT-PCR.

Among the transcripts which were found to be differentially upregulated, histone H2B transcript accumulation was found to be persistently higher in chilli resistant variety Punjab Lal which indicated its role in conferring natural resistance against ChiLCV.

ChiLCV exploits the host histone proteins to form minichromosome in order to initiate the viral transcription. Viruses usually utilize the host machinery to maintain the control of gene expression. Post translational modification is one of such crucial mechanism necessary for regulation of viral gene expression in plant.

Chromatin immune precipitation assay coupled with polymerase chain reaction (ChIP-PCR) assay using H3K4 specific antibody and ChiLCV promoter specific primer from the mock and infected plants of Punjab Lal and *N. benthamiana* revealed high virus titer in *N. benthamiana* and low level in Punjab Lal. These results indicated reduced level of H3K4me3 deposition in the viral promoter region in resistant chilli variety. ChIP-PCR using monoubiquitinated H2B indicated low virus titer in infected Punjab Lal unlike *N. benthamiana* where higher level of viral genome accumulation was observed. Low level of H3K4me3 in virus promoter region was correlated with reduce deposition of monoubiquitinated H2B in Punjab Lal.

The correlation between H3K4me3 and monoubiquitinated H2B was further confirmed by global analysis of H3K4me3 and monoubiquitinated H2B by immunodetection in Punjab Lal and *N. benthamiana*. In the present study, a reproducible and efficient protocol was developed to isolate total histone proteins from diverse solanaceous plant species.

High quantity of modified H2B and steady increasing deposition of H3K4me3 in resistant plants indicated activation of defense related genes could be correlated with decreasing accumulation of virus DNA.

High level of H3K4me3 was observed in both mock inoculated Punjab Lal and *N. benthamiana*. However, in Punjab Lal, at early stage of infection (7 dpi) low level of H3K4me3 was detected which was remarkably increased later on (14 and 21 dpi). At early stage of infection virus presence could not be not detected by host, consequently defense mechanism remains passive during this period. At the later stage of ChiLCV infection, host could sense the virus and activated defense mechanism by triggering and

upregulating expression of several genes involved in defense signal transduction. In Punjab Lal, enhanced level of H3K4me3 level at later stage indicated expression of these genes. In contrary, mock inoculated *N. benthamiana* plants contained higher level of H3K4me3 which was found to be downregulated following ChiLCV infection, implying suppression of defense related gene expression. Further detection of high level of modified H2B in infected Punjab Lal as compared to *N. benthamiana* support role of H2B in imparting resistance in chilli.

Yeast two hybrid assay revealed the interaction between ChiLCV AC1 with CaH2B but not with NbH2B. Interaction of AC1 with CaH2B might prevent the recruitment of monoubiquitination machinery on H2B and inhibit the post translational modification of H2B. This in turn decelerated H3K4me3 methylation and gene expression. Alternatively, CaH2B might block indispensible role of replication initiator protein (Rep encoded by AC1) in ChiLCV replication. Interaction of NbH2B with AV2 suggested formation of compact movement complex for efficient transport of viral genome in susceptible host *N. benthamiana*. Inability of CaH2B to interact with ChiLCV AV2 also adds another level of barrier for efficient movement of viral genome in resistant plant.

The interference of ChiLCV AC1 protein impaired post translational modification of naturally elevated level of H2B which in turn yielded low level of H3K4me3 modification and reduced viral gene expression in resistant chilli var. Punjab Lal. Post translational modification of CaH2B in virus promoter region might be prevented by physical interaction between Rep with H2B, as a result the later could not facilitate the H3K4me3 modification for activation of virus genes. Further, AV2 could not interact with CaH2B to form movement complex for efficient transport of viral genome within the infected plant.

To conclude, natural resistance in chilli is governed by two plausible mechanisms. Firstly elevated accumulation of H2B sequesters majority of the viral Rep protein and hamper the function of Rep in viral replication. Secondly, AC1 can mask H2B present, a

constituents of viral minichromosome and inhibit the activity of monoubiquitination machinery which in turn reduces the efficiency of H3K4me3 deposition. Inability of CaH2B to interact with AV2 and inability to form an efficient movement complex provides another layer of resistance against ChiLCV in chilli.

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	blastxI	**blastn**	**blastxII**	**AtblastP**	**Biological function**	**Molecular function**
A3P1	No homology	CT028854.1 ,Poplar cDNA sequences,2.00E-17	No homology	No homology	unknown	unknown
B11P1	No homology	AK324931.1, *Solanum lycopersicum* cDNA, clone: LEFL1088CH02, HTC in leaf, 2E-53	ABD66505.1 actin depolymerizing factor 3 [*Gossypium hirsutum*] 6e-64	AT5G59880.1 | Symbols: ADF3 | ADF3 (ACTIN DEPOLYMERIZING FACTOR ... 236 3e-63	actin binding	unknown
C3P1	No homology	AC215402.2, *Solanum lycopersicum* chromosome 2 clone C02HBa0161P02, complete sequence, 4E-98	No homology	No homology	unknown	unknown
C6P1	CBI35458.3, unnamed protein product *[Vitis vinifera*], 1E-75	AK323927.1, *Solanum lycopersicum* cDNA, clone: LEFL1067CH09, HTC in leaf,0.0	XP_002267281.1 PREDICTED: hypothetical protein [*Vitis vinifera*], 4e-156	AT3G17800.2 mRNA level of the MEB5.2 gene e-129	response to UV-B	unknown
C9P1	CBI15377.3 unnamed protein product [*Vitis vinifera*], 1E-59	AC238463.1 *Solanum lycopersicum* chromosome 3 clone C03HBa0203K13, complete sequence 1e-101	No homology	No homology	unknown	unknown
D2P1	XP_002271911.1 PREDICTED: hypothetical protein [*Vitis vinifera*],9.0E-08	AK224855.2,*Solanum lycopersicum* cDNA, clone: FC22CB04, HTC in fruit, 3e-85	XP_002271911.1, PREDICTED: hypothetical protein [*Vitis vinifera*] 5e-10	AT2G03440.1 nodulin-related 5e-07	abscisic acid mediated signaling pathway, response toacterium,response to cold, response to heat biological	unknown
D11P1	XP_002282387.1PREDICTED: hypothetical protein [*Vitis vinifera*,9e-27	XM_002314828.1*Populus trichocarpa* predicted protein, mRNA,1e-17	XP_002314864.1predicted protein [*Populus trichocarpa*, 1e-103	AT1G13360.1 unknown protein 3e-29	unknown	unknown

E11P1	No homology	AK327951.1 *Solanum lycopersicum* cDNA, clone: LEFL2041P23, HTC in fruit, 2e-11	XP_002516159.1 pentatricopeptide repeat-containing protein, putative [*Ricinus communis*] 0.0	AT4G33170.1 pentatricopeptide (PPR) repeat-contain0.0	Organelle biogenesis,RNA editing	unknown
F5P1	XP_002268144.1 hypothetical protein [*Vitis vinifera*] 3e-33	AK322040.1*Solanum lycopersicum* cDNA, clone: LEFL1032DB12, HTC in leaf, 1e-141	XP_002268144.1hypothetical protein [*Vitis vinifera*] 4e-30	AT3G17210.1 ATHS1, HS1 | HS1 (HEAT STABLE PROTEIN 1)1e-16	Defense reponse to bacterium, defense response to fungus	unknown
F6P1	No homology	AK319176.1*Solanum lycopersicum* cDNA, clone: LEFL1061DG05, HTC in leaf,3e-34	XP_002515932.1zinc finger protein, putative [*Ricinus communis*, 1e-159	AT3G16370.1 GDSL-motif lipase/hydrolase family, e-143	lipid metabolic process	unknown
F10P1	No homology	Y13862.1, *Nicotiana tabacum* enr-T1 gene, 1e-11	XP_002299155.1predicted protein [*Populus trichocarpa*] 7e-55	AT5G19640.1 proton-dependent oligopeptide transporter, 0.0	oligopeptide transport	unknown
F12P1	CBI15377.3, unnamed protein product [*Vitis vinifera*] 8e-61	XM_002274098.1PREDICTED: *Vitis vinifera* hypothetical protein 2e-15	XP_002274134.1PREDICTED: hypothetical protein [*Vitis vinifera*] 0.0	AT1G74010.1 strictosidine synthase family protein 3e-63	Biosynthetic process	strictosidine synthase activity
G11P1	No homology	AK338797.1*Lotus japonicus* cDNA, clone: LjFL3-010-CE09, HTC 8.0e-61	No homology	No homology	unknown	unknown
H2P1	No homology	AK320991.1 *Solanum lycopersicum* cDNA, clone: LEFL1015DD03, HTC in leaf 3.0e-41	XP_002317708.1 predicted protein [*Populus trichocarpa*] 2e-53	AT3G17780.1 unknown protein 1e-39	unknown	unknown
H5P1	No homology	DQ241862.1 *Solanum tuberosum* clone 027B03 40S ribosomal protein S3a-like mRNA 1.0e-78	ABB87133.1 40S ribosomal protein S3a-like [*Solanum tuberosum*] 3e-119	AT4G34670.1 40S ribosomal protein S3A (RPS3aB)e-101	translation	structural constituent of ribosome
H6P1	XP_002309687.1	BT014174.1 *Lycopersicon*	XP_002324924.1	AT5G10780.1	Unknown	unknown

	predicted protein [*Populus trichocarpa*] 1e-57	*esculentum* clone 133316R, mRNA sequence 0	predicted protein [*Populus trichocarpa* 8e-79	FUNCTIONS IN: molecular_function unknown 1e-65		
H11P1	No homology	CT033829.1 Poplar cDNA sequences 8.0e-4	XP_002313196.1 predicted protein [*Populus trichocarpa*] 6e-20	AT4G33760.1 tRNA synthetase class II (D, K and N) 0.0	aspartyl- tRNA aminoacylation, tRNA aminoacylation for protein translation, translation	ATP binding
A11P2	XP_002322584.1predicted protein [*Populus trichocarpa*]9e-04	BT013510.1,*Lycopersicon esculentum* clone 132201F, mRNA sequence,2e-20	AAL18931.1arabinosidase ARA-1 [*Solanum lycopersicum*]0.0	AT3G10740.1 ALPHA-L-ARABINOFURANOSIDASE- 1 ,0.0	Xylan catabolic process	alpha-N-arabinofuranosidase activiry
A12P2	XP_002516786.1Membrane protein PB1A10.07c, putative [*Ricinus communis*],6e-15	XM_002516740.1*Ricinus communis* Membrane protein PB1A10.07c,1e-28	XP_002516786.1Membrane protein PB1A10.07c, putative [*Ricinus communis*],0.0	AT3G06170.1 TMS membrane family protein / tumour differentially expressed (TDE) family protein e-159	Unknown	Unknown
B1P2	No homology	AK321610.1,*Solanum lycopersicum* cDNA, clone: LEFL1026CH11, 7e-88	CBI33879.3,unnamed protein product [*Vitis vinifera*], 6e-11	No homology	unknown	unknown
D6P2	No homology	DQ306789.1, *Hevea brasiliensis* isolate SSH60 mRNA sequence, 7E-14	CAB62537.1,pseudo-hevein [*Hevea brasiliensis*]1e-72	AT3G04720.1 HEVEIN-LIKE, PATHOGENESIS-RELATED 4 1e-64	Defence response	Chitin binding
D7P2	No homology	AK247242.1,*Solanum lycopersicum* cDNA, clone: LEFL1070DF06,1e-97	CAN67361.1hypothetical protein [*Vitis vinifera*],3e-24	AT2G39530.1 integral membrane protein, putative 1e-10	Unknown	Unknown
D8P2	No homology	AK324984.1,*Solanum lycopersicum* cDNA, clone: LEFL1089CD04,6e-27	ABB29931.1,P40-like protein [*Solanum tuberosum*],1e-114	AT1G72370.2 P40, e-108	mature ribosome assembly, response to osmotic	Structural constituent of ribosome

					stress, response to salt stress, translation	
D9P2	No homology	AK324931.1*Solanum lycopersicum* cDNA, clone: LEFL1088CH02, HTC in leaf,2e-53	ABD66505.1actin depolymerizing factor 3 [*Gossypium hirsutum*] 6e-64	AT5G59880.1 ACTIN DEPOLYMERIZING FACTOR 3,3e-63	response to cadmium ion, response to oxidative stress	actin binding
D12P2	No homology	AC232640.1*Solanum lycopersicum* chromosome 6 clone C06SLm0060E11,5e-18	XP_002330701.1,predicted protein [*Populus trichocarpa*]2e-115	AT3G59690.1 IQ-DOMAIN 13 e-106	unknown	Calmodulin binding
E2P2	No homology	AC212432.2, *Solanum lycopersicum* chromosome 11 clone C11HBa0062I24,1e-130	AAX84649.1,methionine rich arabinogalactan [*Solanum lycopersicum*]2e-09	No homology	No homology	No homology
E4P2	No homology	AK328723.1, *Solanum lycopersicum* cDNA, clone: LEFL3034N14, HTC in root 1e-21	XP_002269068.1,PREDICTED: hypothetical protein [*Vitis vinifera*],4e-106	AT3G05010.1 transmembrane protein e-114	Unknown	Unknown
E11P2	XP_002278361.1 PREDICTED: hypothetical protein [*Vitis vinifera*]3e-20	XM_002278325.1 PREDICTED: Vitis vinifera hypothetical protein,3e-13	XP_002278361.1 PREDICTED: hypothetical protein [*Vitis vinifera*],0.0	AT3G04910.1 WITH NO LYSINE (K) 1 0.0	Protein amino acid phosphorylation	Protein serine/threonine kinase activity
G8P2	CBI38929.3,unnamed protein product [*Vitis vinifera*]3e-50	AK246587.1*Solanum lycopersicum* cDNA, clone: FC16AC01, HTC in fruit,0.0	CBI38929.3unnamed protein product [*Vitis vinifera*],1e-51	AT2G32060.2 40S ribosomal protein S12 2e-50	translation	Structural constituent of ribosome
G11P2	XP_002280398.1PREDICTED: hypothetical protein [*Vitis vinifera*]2e-06	AK323461.1*Solanum lycopersicum* cDNA, clone: LEFL1057AG08,1e-28	XP_002280398.1PREDICTED: hypothetical protein [*Vitis vinifera*]4e-60	AT5G41040.2 feruloyl-CoA transferase 0.0	suberin synthesis.	feruloyl-CoA transferase
H6P2	No homology	AK247270.1 *Solanum lycopersicum* cDNA, clone: LEFL1079AB04, 1e-36	ACS96448.1 unknown protein [*Jatropha curcas*] >gb|ADJ67178.1, 2e-05	AT1G15270.1 Unknown 3e-07	Unknown	Unknown

H12P2	No homology	AK322622.1, *Solanum lycopersicum* cDNA, clone: LEFL1040AB05, 6e-27	XP_002285501.1,PREDICTED: hypothetical protein [*Vitis vinifera*],7e-21	AT1G26355.1 SPIRAL1-LIKE1 7e-15	Plant specific Microtubule localized protein required for directional control ofrapidly expanding cells	unknown
A3P3	XP_002533604.1,conserved hypothetical protein [*Ricinus communis*],8e-45	AK322596.1,*Solanum lycopersicum* cDNA, clone: LEFL1039DA06, 2e-109	XP_002533604.1,conserved hypothetical protein [*Ricinus communis*] 5e-81	AT2G27770.1 unknown protein e-102	unknown	Unknown
B5P3	XP_002285573.1PREDICTED: hypothetical protein [*Vitis vinifera*],3e-50	AK326009.1*Solanum lycopersicum* cDNA, clone: LEFL2001CF10,1e-155	XP_002324924.1predicted protein *[Populus trichocarpa*]6e-78	AT5G10780.1 Unknown 1e-65	unknown	Unknown
B6P3	No homology	AK326009.1,*Solanum lycopersicum* cDNA, clone: LEFL2001CF10,4e-28	XP_002324924.1,predicted protein *[Populus trichocarpa*]6e-78	AT5G10780.1 Unknown 1e-65	unknown	Unknown
B11P3	No homology	AK324646.1*Solanum lycopersicum* cDNA, clone: LEFL1080BH08, 1e-63	ABB16967.1unknown [*Solanum tuberosum*]1e-155	AT3G27090.1 Unknown e-107	unknown	Unknown
C3P3	No homology	EZ325049.1TSA: *Artemisia annua* strain Madagascar Contig11595.,8e-07	XP_002515244.1long-chain-fatty-acid CoA ligase, putative [*Ricinus communis*]7e-163	AT2G47240.2 long-chain-fatty-acid--CoA ligase, 0.0	fatty acid biosynthetic process	Long-chain fatty acid-CoA ligase activity,
E12P3	XP_002299901.1,predicted protein [*Populus trichocarpa*]2e-42	AK329145.1 *Solanum lycopersicum* cDNA, clone: LEFL3105K09,2e-99	XP_002299901.1,redicted protein [*Populus trichocarpa*]2e-42	AT5G12470.1 unknown protein e-121	unknown	Unknown
F11P3	No homology	AC238435.1,*Solanum lycopersicum* chromosome 3 clone C03HBa0001E15,1e-31	unknown	unknown	unknown	Unknown
G5P3	XP_002309687.1,predict	AK326009.1*Solanum*	XP_002324924.1predicte	AT5G10780.1	Unknown	unknown

	ed protein [*Populus trichocarpa]* 1e-37	*lycopersicum* cDNA, clone: LEFL2001CF10,5e-140	d protein [*Populus trichocarpa*] 6e-78	Unknown protein 1e-65		
G9P3	XP_002521904.1conserved hypothetical protein [*Ricinus communis*]1e-09	AK247242.1*Solanum lycopersicum* cDNA, clone,1e-97	CAN67361.1hypothetical protein [*Vitis vinifera*]3e-24	AT2G39530.1 integral membrane protein 1e-10	unknown	unknown
H3P3	CBI27029.3unnamed protein product [*Vitis vinifera*]4e-05	AK323748.1*Solanum lycopersicum* cDNA, clone:2e-50	XP_002282387.1PREDICTED: hypothetical protein [*Vitis vinifera*]8e-32	AT1G13360.1 unknown protein 3e-19	unknown	unknown
B10P4	BAG16529.1putative Ran/TC4 protein [*Capsicum chinense*]6e-10	DQ222522.1*Solanum tuberosum* clone 129C03 Ran/TC4-like protein mRNA,2e-88	P38546.1RecName: Full=GTP-binding nuclear protein Ran1;5e-126	AT5G55190.1 RAN3, ATRAN3 | RAN3 (RAN GTPASE 3 e-127	protein import into nucleus	GTPase activity
C1P4	No homology	AK319176.1*Solanum lycopersicum* cDNA, clone: LEFL1061DG05,3e-34	XP_002515932.1,zinc finger protein, putative [*Ricinus communis*]1e-159	AT3G16370.1 GDSL-motif lipase/hydrolase e-143	lipid metabolic process	carboxylesterase activity, hydrolase activity, acting on ester bonds
C8P4	No homology	AK323427.1,*Solanum lycopersicum* cDNA, clone,4e-11	CAD13178.1alpha-tubulin [*Nicotiana tabacum*], 0.0	AT4G14960.2 alpha-tubulin isoform 0.0	microtubule cytoskeleton organization	structural constituent of cytoskeleton
C11P4	No homology	AK247242.1,*Solanum lycopersicum* cDNA, clone: LEFL1070DF06,2e-06	CAN67361.1,hypothetical protein [*Vitis vinifera*]3e-24	AT2G39530.1 integral membrane protein 2e-08	Unknown	unknown
D4P4	No homology	AK319176.1*Solanum lycopersicum* cDNA, clone: LEFL1061DG05,3e-34	XP_002515932.1,zinc finger protein, putative [*Ricinus communis*] , 1e-159	AT3G16370.1 GDSL-motif lipase/hydrolase e-143	Lipid metabolism	carboxylesterase activity, hydrolase activity, acting on ester bonds
D7P4	No homology	AC238435.1*, Solanum lycopersicum* chromosome 3 clone C03HBa0001E15, 1e-31	No homology	No homology	No homology	No homology
D8P4	No homology	CT028990.1 Poplar cDNA sequences, 7e-12	ACA04028.1 galactinol synthase 1 [*Populus trichocarpa* x *Populus*	AT1G60470.1 Arabidopsis thaliana galactinol synthase 4	carbohydrate biosynthetic process	transferase activity

			deltoids, 6e-79	(AtGolS4) e-150		
F12P4	No homology	AK325463.1 *Solanum lycopersicum* cDNA, clone: LEFL1096DH05, 9e-41	XP_002516111.1 DNA binding protein, putative [*Ricinus communis*] 2e-93	AT5G20510.1 Alfin-Like family of nuclear-localized PhD domain containing homeodomain proteins 2e-90	regulation of transcription, DNA-dependent	DNA binding, methylated histone residue binding
A11P4	ABC61505.1 AGO4-2 [*Nicotiana benthamiana*] 4e-45	DQ321491.1 *Nicotiana benthamiana* AGO4-2 mRNA, partial cds 2e-83	ABC61505.1 AGO4-2 [*Nicotiana benthamiana*] 0.0	AT2G27040.1 ARGONAUTE 4 0.0	DNA methylation, chromat in silencing,	siRNA binding
B5P5	No homology	AK321610.1 *Solanum lycopersicum* cDNA, clone: LEFL1026CH11, 1e-83	CBI33879.3 unnamed protein product [*Vitis vinifera*], 6e-11	No homology	No homology	No homology
B10P5	No homology	BT012800.1 *Lycopersicon esculentum* clone 113815R, mRNA sequence 1e-22	XP_002524629.1 chaperonin containing t-complex protein 1, 0.0	AT3G20050.1 ATTCP-1 | ATTCP-1; ATP binding / protein 0.0	cellular protein metabolic process, protein folding	ATP binding, unfolded protein binding
C5P5	ACB05668.1 cyclophilin [*Capsicum annuum*] 4e-50	EU401723.1 *Capsicum annuum* cyclophilin mRNA, complete cds, 0.0	ACB05668.1 cyclophilin [*Capsicum annuum*] 4e-50	AT2G21130.1 peptidyl-prolyl cis-trans isomerase / cyclophilin (CYP2) / rotamase 1e-69	protein folding	peptidyl-prolyl cis-trans isomerase activity
C8P5	No homology	CT028140.1 Poplar cDNA sequences, 1e-04	XP_002513006.1 aldo-keto reductase, putative [*Ricinus communis*] 2e-14	AT2G21250.1 mannose 6-phosphate reductase (NADPH-dependent), putative e-162	Response to cadmium ion	oxidoreductase activity
D1P5	No homology	AK325991.1 *Solanum lycopersicum* cDNA, clone: LEFL2001CB07, 6e-16	ADM26718.1, glycolate oxidase [*Nicotiana benthamiana*], 3e-180	AT3G14420.2 (S)-2-hydroxy-acid oxidase 0.0	metabolic process	glycolate oxidase activity
D5P5	No homology	AC239432.2 *Solanum lycopersicum* strain Heinz 1706 chromosome 1 clone,	No homology	No homology	No homology	No homology

		1e-08				
D11P5	ACL54967.1, MADS FLC-like protein 3 [*Cichorium intybus*], 2e-07	FJ347970.1*Cichorium intybus* MADS FLC-like protein 3 mRNA, partial cds, 7e-84 [tblastx]	ACL54967.1MADS FLC-like protein 3 [*Cichorium intybus*],6e-80	AT5G10140.1 FLOWERING LOCUS C, FLOWERING LOCUS F 6e-52	Negative regulation of flower development, regulation of circadian rhythm,response to temperature stimulus	specific transcriptional repressor activity
E1P5	ACJ84880.1unknown [*Medicago truncatula*]1e-44	DQ235176.1 *Solanum tuberosum* clone 156H10 P0 ribosomal protein-like mRNA, complete cds, 3e-161	ABB29933.1P0 ribosomal protein-like [*Solanum tuberosum*]2e-144	AT3G09200.1 60S acidic ribosomal protein P0 e-125	response to cadmium ion, response to cold, response to salt stress, response to zinc ion, translation, translational elongation	Copper ion binding
E4P5	No homology	AK326276.1*Solanum lycopersicum* cDNA, clone: LEFL2003DB09,1e-07	NP_179244.1vacuolar ATP synthase 16 kDa proteolipid subunit 5 / V-ATPase ,8e-55	AT4G34720.1 vacuolar H+-pumping ATPase 16 kDa proteolipid (ava-p1) 9e-47	ATP synthesis coupled proton transport, protein transport	ATPase activity, proton-transporting ATPase activity, rotational mechanism
E5P5	XP_002271573.1,PREDICTED: hypothetical protein [*Vitis vinifera*],3e-11	AK324686.1,*Solanum lycopersicum* cDNA, clone: LEFL1081AA06,5e-89	XP_002321787.1predicted protein [*Populus trichocarpa*],8e-113	AT5G45170.1 CbbY protein-related e-103	unknown	Unknown
A1P1	BAF47116.1G protein beta-subunit-like protein [*Nicotiana tabacum*]2e-24	Y09514.1*N.plumbaginifolia* mRNA for G protein beta subunit-like protein,2e-47	ACR77528.1heterotrimeric G protein beta 1 subunit [*Nicotiana benthamiana*]0.0	AT3G18130.1 RACK1C_AT, RECEPTOR FOR ACTIVATED C KINASE 1 C e-146	root development, shoot development	Nucleotide binding
A11P1	ABY66953.1thionin-like protein [*Capsicum annuum*]3e-17	EU367112.1*Capsicum* annuum thionin-like protein mRNA, complete cds,4e-173	ABY66953.1thionin-like protein [*Capsicum annuum*]9e-48	No homology	defence	unknown
A11P3	AAR17080.1heat shock protein 70-3 [*Nicotiana*	AY372071.1*Nicotiana tabacum* heat shock protein	AAR17080.1heat shock protein 70-3 [*Nicotiana*	AT5G02500.1 HEAT SHOCK COGNATE	protein folding, response to	ATP binding

	tabacum]8e-71	70-3,7e-164	*tabacum*],0.0	PROTEIN 70-1,0.0	cadmium ion, response to cold, response to heat, response to virus	
A5P1	XP_002521801.1Patellin-3, putative [*Ricinus communis*],7e-13	XM_002521755.1,*Ricinus communis* Patellin-3, putative, mRNA,8e-11	XP_002521801.1, Patellin-3, putative [*Ricinus communis*],0.0	AT1G72160.1 SEC14 cytosolic factor family protein e-120	transport	transporter activity
A6P1	ACB05671.1chloroplast ferredoxin [*Capsicum annuum*]7e-22	EU401726.1*Capsicum annuum* chloroplast ferredoxin mRNA,1e-74	ACB05671.1chloroplast ferredoxin [*Capsicum annuum*],2e-78	AT1G60950.1 encodes a major leaf ferredoxin 1e-43	photosynthetic electron transport chain, response to light stimulus	2 iron, 2 sulphor cluster binding, electron carrier activity, iron-sulfur cluster binding
A7P1	BAG16516.1putative Cu/Zn superoxide dismutase [*Capsicum chinense*].8e-28	AF009734.1*Capsicum annuum* Cu/Zn superoxide dismutase (SOD) mRNA,2e-157	O22373.1RecName: Full=Superoxide dismutase [Cu-Zn]1e-84	AT1G08830.2 copper/zinc superoxide dismutase CSD1 6e-56	Defence response to bacterium, removal of superoxide radicals, response to copper ion, response to iron ion, response to oxidative stress, response to ozone, response to salt stress	Superoxide dismutase activity
A8P1	CAA63482.1,glycolate oxidase [*Solanum lycopersicum*],9e-16	AK325991.1*Solanum lycopersicum* cDNA, clone: LEFL2001CB07, 1e-46	ADM26718.1glycolate oxidase [*Nicotiana benthamiana*],3e-180	AT3G14420.2 2-hydroxy-acid oxidase, peroxisomal, putative / glycolate oxidase,0.0	metabolic process	Glycolate oxidase activity
E7P2	ACI66048.1Histone H3.3 [*Salmo salar*] 8e-07	AB355993.1*Nicotiana tabacum* mRNA for histone H3.2, partial cds,2e-70	ACG38891.1histone H3 [*Zea mays*]6e-56	AT4G40030.2 histone H3.2 2e-59	Nucleosome assembly	DNA binding
B1P1	CAA06154.1arcA 3 [*Nicotiana tabacum*]3e-25	AJ004807.1*Nicotiana tabacum* xanthi arcA 3 gene, partial4e-49	CAA06154.1arcA 3 [*Nicotiana tabacum*]8e-169	AT1G18080.1 RECEPTOR FOR ACTIVATED C KINASE 1	response to cadmium ion	Nucleotide binding

				A e-129		
B2P1	XP_002888582.1,proteasome maturation factor UMP1 family protein [Arabidopsis,,2e-46	AK321247.1*Solanum lycopersicum* cDNA, clone:3e-174	XP_002283197.1REDICTED: hypothetical protein [*Vitis vinifera*]4e-58	AT1G67250.1 proteasome maturation factor UMP1 family protein 1e-57	Protein metabolism	unknown
B5P1	No homology	AY256915.1,*Petunia x hybrida* S-adenosyl-L-homocystein hydrolase mRNA,3e-54	AAP92452.1S-adenosyl-L-homocystein hydrolase [*Petunia x hybrida*],0.0	AT3G23810.1 S-ADENOSYL-L-HOMOCYSTEINE (SAH) HYDROLASE 2	metabolic process, one-carbon metabolic process	adenosylhomocysteinase activity, binding, catalytic activity
B6P1	XP_002532822.1Protein P21, putative [*Ricinus communis*]7e-103	XM_002532776.1*Ricinus communis* Protein P21, putative, mRNA,4e-118	XP_002532822.1Protein P21, putative [*Ricinus communis*]7e-103	AT1G73620.1 thaumatin-like protein e-104	response to other organism	unknown
D1P2	ACX50406.1histone H4 [*Eriobotrya japonica*]1e-36	AF038387.1*Capsicum annuum* histone H4 mRNA, complete cds,0.0	ACX50406.1histone H4 [*Eriobotrya japonica*]1e-36	AT5G59970.1 histone H4 3e-34	nucleosome assembly	DNA binding
B8P1	No homology	Y18312.1*Solanum tuberosum* mRNA for major intrinsic protein 2,4e-52	CAB46351.1major intrinsic protein 2 [*Solanum tuberosum*]4e-163	AT2G37170.1 a member of the plasma membrane intrinsic protein subfamily PIP2. e-122	Response to water deprivation, transport, water transport	water channel activity
B4P4	NP_199330.2CbbY protein-related [*Arabidopsis thaliana*]3e-18	AK324686.1*Solanum lycopersicum* cDNA, clone: LEFL1081AA06,6e-108	NP_199330.2CbbY protein-related [*Arabidopsis thaliana*]3e-18	AT5G45170.1 CbbY protein-related 0.0	proteinase activated receptor binding	unknown
B10P1	No homology	Y14032.1*Nicotiana tabacum* mRNA for ferredoxin-NADP reductase,8e-46	CAB71293.1 chloroplast ferredoxin-NADP+ oxidoreductase precursor [*Capsicum annuum*] 0.0	AT4G05390.1 ferredoxin:NADP(H) oxidoreductase. 2e-81	oxidation reduction	oxidoreductase activity
G1P3	XP_002284281.1similar to Histone H2B isoform 2	AK228063.1Arabidopsis thaliana mRNA for histone	BAF00024.1histone H2B like protein [*Arabidopsis*	AT5G22880.1 histone 2B (H2B)	Nucleosome assembly	DNA binding

	[*Vitis vinifera*],5e-43	H2B like protein,4e-110	*thaliana*],2e-43	protein e-41		
C2P1	AAT08691.1asparaginyl-tRNA synthetase [*Hyacinthus orientalis*]6e-15	AK320582.1*Solanum lycopersicum* cDNA, clone: LEFL1011AB11,4e-83	XP_002330501.1predicted protein [*Populus trichocarpa*,0.0	AT5G56680.1 cytosolic asparaginyl-tRNA synthetase0.0	embryo development ending in seed dormancy, response to cadmium ion	ATP binding, aminoacyl-tRNA ligase activity, asparagine-tRNA ligase activity,aspartate-tRNA ligase activity, nucleic acid binding, nucleotide binding
C4P1	XP_002516809.1Transmembrane BAX inhibitor motif-containing protein, putative,6e-50	XM_002516763.1 *Ricinus communis* Transmembrane BAX inhibitor motif-containing protein, putative, mRNA 5e-72	XP_002516809.1Transmembrane BAX inhibitor motif-containing protein,1e-97	AT4G14730.1 transmembrane protein-related 4e-65	Transmembrane BAX inhibitor	Glutamate binding
C5P1	XP_002518847.1n-rich protein, putative [*Ricinus communis*]3e-26	XM_002518801.1*Ricinus communis* n-rich protein, putative, mRNA,1e-42	XP_002518847.1n-rich protein, putative [*Ricinus communis*]3e-26	AT5G42050.1 function unknown5e-85	signal transduction pathway of programmed cell death	
C8P1	XP_002322154.1nucleobase ascorbate transporter [*Populus trichocarpa*]2e-43	XM_002511400.1*Ricinus communis* purine permease, putative, mRNA1e-65	XP_002511446.1purine permease, putative [*Ricinus communis*] 0.0	AT5G62890.3 Permease 0.0	Transport	Transmembrane transporter activity
C11P1	AAF66824.1poly(A)-binding protein [*Nicotiana tabacum*]8e-17	AF190656.1*Nicotiana tabacum* clone 1 poly(A)-binding protein,2e-82	AAF66824.1poly(A)-binding protein [*Nicotiana tabacum*]2e-126	AT1G49760.1 polyadenylate-binding protein, putative / PABP, 1e-44	Response to cadmium ion	RNA binding,translation initiation factor activity
C12P1	NP_177893.1 pathogenesis-related thaumatin family protein	NM_106419.1 *Arabidopsis thaliana* pathogenesis-related	NP_177893.1 pathogenesis-related thaumatin family protein	AT1G77700.1 pathogenesis-related thaumatin family	Response to other organism	No homology

	[*Arabidopsis thaliana*] >gb|AAG51631.1|AC012 193_13 thaumatin-like protein; 12104-13574 *[Arabidopsis thaliana*] 1e-62	thaumatin family protein (AT1G77700) mRNA, complete cds 4e-56	[*Arabidopsis thaliana*] >gb|AAG51631.1|AC0121 93_13 thaumatin-like protein; 12104-13574 [Arabidopsis thaliana] 0.0	0.0		
D3P1	ABK42078.114-3-3 protein [*Capsicum annuum*]1e-69	DQ991045.1*Capsicum annuum* 14-3-3 protein mRNA, complete cds,0.0	ABK42078.114-3-3 protein [*Capsicum annuum*],5e-96	AT4G09000.2 GRF1, GF14 CHI | 14-3-3-like protein 2e-77	Response to cadmium ion	Protein phosphorylated amino acid binding
F6P3	AAN63819.1ankyrin domain protein [*Nicotiana tabacum*]2e-62	BT013193.1*Lycopersicon esculentum* clone 134344R, mRNA sequence,0.0	AAK18619.1ankyrin-repeat protein HBP1 [*Nicotiana tabacum*]3e-160	AT4G35450.4 ANKYRIN REPEAT-CONTAINING PROTEIN 2 e-103	defense response to bacterium, protein folding	Protein binding
D5P1		EF522839.1*Nicotiana tabacum* histone H1E (H1e) mRNA, complete cds,8e-11	ABP87679.1histone H1E [*Nicotiana tabacum*],1e-21	AT1G06760.1 histone H1, putative 1e-10	Nucleosome assembly	DNA binding
D6P1	AAR83875.1mary storys protein [*Capsicum annuum*] 1e-22	AY484392.1*Capsicum annuum* eukaryotic initiation factor 5A2 (eIF5A2) 0.0	AAR83875.1mary storys protein [Capsicum annuum] 2e-77	AT1G13950.1 EUKARYOTIC ELONGATION FACTOR 5A-1 8e-65	translational initiation, xylem development	Translation initiation factor activity
D7P1	ABB72814.1putative 40S ribosomal protein S8-like protein,2e-53	AK323126.1Solanum lycopersicum cDNA, clone: LEFL1049DC02,3e-157	ABB72814.1 putative 40S ribosomal protein S8-like protein [*Solanum tuberosum*]4e-96	AT5G20290.1 0S ribosomal protein S8 (RPS8A) 8e-73	Ribosome biogenesis, translatio n	structural constituent of ribosome
D9P1	AAF28382.1DnaJ-like protein [*Solanum lycopersicum*]2e-58	AF124139.1 *Lycopersicon esculentum* DnaJ-like protein mRNA, complete cds 0.0	AAF28382.1DnaJ-like protein [*Solanum lycopersicum*]0.0	AT3G44110.1 ATJ3, ATJ | ATJ3; protein binding e-142	protein folding, regulation of ATPase activity, response to salt stress	Protein binding
D12P1	CAI48073.160S ribosomal protein L37a [*Capsicum chinense*]1e-	AJ879068.1*Capsicum chinense* mRNA for 60S ribosomal protein L37a,2e-	CAI48073.160S ribosomal protein L37a [*Capsicum chinense*],1e-46	AT3G10950.1 60S ribosomal protein L37a (RPL37aB	Ribosome biogenesis, translatio n	structural constituent of ribosome

	24	47		4e-34		
E1P1	CAA63710.1 annexin [*Capsicum annuum*], 1e-67	X93308.1 *C.annuum* mRNA for annexin, 0.0	CAA63710.1 annexin [*Capsicum annuum*], 2e-175	AT1G35720.1 ANNEXIN ARABIDOPSIS 1 e-112	response to abscisic acid stimulus response to osmotic stress, response to oxidative stress	peroxidase activity, protein homodimerization activity,A TP binding, calcium ion binding, calcium-dependent phospholipid binding,copper ion binding, zinc ion binding
E2P1	AAO89565.2, ZIP [*Nicotiana tabacum*], 3e-52	AY221168.2 *Nicotiana* tabacum ZIP mRNA, complete cds, 1e-161	AAO89565.2, ZIP [*Nicotiana tabacum*] 0.0	AT3G56940.1 putative ZIP protein, e-163	chlorophyll biosynthetic process, photosynthesis	DNA binding
E3P1	XP_002529218.1 methionine aminopeptidase, putative [*Ricinus communis*] 8e-80	XM_002529172.1 *Ricinus communis* methionine aminopeptidase, putative, mRNA, 8e-127	XP_002529218.1 methionine aminopeptidase, putative [Ricinus communis], 0.0	AT3G59990.3 MAP2 like methionine aminopeptidase,0.0	protein processing	aminopeptidase activity, metalloexopeptidase activity
E8P1	AAN62015.2 leucine-rich repeat protein [*Capsicum annuum*] 2e-74	AY237117.1 *Capsicum annuum* leucine-rich repeat protein (LRR1) mRNA, 0.0	AAN62015.2leucine-rich repeat protein [*Capsicum annuum*]1e-96	AT1G34210.1 Plasma membrane LRR receptor-like serine threonine kinase	microsporogenesis, pollen maturation	kinase activity
E9P1	XP_002509493.1transcription factor, putative [*Ricinus communis*] 3e-23	XM_002509444.1*Ricinus communis* DNA binding protein, putative, mRNA,3e-30	XP_002509490.1,DNA binding protein, putative [*Ricinus communis*] 4e-49	AT4G39250.1 RAD-LIKE 1 (ATRL1) 4e-27	regulation of transcription, DNA-dependent	DNA binding
E10P1	XP_002528712.1carboxylic ester hydrolase, putative [*Ricinus communis*],7e-60	XM_002528666.1*Ricinus communis* carboxylic ester hydrolase, putative, mRNA,4e-49	XP_002528712.1carboxylic ester hydrolase, putative [*Ricinus communis*]0.0	AT5G03610.1 GDSL-motif lipase/hydrolase family e-126	lipid metabolic process	carboxylesterase activity, hydrolase activity, acting on ester bonds

F2P1	XP_002886106.1phosphate-responsive 1 family protein [Arabidopsis],1e-13	AK324232.1*Solanum lycopersicum* cDNA, clone: LEFL1074AD02,2e-44	XP_002886106.1phosphate-responsive 1 family protein [Arabidopsis],2e-133	AT2G17230.1, EXORDIUM LIKE 5 (EXL5), 0.0	signal transduction	unknown
F7P1	AAG44479.1,isocitrate lyase [*Ipomoea batatas*],2e-123	AF243525.1,*Ipomoea batatas* isocitrate lyase (ICL) mRNA, complete cds,0.0	AAG44479.1isocitrate lyase [*Ipomoea batatas*],0.0	AT3G21720.1 ICL (ISOCITRATE LYASE) 0.0	carboxylic acid metabolic process, metabolic process	isocitrate lyase activity
G1P1	XP_002519082.1phosphatidylinositol 4-kinase, putative [*Ricinus communis*],4e-42	XM_002519036.1*Ricinus communis* phosphatidylinositol 4-kinase, putative, mRNA,5e-79	XP_002519082.1phosphatidylinositol 4-kinase, putative [*Ricinus communis*] 0.0	AT1G49340.1 phosphatidylinositol 4-kinase, 0.0	Protein amino acid phosphorylation	1-phosphatidylinositol 4-kinase activity, inositol or phosphatidylinositol kinase activity
G2P1	NP_177829.4 ATP binding / ATP-dependent helicase/ RNA binding / helicase/ hydrolase, acting on acid anhydrides, in phosphorus-containing anhydrides / nucleic acid binding [Arabidopsis thaliana] 4e-05	XM_002531848.1*Ricinus communis* dead box ATP-dependent RNA helicase,,2e-09	XP_002531894.1 dead box ATP-dependent RNA helicase, putative [*Ricinus communis*] >gb|EEF30493.1| dead box ATP-dependent RNA helicase, putative [*Ricinus communis*]0.0	AT1G77030.1 ATP binding / ATP-dependent helicase/ RNA binding / helicase/ hydrolase,0.0	unknown	ATP binding, ATP-dependent helicase activity, RNA binding, helicase activity
G3P1	ABB72823.1adenosine kinase isoform 1T-like protein [*Solanum tuberosum*]1e-28	AY695052.1 *Nicotiana tabacum* adenosine kinase isoform 1S mRNA, complete cds 2e-72	AAU14832.1adenosine kinase isoform 1S [*Nicotiana tabacum*],0.0	AT3G09820.1 ADENOSINE KINASE 1 e-172	Adenosine salvage, response to cadmium ion, response to trehalose-6-phosphate stimulus	adenosine kinase activity
G5P1	ABR25730.1ubiquitin fusion protein [*Oryza sativa* Indica Group],1e-	AY487808.1 *Capsicum annuum* ubiquitin-conjugating enzyme mRNA,	XP_002282071.1 similar to ubiquitin fusion protein [*Vitis vinifera*],1e-67	AT3G52590.1 UBIQUITIN EXTENSION PROTEIN 1, 8e-63	protein ubiquitination	protein binding

	16	partial sequence, 1e-95				
G7P1	XP_002527783.1phosphoglucomutase, putative [*Ricinus communis*] ,2e-110	AJ240054.1*Solanum tuberosum* mRNA for cytosolic phosphoglucomutase,0.0	XP_002527783.1phosphoglucomutase, putative [*Ricinus communis]*,0.0	AT1G23190.1 phosphoglucomutase 0.0	carbohydrate metabolic process, response to cadmium ion	intramolecular transferase activity, phosphotransferases, magnesium ion binding, phosphoglucomutase activity
G8P1	BAA33810.1phi-1 [*Nicotiana tabacum*]1e-98	AM040267.1,*Capsicum chinense* partial mRNA for Phi-1 protein 0.0	CAJ13706.1Phi-1 protein [*Capsicum chinense*]4e-114	AT4G08950.1 PHI-1 (PHOSPHATE-INDUCED 1) 6e-80	response to brassinosteroid stimulus	unknown
G10P1	XP_002894444.1mucilage-modified 4 [*Arabidopsis lyrata* subsp. lyrata],6e-12	GQ292791.1,*Gossypium hirsutum* rhamnose synthase (RHM2) mRNA,8e-24	ADB24772.1rhamnose synthase [*Gossypium hirsutum*]0.0	AT1G78570.1 UDP-L-Rhamnose synthase0.0	UDP-rhamnose biosynthetic process, auxin efflux, flavonol biosynthetic process,nucleotide-sugar metabolic process	UDP-L-rhamnose synthase activity, UDP-glucose 4,6-dehydratase activity,catalytic activity
H3P1	BAD99512.1calnexin-like protein [*Solanum lycopersicum*]8e-109	XM_002511020.1*Ricinus communis* calnexin, putative, mRNA,0.0	XP_002511066.1calnexin, putative [*Ricinus communis*]0.0	AT5G61790. calnexin 1 (CNX1); e-177	protein folding	unfolded protein binding
H4P1	CAA41632.1AT-LS1 product [*Arabidopsis thaliana*] 1e-19	XM_002526639.1*Ricinus communis* light-inducible protein atls1, putative, mRNA,5e-38	XP_002526685.1light-inducible protein atls1, putative [*Ricinus communis*]9e-58	AT5G01650.1 macrophage migration inhibitory factor family protein / MIF family protein, 3e-48s	inflammatory response, response to other organism	unknown
H7P1	NP_173786.1oxidoreductase, zinc-binding dehydrogenase family protein ,2e-28	XM_002525333.1*Ricinus communis* alcohol dehydrogenase, putative, mRNA,8e-67	XP_002525379.1alcohol dehydrogenase, putative [*Ricinus communis*],0.0	AT1G23740.1 oxidoreductase, zinc-binding dehydrogenase family protein e-135	Response to cold	binding, catalytic activity, oxidoreductase activity, zinc ion binding
H8P1	ABD65459.1putative	AK246432.1*Solanum*	ABD65459.1putative	AT4G29410.2	Ribosome	structural

	ribosomal protein L28 [*Gossypium hirsutum*]4e-48	*lycopersicum* cDNA, clone: FC11AA09, HTC in fruit,0.0	ribosomal protein L28 [*Gossypium hirsutum*]9e-62	60S ribosomal protein L28 6e-49	biogenesis, translatio n	constituent of ribosome
H9P1	NP_564399.1TOM2A (TOBAMOVIRUS MULTIPLICATION 2A)6e-49	AK323532.1*Solanum lycopersicum* cDNA, clone: LEFL1059AC03,2e-165	NP_564399.1TOM2A (TOBAMOVIRUS MULTIPLICATION 2A)1e-101	AT1G32400.3 TOBAMOVIRUS MULTIPLICATION 2A e-124	viral replication complex formation and maintenance	protein binding
H10P1	XP_002512831.1n-acetyltransferase mak3, putative [*Ricinus communis*]3e-29	AK325261.1 *Solanum lycopersicum* cDNA, clone: LEFL1094AF10, HTC in leaf 7e-85	XP_002512831.1n-acetyltransferase mak3, putative [*Ricinus communis*1e-79	AT2G38130.2 N-terminal acetyltransferase complex C4e-80	Metabolic process	N-acetyltransferase activity
A1P2	ADK66339.1alpha-mannosidase [*Solanum lycopersicum*]4e-77	GU434316.1*Solanum lycopersicum* alpha-mannosidase mRNA, complete cds,0.0	ADK66339.1alpha-mannosidase [*Solanum lycopersicum*]0.0	AT5G13980.2 glycosyl hydrolase family 38 protein 0.0	carbohydrate metabolic process, mannose metabolic process	alpha-mannosidase activity,
A5P2	XP_002529802.1geranyl geranyl pyrophosphate synthase, putative2e-26	AB294714.1 *Hevea brasiliensis* HbGGPS mRNA for geranylgeranyl-diphosphate synthase, complete cds, 7e-42	BAF98303.1geranylgeranyl-diphosphate synthase [*Hevea brasiliensis*]7e-155	AT4G38460.1 geranylgeranyl reductase (GGR e-103	isoprenoid biosynthetic process	Farnesyltranstransferase activity
A8P2	XP_002515795.1 Pre-mRNA-processing protein PRP40, putative [*Ricinus communis*] >gb|EEF46576.1| Pre-mRNA-processing protein PRP40, putative [*Ricinus communis*] 2e-49	AK325713.1*Solanum lycopersicum* cDNA, clone: LEFL2015K23, HTC in fruit 0.0	XP_002515795.1 Pre-mRNA-processing protein PRP40, putative [*Ricinus communis*] >gb|EEF46576.1| Pre-mRNA-processing protein PRP40, putative [*Ricinus communis*] 0.0	AT3G19840.1 PRE-MRNA-PROCESSING PROTEIN 40C e-170	RNA splicing	RNA polymerase binding
A10P2	CAI51314.2glutathione S-transferase GST1 [*Capsicum chinense*]7e-27	AJ879121.2*Capsicum chinense* mRNA for glutathione S-transferase GST,2e-144	CAI51314.2glutathione S-transferase GST1 [*Capsicum chinense*]6e-119	AT2G47730.1 GLUTATHIONE S-TRANSFERASE (CLASS PHI) 51e-67	N-terminal protein myristoylation	Glutathione transferase activity

A12P2	XP_002516786.1Membrane protein PB1A10.07c, putative [*Ricinus communis*] 6e-15	XM_002516740.1*Ricinus communis* Membrane protein PB1A10.07c,1e-28	XP_002516786.1Membrane protein PB1A10.07c, putative [*Ricinus communis*] 0.0	AT3G06170.1 TMS membrane family protein / tumour differentially expressed (TDE) family proteine-159	Endomembrane system,	unknown
B3P2	ACN58228.1 mercaptopyruvate sulfurtransferase-like protein [*Solanum lycopersicum*] 1e-82	FJ711706.1 *Solanum lycopersicum* mercaptopyruvate sulfurtransferase-like protein (MST1) mRNA, complete cds 0.0	ACN58228.1 mercaptopyruvate sulfurtransferase-like protein [*Solanum lycopersicum*] 0.0	AT1G79230.3 SULFURTRANSFERASE 1, e-153	sulfate transport	3-mercaptopyruvate sulfurtransferase activity,
B6P2	AAL18931.1arabinosidase ARA-1 [*Solanum lycopersicum*]6e-47	BT013510.1*Lycopersicon esculentum* clone 132201F, mRNA sequence1e-128	AAL18931.1arabinosidase ARA-1 [*Solanum lycopersicum*]0.0	AT3G10740.1 ALPHA-L-ARABINOFURANOSIDASE 10.0	xylan catabolic process	alpha-N-arabinofuranosidase activity,
B8P2	BAA85117.1histone H2A-like protein [*Solanum melongena*]4e-23	AY501384.1*Capsicum annuum* histone H2A-like protein mRNA2e-167	XP_002533104.1histone h2a, putative [*Ricinus communis*]4e-46	AT5G02560.1 HISTONE H2A 121e-34	nucleosome assembly	DNA binding
B9P2	No homology	GU373985.1*Capsicum annuum* stress-related protein 1 (SRP1) mRNA,7e-47	ADI60300.1stress-related protein 1 [*Capsicum annuum*]2e-114	AT3G05500.1 rubber elongation factor (REF) family protein 3e-53	stress	unknown
B11P2	AAS13435.1lipid-transfer protein [*Nicotiana attenuata*]3e-29	AY621632.1*Nicotiana attenuata* lipid transfer protein 1-like (LTP1) mRNA,2e-64	AAS13435.1lipid-transfer protein [*Nicotiana attenuata*]5e-51	AT5G59310.1 lipid transfer protein family, 1e-20	lipid transport, response to abscisic acid stimulus, response to water deprivation	Lipid binding
B12P2	No homology	AM748466.1 *Vigna unguiculata* partial mRNA for putative single-stranded nucleic acid	CAO02553.1 putative single-stranded nucleic acid binding R3H [*Vigna* unguiculata] 1e-61	AT2G40960.1 nucleic acid binding, 6e-38	unknown	Nucleic acid binding

		binding R3H , 2e-05				
C1P2	ACH68563.1alpha-tubulin [*Solanum tuberosum*]1e-52	EU935741.1*Solanum tuberosum* alpha-tubulin mRNA, partial cds2e-132	ACH68563.1alpha-tubulin [*Solanum tuberosum*]0.0	AT4G14960.2 alpha-tubulin0.0	Microtubule cytoskeleton organization	protein binding
C5P2	CAB46641.1cyclin A1 [*Solanum lycopersicum*]3e-99	X92967.1*N.tabacum* mRNA for cyclin A-like protein (clone 30	CAA63543.1cyclin A-like protein [*Nicotiana tabacum*]0.0	AT1G44110.1 Cyclin A1;1 e-138	regulation of cell cycle	cyclin-dependent protein kinase regulator activity
C6P2	CAQ57979.1profilin [*Triticum aestivum*]2e-13	XM_002514153.1*Ricinus communis* profilin, putative, mRNA3e-42	XP_002514199.1profilin, putative [*Ricinus communis*]4e-61	AT4G29350.1 profilin27e-50	actin polymerization or depolymerization, cytoskeleton organization	actin binding, protein binding
C8P2	No homology	GU177459.1 *Nicotiana tabacum* glycine-rich protein precursor (GRP-CD5) mRNA, complete cds 3e-19	ACZ73649.1glycine-rich protein precursor [*Nicotiana tabacum*]4e-10	AT2G05540.1 glycine-rich protein 0.66	unknown	unknown
C10P2	No homology	U19098.1*Lycopersicon chilense* proline-rich protein (PRP13) gene 2e-07	No homology	No homology	cell wall protein, wounding and environmental stress protein	unknown
C11P2	XP_002887944.1 protease-associated domain-containing protein [*Arabidopsis lyrata* subsp. *lyrata*] 4e-59]	BT013721.1*Lycopersicon esculentum* clone 132569F, mRNA sequence2e-172	XP_002887944.1 protease-associated domain-containing protein [*Arabidopsis lyrata* subsp. lyrata] 0.0	AT1G63690.1 protease-associated (PA) domain-containing protein 0.0	proteolysis	aspartic-type endopeptidase activity;
D5P2	XP_002524341.1Protein GIGANTEA, putative [*Ricinus communis*] 5e-11	AC212791.2 *Solanum lycopersicum* DNA sequence from clone LE_HBa-193C3 on chromosome 12, complete sequence, 4e-55	No homology	No homology	regulator of photoperiodic flowering	unknown

D10P2	XP_002529559.1zinc finger protein, putative [*Ricinus communis*] 8e-84	EF678199.1Picea sitchensis clone WS0283_D20 unknown mRNA 2e-53	XP_002512671.1zinc finger protein, putative [Ricinus communis] 0.0	AT1G57820.1 methylcytosine-binding protein with a PHD domain 0.0	DNA methylation on cytosine,	ubiquitin-protein ligase activity
D11P2	ABC61505.1AGO4-2 [*Nicotiana benthamiana*]4e-45	DQ321491.1*Nicotiana benthamiana* AGO4-2 mRNA, partial cds 2e-83	ABC61505.1AGO4-2 [*Nicotiana benthamiana*]0.0	AT2G27040.1 AGO4 0.0	DNA methylation, chromatin silencing, chromatin silencing by small RNA,	siRNA binding
H5P3	ACB05668.1cyclophilin [*Capsicum annuum*]8e-50	EU401723.1*Capsicum annuum* cyclophilin mRNA, complete cds 0.0	ACB05668.1yclophilin [*Capsicum annuum*]1e-88	AT2G21130.1 peptidyl-prolyl cis-trans isomerase / cyclophilin (CYP2) / rotamase 1e-69	protein folding	peptidyl-prolyl cis-trans isomerase activity;
E6P2	AAR83848.1ribosomal protein PETRP [*Capsicum annuum*]5e-08	AY496096.1*Capsicum annuum* ribosomal protein PETRP mRNA, 1e-28	AAR83848.1ribosomal protein PETRP [*Capsicum annuum*]2e-86	AT1G27400.1 60S ribosomal protein L17 (RPL17A) 6e-70	structural constituent of ribosome	translation
E9P2	BAG16527.1putative aconitase [*Capsicum chinense*]6e-15	X97012.1*S.tuberosum* mRNA for aconitase/aconitate hydratase 2e-140	BAG16527.1putative aconitase [*Capsicum chinense*]0.0	AT2G05710.1 Aconitase 0.0	citrate metabolic process, isocitrate metabolic process, response to abscisic acid stimulus	aconitate hydratase activity
E10P2	XP_002510912.1FK506-binding protein, putative [*Ricinus communis*]6e-23	NM_120624.1 *Arabidopsis thaliana* immunophilin, putative / FKBP-type peptidyl-prolyl cis-trans isomerase, putative	NP_196161.1immunophilin, putative / FKBP-type peptidyl-prolyl cis-trans isom3e-76	AT5G05420.1 immunophilin, putative / FKBP-type peptidyl-prolyl cis-trans isomerase, putative 5e-71	unknown	cis-trans isomerise activity
E12P2	CAA65268.1 13-lipoxygenase [*Solanum tuberosum*] 3e-104	X96405.1 *S.tuberosum* mRNA for 13-lipoxygenase, clone H1 0.0	CAA65268.1 13-lipoxygenase [*Solanum tuberosum*] 0.0	AT1G17420.1 Lipoxygenase 0.0	defense response,	Electron carrier activity, iron ion binding, lipoxygenase activity

B4P5	AAR83865.1 elongation factor 1-alpha [*Capsicum annuum*] 1e-08	D63396.1 *Nicotiana tabacum* mRNA for elongation factor-1 alpha, complete cds 9e-49	BAA09709.1 elongation factor-1 alpha [*Nicotiana tabacum*] 0.0	AT5G60390.3 elongation factor 1-alpha / EF-1-alpha 0.0	translational elongation	translation elongation factor activity
F3P2	XP_002523592.1 RNA-binding protein, putative [*Ricinus communis*] 5e-49	XM_002523546.1 *Ricinus communis* RNA-binding protein, putative, mRNA 3e-75	XP_002523592.1 RNA-binding protein, putative [*Ricinus communis*] 0.0	AT4G36960.2 RNA recognition motif (RRM)-containing protein e-147	RNA binding, nucleotide binding, nucleic acid binding	unknown
F5P2	No homology	AY829651.1 *Capsicum annuum* CPRD49-like protein mRNA, complete cds 2e-59	AAX20048.1CPRD49-like protein [*Capsicum annuum*]1e-149	AT3G11210.1 GDSL-motif lipase/hydrolase family protein, 8e-60	lipid metabolic process	carboxylesterase activity, hydrolase activity,
F6P2	ABB29931.1P40-like protein [*Solanum tuberosum*]5e-51	AB012702.1*Daucus carota* mRNA for P40-like protein, complete cds 3e-79	3e-79P40-like protein [*Solanum tuberosum*]3e-109	AT1G72370.2 P40acidic protein associated to 40S ribosomal subunit of ribosomes., e-108	mature ribosome assembly, response to osmotic stress, response to salt stress, translation	Structural constituent of ribosome
F8P2	AAC62625.1rac GTPase activating protein 2 [*Lotus japonicus*]3e-11	XM_002529918.1*Ricinus communis* gtpase activating protein, putative, mRNA 3e-21	XP_002529964.1, gtpase activating protein, putative [*Ricinus communis*] 0.0	AT2G46710.1 rac GTPase activating protein e-126	Signal transduction	Rac GTPase activator activity
F10P2	XP_002512999.1NADH dehydrogenase, putative [*Ricinus communis*]1e-32	XM_002512953.1*Ricinus communis* NADH dehydrogenase, putative, mRNA7e-38	XP_002512999.1NADH dehydrogenase, putative [*Ricinus communis*]3e-63	AT4G34700.1 complex 1 family protein / LVR family protein; 4e-51	photorespiration	Catalytic activity
G3P2	ABE96885.1 polyphenol oxidase [*Nicotiana tabacum*] 4e-24	M95197.1 Potato polyphenol oxidase mRNA, 3' end 2e-90	AAA02877.1 propolyphenol oxidase [*Solanum tuberosum*] 0.0	No homology	Biosynthesis and defence	unknown
G5P2	XP_002517060.1 eukaryotic translation initiation factor 2c,	XM_002517014.1 *Ricinus communis* eukaryotic translation initiation factor	XP_002517060.1 eukaryotic translation initiation factor 2c,	AT5G43810.1 elongation initiation factor 2c, 0.0	miRNA metabolic process	translation initiation factor activity

	putative [*Ricinus communis*] 4e-44	2c, putative, mRNA 2e-58	putative [*Ricinus communis*] 0.0			
G7P2	ACV74414.1 putative cinnamyl alcohol dehydrogenase [*Camellia sinensis*] 5e-36	GQ438848.1 *Camellia sinensis* putative cinnamyl alcohol dehydrogenase mRNA, complete cds2e-49	ACV74414.1 putative cinnamyl alcohol dehydrogenase [*Camellia sinensis*] 0.0	AT4G39330.1 CINNAMYL ALCOHOL DEHYDROGENASE 9 (CAD9 e-113	metabolic process,	oxidoreductase activity
G10P2	AAN73052.2 mini-chromosome maintenance protein MCM6 [*Pisum sativum*]4e-12	AY169793.2 *Pisum sativum* mini-chromosome maintenance protein MCM6 6e-31	AAN73052.2 mini-chromosome maintenance protein MCM6 [*Pisum sativum*] 0.0	AT5G44635.1 minichromosome maintenance family protein / MCM family protein 0.0	cell proliferation	ATP binding, DNA binding, DNA-dependent ATPase activity
H4P2	XP_002885354.1 t-complex protein alpha subunit of chaperonin [*Arabidopsis lyrata* subsp. *lyrata*] , 6e-46	XM_002524583.1 *Ricinus communis* chaperonin containing t-complex protein 1, alpha subunit, tcpa, putative, mRNA 9e-64	XP_002524629.1, chaperonin containing t-complex protein 1, alpha subunit, tcpa, putative [*Ricinus communis*] 0.0	AT3G20050.1 cytoplasmic chaperonin 0.0	cellular protein metabolic process, protein folding	ATP binding, unfolded protein binding
H7P2	XP_002511724.1 roteasome subunit beta type, putative [*Ricinus communis*] 3e-51	AJ291742.1 *Nicotiana tabacum* partial mRNA for putative beta6 proteasome subunit (b6 gene) 5e-121	CAC43327.1 putative beta6 proteasome subunit [*Nicotiana tabacum*] 8e-101	AT3G60820.2 20S proteasome beta subunit PBF1 (PBF1) 1e-67	defense response to fungus, incompatible interaction, ubiquitin -dependent protein catabolic process	peptidase activity, threonine-type endopeptidase activity
F9P4	AAR83898.1 ubiquitin-conjugating protein [*Capsicum annuum*] 6e-05	DQ294262.1 *Solanum tuberosum* clone 130D10 ubiquitin-conjugating enzyme E2-like protein mRNA, complete cds2e-116	XP_002532653.1 ubiquitin-conjugating enzyme E2, putative [*Ricinus communis*, 1e-81	AT4G27960.2 ubiquitin conjugating enzyme 8e-79	ubiquitin-dependent protein catabolic process	ubiquitin-protein ligase activity
A11P4	XP_002529148.1Transmembrane protein TPARL, putative [*Ricinus communis*]2e-77	DQ672569.1*Capsicum annuum* CCR4 associated factor 1-related protein2e-122	ABG66307.1CCR4 associated factor 1-related protein [*Capsicum annuum*]1e-151	AT3G44260.1 CCR4-NOT transcription complex proteine-112	RNA modification, response to biotic stimulus, response to	nucleic acid binding, ribonuclease activity

					wounding	
A1P3	ACC95444.1beta-ketoacyl-CoA synthase [*Helianthus annuus*]1e-24	XM_002324240.1 *Populus trichocarpa* beta-ketoacyl-coa synthase family protein, mRNA 2e-30	XP_002324276.1 beta-ketoacyl-coa synthase family protein [*Populus trichocarpa*] 0.0	AT2G26640.1 3-ketoacyl-CoA synthase	response to cold, response to light stimulus	acyltransferase activity
A7P3	AAF33112.1RPT2 [*Arabidopsis thaliana*]5e-15	XM_002533834.1*Ricinus communis* Root phototropism protein, putative, mRNA1e-04	XP_002533880.1Root phototropism protein, putative [*Ricinus communis*]0.0	AT2G30520.1 ROOT PHOTOTROPISM 2, 0.0	Phototropism	protein binding
A10P3	No homology	AY488031.1*Capsicum annuum* ribosomal protein L19 mRNA,1e-114	AAR83877.160S ribosomal protein L19 [*Capsicum annuum*]4e-47	AT3G16780.1 60S ribosomal protein L19 (RPL19B)	ribosome biogenesis, translation	Structural constituent of ribosome
A12P3	XP_002865215.1 GDSL-motif lipase/hydrolase family protein [*Arabidopsis lyrata* subsp. *lyrata*], 5e-23	BT009458.1 *Triticum aestivum* clone wlsu2.pk0001.h3:fis, full insert mRNA sequence 1e-26	NP_974770.1 geranylgeranyl transferase type II beta subunit, putative / RAB geranylgeranyltransferase beta subunit, putative [*Arabidopsis thaliana*] 3e-122	AT5G12210.2 RAB GERANYLGERANYL TRANSFERASE BETA SUBUNIT 1	catalytic activity	unknown
B2P3	No homology	AY256915.1*Petunia x hybrida* S-adenosyl-L-homocystein hydrolase mRNA,9e-54	AAP92452.1S-adenosyl-L-homocystein hydrolase [*Petunia x hybrida*]0.0	AT3G23810.1 S-ADENOSYL-L-HOMOCYSTEINE (SAH) HYDROLASE 2	metabolic process, one-carbon metabolic process	adenosylhomocysteinase activity, binding, catalytic activity
B7P3	XP_002512065.1SP1L, putative [*Ricinus communis*] 5e-28	XM_002512019.1*Ricinus communis* SP1L, putative, mRNA,4e-32	XP_002512065.1SP1L, putative [*Ricinus communis*] 8e-42	AT1G26355.1 SPIRAL1-LIKE1	growth and development	unknown
B10P3	XP_002514526.1importin, putative [*Ricinus communis*]1e-09	XM_002514480.1*Ricinus communis* importin, putative, mRNA,6e-29	XP_002514526.1importin, putative [*Ricinus communis*]0.0	AT1G26170.1 binding / protein transporter; 0.0	intracellular protein transport, protein import into nucleus, docking	Binding, protein transporter activity
B12P3	ABU49726.1ferritin [*Solanum tuberosum*]6e-	AY083924.1*Nicotiana tabacum* ferritin mRNA,	ACV50433.1chloroplast ferritin 2 precursor	AT3G11050.1 ferritin 2 (ATFER2)2e-	cellular iron ion homeostasis, iron ion	ferric iron binding

	17	complete cds 4e-45	[*Jatropha curcas*]2e-98	90	transport,	
C1P3	No homology	DQ098654.1 *Lycopersicon esculentum* xyloglucan endotransglycosylase/hydrolase 16 protein (XTH16) mRNA, partial cds1e-32	AAZ08349.1 xyloglucan endotransglycosylase/hydrolase 16 protein [*Solanum lycopersicum*] 9e-162	AT4G03210.1 encodes a member of xyloglucan endotransglucosylase/hydrolases (XTHs) e-121	cellular glucan metabolic process	Hydrolase activity, acting on glycosyl bonds, xyloglucan:xyloglucosyl transferase activity
C2P3	ABY21250.1metallothionein-like protein [*Solanum tuberosum*]2e-16	AJ879116.1*Capsicum chinense* mRNA for metallothionein-like protein 7e-70	CAI51310.1metallothionein-like protein [*Capsicum chinense*]6e-08	No homology	heavy metal detoxification	unknown
C4P3	XP_002317554.1nbs-lrr resistance protein [*Populus trichocarpa*]3e-14	AK320048.1*Solanum lycopersicum* cDNA, clone: LEFL1004BH01, HTC in leaf3e-90	XP_002322892.1nbs-lrr resistance protein [*Populus trichocarpa*]0.0	AT3G50950.1 disease resistance protein (CC-NBS-LRR class)	apoptosis, defense response	ATP binding
C6P3	AAT08712.1nucleoside diphosphate kinase [*Hyacinthus orientalis*]7e-08	AF108881.1*Capsicum annuum* nucleoside diphosphate kinase mRNA,2e-57	AAZ85394.1cytosolic nucleoside diphosphate kinase [*Solanum chacoense*]2e-76	AT4G09320.1 nucleoside diphosphate kinase type 1 (NDPK1) gene	response to cadmium ion, response to salt stress	ATP binding, nucleoside diphosphate kinase activity
C8P3	CAA78702.1ribulose bisphosphate carboxylase activase [*Nicotiana tabacum*]8e-20	Z14979.1 *N.tabacum* Rca gene for ribulose bisphosphate carboxyase activase (pJQ11) 1e-53	CAA78702.1ribulose bisphosphate carboxylase activase [*Nicotiana tabacum*]3e-115	AT2G39730.3 Rubisco activase 4e-99	defense response to bacterium, response to cold, response to light stimulus	ATP binding, ATP binding
C10P3	XP_002516841.1NADH dehydrogenase, putative [*Ricinus communis*]5e-32	XM_002871795.1*Arabidopsis lyrata* subsp. *lyrata* NADH-ubiquinone oxidoreductas 6e-42	NP_197381.1 NADH-ubiquinone oxidoreductase 19 kDa subunit (NDUFA8) family protein [*Arabidopsis thaliana*]	AT5G18800.2 NADH-ubiquinone oxidoreductase 1e-53	mitochondrial electron transport, NADH to ubiquinone	NADH dehydrogenase (ubiquinone) activity
C12P3	BAG16529.1putative Ran/TC4 protein [*Capsicum chinense*]3e-	DQ222522.1*Solanum* tuberosum clone 129C03 Ran/TC4-like protein	P38546.1RecName: Full=GTP-binding nuclear protein Ran1;5e-126	AT5G55190.1 RAN GTPasee-127	protein import into nucleus	GTPase activity

	10	mRNA6e-87				
D1P3	CAA65186.1aquaporin [*Helianthus annuus*]5e-20	AF290618.1*Nicotiana glauca* putative delta TIP (MIP2) mRNA4e-52	AAG44945.1putative delta TIP [*Nicotiana glauca*]2e-111	AT3G16240.1 DELTA TONOPLAST INTEGRAL PROTEIN2e-76	Water transporrt	Ammonia transmembrane transporter activity,
D4P3	AAY21068.1cytosolic ascorbate peroxidase [*Capsicum annuum*] 4e-86	DQ002888.1 *Capsicum annuum* cytosolic ascorbate peroxidase (APX1) mRNA, complete cds0.0	AAY21068.1cytosolic ascorbate peroxidase [*Capsicum annuum*]2e-123	AT1G07890.8 cytosolic ascorbate peroxidase APX1, e-107	unknown	L-ascorbate peroxidase activity
D10P3	AAA34198.1proteinase inhibitor I [*Solanum peruvianum*]4e-05	M74102.1*Nicotiana sylvestris* serine proteinase inhibitor I mRNA, complete1e-19	AAA34198.1proteinase inhibitor I [*Solanum peruvianum*]8e-22	AT2G38870.1 Predicted to encode a PR (pathogenesis-related) 4e-13	response to wounding	Serine-type endopeptidase inhibitor activity
E1P3	AAT68265.1lipid transfer protein precursor [*Nicotiana glauca*]4e-12	AY621634.1*Nicotiana glauca* lipid transfer protein precursor (LTP2) gene,5e-27	AAT68265.1lipid transfer protein precursor [*Nicotiana glauca*]6e-47	AT5G59320.1 LIPID TRANSFER PROTEIN 37e-21	response to abscisic acid stimulus, response to water deprivation	Lipid binding
E3P3	AAB63381.2beta-tubulin 3 [*Nicotiana tabacum*]2e-10	U91563.2*Nicotiana tabacum* beta-tubulin 2 mRNA, complete cds6e-75	XP_002510784.1tubulin beta chain, putative [*Ricinus communis*]2e-05	AT5G23860.1 beta-tubulin 0.0	microtubule-based process, response to salt stress	structural constituent of cytoskeleton
E4P3	AAX83944.1Skp1 [*Capsicum annuum*]1e-20	AY899281.1*Capsicum annuum* Skp1 mRNA, complete cds1e-81	AAX83944.1kp1 [*Capsicum annuum*]1e-76	AT5G42190.1 ARABIDOPSIS SKP-LIKE 2	SCF ubiquitin ligase complex	ubiquitin-protein ligase activity
E5P3	NP_001147611.1agmatine coumaroyltransferase [*Zea mays*] 2e-16	AK327374.1*Solanum lycopersicum* cDNA, clone: LEFL2028C04, HTC in fruit2e-38	XP_002533025.1 Anthranilate N-benzoyltransferase protein, putative [*Ricinus communis*] 1e-136	AT5G48930.1 HYDROXYCINNAMOYL-COA SHIKIMATE/QUINATE HYDROXYCINNAMOYL TRANSFERASE 6e-47	auxin homeostasis, lignin biosynthetic process, positive regulation of flavonoid biosynthetic process	quinate O-hydroxycinnamoyltransferase activity
E8P3	NP_197954.1exostosin family protein [*Arabidopsis thaliana*]2e-	AK328164.1*Solanum lycopersicum* cDNA, clone: LEFL2048D18, HTC in	XP_002869243.1exostosin family protein [*Arabidopsis lyrata* subsp.	AT4G32790.1 exostosin family protein0.0	catalytic activity	unknown

	17	fruit2e-59	*lyrata*] 8e-149			
F3P3	No homology	AB107692.1 *Nicotiana tabacum* AGP4 mRNA for AG-motif binding protein-4, complete cds8e-05	BAC98494.1AG-motif binding protein-4 [*Nicotiana tabacum*]7e-148	AT5G66320.2 GATA transcription factor gene GNC1e-39	regulation of transcription, DNA-dependent	transcription factor activity
F5P3	CAD27343.1protein kinase 2 beta chain [*Nicotiana tabacum*]4e-65	AJ438265.1*Nicotiana tabacum* mRNA for protein kinase 2 beta chain 0.0	CAD27343.1protein kinase 2 beta chain [*Nicotiana tabacum*]4e-65	AT2G44680.1 Nuclear-localized casein kinase II beta chain, a CK2 regulatory subunit.	protein ubiquitination	protein serine/threonine kinase activity
F8P3	No homology	AB158477.1 *Arabidopsis thaliana* JAC1 mRNA for auxilin-like J-domain protein, complete cds 6e-08	NP_565101.1 AC1 (J-DOMAIN PROTEIN REQUIRED FOR CHLOROPLAST ACCUMULATION RESPONSE 1); heat shock protein binding [*Arabidopsis thaliana*] 0.0	AT1G75100.1 J-DOMAIN PROTEIN REQUIRED FOR CHLOROPLAST ACCUMULATION RESPONSE 1, 0.0	chloroplast accumulation movement	Heat shock protein binding
G3P3	No homology	EU558534.1 *Arabidopsis lyrata* clone SINE9 transposon-insertion display band genomic sequence 5e-47	CAQ43070.2 putative puroindoline b protein [*Triticum aestivum* subsp. *macha*] 3e-36	No homology	genome organization and expression	unknown
G10P3	No homology	XM_002517840.1*Ricinus communis* ADP,ATP carrier protein, putative, mRNA,3e-95	XP_002517886.1ADP,ATP carrier protein, putative [*Ricinus communis*]1e-69	AT4G28390.1 mitochondrial ADP/ATP carrier protein e-158	Mitochondrial transporter	ATP:ADP antiporter activity
G12P3	XP_002532594.1strictosidine synthase, putative [*Ricinus communis*]2e-28	XM_002274098.1 PREDICTED: *Vitis vinifera* hypothetical protein LOC100259372 (LOC100259372), mRNA 1e-15	NP_177542.1 SS2 (STRICTOSIDINE SYNTHASE 2); strictosidine synthase [*Arabidopsis thaliana*] *thaliana*] 7e-67	AT1G74010.1 strictosidine synthase family protein; e-156	biosynthetic process	Strictosidine synthase activity

H6P3	No homology	AY169238.2*Nicotiana tabacum* DNA topoisomerase II mRNA, complete cds1e-46	AAN85207.1DNA topoisomerase II [*Nicotiana tabacum*]0.0	AT3G23890.2 topoisomerase II,0.0	DNA metabolic process, DNA topological change,	ATP binding, DNA binding
H9P3	No homology	NM_119189.1 *Arabidopsis thaliana* TET9 (TETRASPANIN9) (TET9) mRNA, complete cds8e-05	NP_194772.1TET9 (TETRASPANIN9) [*Arabidopsis thaliana*]2e-141	AT4G30430.1 TETRASPANIN	aging	signaling complexes controlling cell differentiation
H10P3	AAG44945.1putative delta TIP [*Nicotiana glauca*],9e-43	AF290618.1*Nicotiana glauca* putative delta TIP (MIP2) mRNA, complete cds,5e-103	AAG44945.1putative delta TIP [*Nicotiana glauca*] 2e-111	AT3G16240.1 Delta tonoplast intrinsic protein, 2e-76	transport, urea transport, water transport	mmonia transmembrane transporter activity, methylammonium transmembrane transporter activity
C9P4	XP_002520383.1copper ion binding protein, putative [*Ricinus communis*] 3e-19	XM_002520337.1*Ricinus communis* copper ion binding protein, putative, mRNA,4e-27	XP_002520383.1copper ion binding protein, putative [*Ricinus communis*]9e-60	AT2G42840.1 extracellular proline-rich protein,2e-40	unknown	maintaining copper homeostatic
D10P4	XP_002515889.1chmp1, putative [*Ricinus communis*]5e-22	NM_105961.2*Arabidopsis thaliana* VPS46.2 (VPS46.2) mRNA, complete cds,8e-35	NP_565053.1VPS46.2 [*Arabidopsis thaliana*]2e-93	AT1G73030.1 CHARGED MULTIVESICULAR BODY PROTEIN/CHROMATIN MODIFYING PROTEIN1A, 2e-82	endosome transport via multivesicular body sorting pathway, growth, vesicle-mediated transport	unknown
E5P4	No homology	AY496102.1*Capsicum annuum* Vip2 protein mRNA, complete cds,2e-16	AAL49748.1channel-like protein [*Petunia x hybrida*]3e-24	AT4G00430.1 RANSMEMBRANE PROTEIN Ce-142	response to water deprivation, transport	Water channel activity
E7P4	ACS12837.1chalcone synthase [*Nicotiana alata*]2e-107	U47740.1*Solanum tuberosum* chalcone synthase 1b mRNA, complete cds0.0	AAK49457.1chalcone synthase [*Nicotiana tabacum*]0.0	AT5G13930.1 CHALCONE SYNTHASE0.0	auxin polar transport, chalcone biosynthetic process	naringenin-chalcone synthase activity
E12P4	XP_002527783.1phosphoglucomutase, putative	XM_002527737.1*Ricinus communis*	XP_002527783.1phosphoglucomutase, putative	AT1G23190.1 phosphoglucomutase0.0	carbohydrate metabolic	intramolecular transferase activity,

	[*Ricinus communis*]5e-111	phosphoglucomutase, putative, mRNA4e-169	[*Ricinus communis*]0.0		process, response to cadmium ion	phosphotransferases
F2P4	XP_002537268.1endo-1,3(4)-beta-glucanase, putative [*Ricinus communis*]4e-13	DQ190943.1 *Medicago truncatula* beta-glucan-binding protein 4 (GBP4) mRNA, complete cds 2e-20	ABB69784.1beta-glucan-binding protein 4 [*Medicago truncatula*]0.0	AT5G15870.1 glycosyl hydrolase family 81 protein,0.0	endo-1,3(4)-beta-glucanase activity	unknown
F4P4	NP_200894.1AtRABA1f (Arabidopsis Rab GTPase homolog A1f)2e-27	AK326360.1*Solanum lycopersicum* cDNA, clone: LEFL2005I16, HTC in fruit3e-118	NP_200894.1AtRABA1f (Arabidopsis Rab GTPase homolog A1f)1e-111	AT5G60860.1 Rab GTPase homolog A1f (AtRABA1f) e-116	protein transport, small GTPase mediated signal transduction	GTP binding
F5P4	XP_002515572.1syntaxin, putative [*Ricinus communis*] 2e-31	AK326056.1 *Solanum lycopersicum* cDNA, clone: LEFL2002AB01, HTC in fruit 2e-137	XP_002515572.1syntaxin, putative [*Ricinus communis*]1e-93	AT3G24350.1 SYNTAXIN OF PLANTS 32, 9e-74	cellular membrane fusion, intracellular protein transport	SNAP receptor activity
F6P4	No homology	M95201.1Potato 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase,2e-25	CAA75386.1 2-dehydro-3-deoxyphosphoheptonate aldolase; 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase [*Morinda citrifolia*] 0.0	AT4G39980.1 2-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, 0.0	aromatic amino acid family biosynthetic process, chorismate biosynthetic process, response to bacterium, response to wounding	protein binding
F7P4	ACR09632.1chitinase [*Capsicum chinense*]2e-60	FJ895872.1*Capsicum chinense* chitinase mRNA, partial cds7e-101	ACR09632.1chitinase [*Capsicum chinense*]5e-147	AT3G12500.1 PATHOGENESIS-RELATED 3, 1e-72	defense response to fungus, jasmonic acid and ethylene-dependent systemic resistance	chitinase activity
F10P4	No homology	AF215853.1 *Solanum tuberosum* hexose transporter (pGlcT) mRNA, partial cds; nuclear gene for chloroplast product 3e-44	AAF74567.1hexose transporter [*Solanum tuberosum*]0.0	AT5G16150.3 glucose transporter. e-178	response to trehalose stimulus	Carbohydrate transmembrane transporter activity, sugar:hydrogen symporter activity
G8P4	ABD32677.1Like-Sm	AK322824.1	NP_179971.1	AT2G23930.1	nucleus, small	Unknown

	ribonucleoprotein-related, core [*Medicago truncatula*]4e-32	*Solanum lycopersicum* cDNA, clone: LEFL1043DD06, HTC in leaf 6e-126	SNRNP-G (PROBABLE SMALL NUCLEAR RIBONUCLEOPROTEIN G) [*Arabidopsis thaliana*] 2e-33	PROBABLE SMALL NUCLEAR RIBONUCLEOPROTEIN G, 1e-34	nucleolar ribonucleoprotein complex	
A1P5	XP_002512999.1NADH dehydrogenase, putative [*Ricinus communis*]1e-32	XM_002512953.1*Ricinus communis* NADH dehydrogenase, putative, mRNA7e-38	XP_002512999.1NADH dehydrogenase, putative [*Ricinus communis*]3e-63	AT4G34700.1 complex 1 family protein / LVR family protein, 4e-51	photorespiration	catalytic activity
A4P5	ACT32032.1 putative glycerol-3-phosphate acyltransferase [*Vernicia fordii*] 3e-26	XM_002511827.1 *Ricinus communis* ER glycerol-phosphate acyltransferase, mRNA 6e-45	XP_002511873.1ER glycerol-phosphate acyltransferase [*Ricinus communis*]0.0	AT4G00400.1 GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 8, 0.0	cutin biosynthetic process, metabolic process	acyltransferase activity, glycerol-3-phosphate O-acyltransferase activity
A10P5	No homology	AM748435.1 *Vigna unguiculata* partial mRNA for putative plant disease resistance response protein family (Os11g0179700 gene), clone 49 5e-11	CAO02551.1 putative plant disease resistance response protein family [*Vigna unguiculata*] 3e-52	No homology	No homology	Resistant to pathogen
B6P5		CU228024.1Populus EST from severe drought-stressed leaves,2e-14	XP_002298214.1predicted protein [*Populus trichocarpa*]2e-18	No homology	No homology	Drought –stress protein
B9P5	ACL50298.1SUMO1b protein [*Zea mays*]1e-11	NM_118818.3*Arabidopsis thaliana* SUMO1 (SMALL UBIQUITIN-LIKE MODIFIER,1e-23	NP_194414.1 SUMO1 (SMALL UBIQUITIN-LIKE MODIFIER 1); protein binding / protein tag [*Arabidopsis thaliana*] 5e-45	AT4G26840.1 SMALL UBIQUITIN-LIKE MODIFIER 1	protein sumoylation, response to heat	protein binding
B12P5	AAF28382.1DnaJ-like protein [*Solanum*	AY491517.1*Capsicum annuum* DnaJ-like protein	AAF28382.1DnaJ-like protein [*Solanum*	AT3G44110.1 co-chaperon DNAJ	protein folding, regulation of	protein binding

	lycopersicum],2e-58	mRNA, partial sequence,4e-175	*lycopersicum*]1e-92	protein e-142	ATPase activity, response to salt stress	
C9P5	AAL29690.1,profilin [*Solanum lycopersicum*]3e-10	XM_002514153.1,*Ricinus communis* profilin, putative, mRNA,4e-35	XP_002514199.1profilin, putative [*Ricinus communis*]4e-61	AT4G29350.1 profilin2 7e-50	Actin polymerization or depolymerisation, cytoskeleton organization	actin binding, protein binding
C11P5	XP_002299901.1predicted protein [*Populus trichocarpa*] 2e-42	AK329145.1 *Solanum lycopersicum* cDNA, clone: LEFL3105K09, HTC in root 3e-126	XP_002299901.1predicted protein [*Populus trichocarpa*]5e-24	AT5G12470.1 unknown protein e-121	No homology	No homology
D10P5	AAP03872.1 putative photosystem I subunit III precursor [*Nicotiana tabacum*] 8e-26	AY220077.1 *Nicotiana tabacum* putative photosystem I subunit III precursor, mRNA, complete cds; nuclear gene for chloroplast product 6e-62	AAP03872.1 putative photosystem I subunit III precursor [*Nicotiana tabacum*] 8e-102	AT1G31330.1 subunit F of photosystem I. 5e-76	Photosynthesis	Unknown
E6P5	AAX59986.2violaxanthin de-epoxidase [*Zingiber officinale*]9e-21	FJ648424.1 *Solanum lycopersicum* violaxanthin de-epoxidase mRNA, complete cds 4e-108	ACM92036.1violaxanthin de-epoxidase [*Solanum lycopersicum*]0.0	AT1G08550.2 Violaxanthin deepoxidase, e-168	chlorophyll metabolic process, fatty acid metabolic process, response to heat,xanthophyll cycle, xanthophyll metabolic process	violaxanthin de-epoxidase activity
E8P5	NM_115038.2 *Arabidopsis thaliana* ATG1 (ARABIDOPSIS TRANSMEMBRANE PROTEIN G1P-RELATED 1) (ATG1) mRNA, complete cds 1e-23	NP_190747.1 ATG1 (ARABIDOPSIS TRANSMEMBRANE PROTEIN G1P-RELATED 1) [*Arabidopsis thaliana*] 5e-18	NP_190747.1 ATG1 (ARABIDOPSIS TRANSMEMBRANE PROTEIN G1P-RELATED 1) [*Arabidopsis thaliana*] 7e-114	AT3G51790.1 ARABIDOPSIS TRANSMEMBRANE PROTEIN G1P-RELATED 1 e-115	Cytochrome complex assembly	Unknown

C11P3	AAQ08191.1 eukaryotic translation initiation factor 5A isoform I [*Hevea brasiliensis*] 9e-19	AY484392.1 *Capsicum annuum* eukaryotic initiation factor 5A2 (eIF5A2) mRNA, complete cds 9e-142	AAQ08191.1 eukaryotic translation initiation factor 5A isoform I [*Hevea brasiliensis*] 1e-71	AT1G13950.1 EUKARYOTIC ELONGATION FACTOR 5A, 9e-74	Translational initiation, xylem development	Translational initiation factor activity

PREPARATION OF REAGENTS, BUFFERS AND MEDIA

Buffers/Reagents/Media	Method of preparation
1. Agarose gel electrophoresis	
i. 50X Tris acetate EDTA (TAE)	240g of Tris base, 57.1 ml of Glacial acetic acid, 0.5M EDTA was added and the volume was made upto to 100 ml with distilled H_2O. The pH was adjusted to 8.0.
ii. 6X DNA loading dye	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol was dissolved in distilled H_2O and stored at 4ºC.
iii. DNA Molecular Marker	Fermentas DNA marker-(ladder size in bp) 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500 and 250.
iv. Ethidium Bromide (10mg/ml)	1g Ethidium Bromide was added to 100ml of distilled H_2O, stirred at magnetic stirrer for several hours, transferred to a dark bottle and stored at room temperature.
2. Cloning	
i. Ampicillin	Stock solution (100mg/ml) was made in double distilled H_2O, filter sterilized (through 0.22µ filter) and aliquoted in 1.5ml tubes and stored at -20ºC.
ii. Kanamycin	Stock solution (50mg/ml) was made in double distilled H_2O, filter sterilized (through 0.22µ filter) and aliquoted in 1.5ml tubes and stored at -20ºC.
iii. Luria broth media	1g of bacto-tryptone, 0.5g of yeast extract and 1g of NaCl was dissolved in 95 ml of H_2O and the pH was adjusted to 7 with 5N NaOH. The volume was adjusted to 100 ml and sterilized by autoclaving.

iv. Luria agar media	1g of Bacto-tryptone, 0.5 g of yeast extract and 1g of NaCl was dissolved in 95 ml of water and the pH was adjusted to 7 with 5 N NaOH. The volume was adjusted to 100 ml. Then 1.5g of bacto-agar was added and sterilized by autoclaving.
v. X-gal (5-bromo- 4 chloro-3-indolyl-b D galactopyranoside)	Stock solution (20mg/ml) was prepared by dissolving X-gal in dimethyl foramide, and stored at -20ºC.
vi. IPTG (Isopropyl thio- a-galactoside)	2g of IPTG was dissolved in distilled H_2O, and volume was adjusted to 10ml then filter sterilized through 0.22 disposable filters and was stored at -20ºC.
vii. 100mM MgCl2	2.03g of $MgCl_2.6H_2O$ was dissolved in distilled H2O, volume adjusted to100ml and sterilized by autoclaving.
viii. 100mM CaCl2	1.47g of $CaCl2.2H_2O$ was dissolved in distilled H2O, volume adjusted to100ml and sterilized by autoclaving.

3. Plasmid Isolation

i. Solution I (Resuspension buffer)	It was prepared by adjusting final concentration to 1M Tris-HCl (25mM), (pH 8.0)- 2.5ml 20% Glucose (50mM) -4.5 ml 0.5M EDTA (10mM) - 2.0ml and sterile distilled H_2O- up to 100ml.
ii. Solution II (Lysis buffer)	It was prepared freshly by mixing 10N NaOH (0.2N)- 2.0ml, 1% SDS- 5.0ml, distilled H_2O up to 100ml.
iii. Solution III (pH 4.8) (Neutralization buffer)	40.81g of sodium acetate (3M) was dissolved in minimum volume of distilled H_2O, and pH was adjusted to 4.8 with glacial acetic acid, now volume adjusted to 100ml with H_2O and sterilized by autoclaving.
iv.Phenol:Chloroform: Isoamylalcohol	It was prepared by mixing Tris-saturated Phenol (pH 7.5) 25ml, chloroform 24ml, and Isoamylalcohol 1ml.

4. Total DNA Extraction

CTAB method	Extraction buffer was prepared by adjusting final concentration to 100mM TrisCl pH 8.0, 100m EDTA pH 8.0, 1.4M NaCl and 2% CTAB (w/v). Solution was incubated this solution at 65C for 30-60 min (till CTAB completely dissolves) and 1% 2-mercapto-ethanol was added just before use.

5. Southern hybridization

i. 20X SSC	It was prepared by adding 175.3g NaCl, 88.2g sodium citrate (pH adjusted to 7.0 by adding 1.4N HCl) in 1 litre of H_2O.
ii. Denauration solution	It was prepared by adjusting final concentration of 1.5M NaCl and 0.5M NaOH in H_2O.
iii. Denhardt's solution (50X)	It was prepared by adding 1% (W/V) ficol, 1% (W/V) polyvinylpyrrolidone and 1% (w/v) BSA and was dissolved in H_2O to prepare a 50X solution.
iv. Depurination solution	It was prepared by adding 2.8ml of HCl in 197.2 ml of sterile H_2O.
v. Neutralization Solution	It was prepared by adding 1.5M NaCl and 1M TrisCl pH 7.2.
vi. Herring sperm	100mg of DNA was dissolved in 10 ml of sterile distilled H_2O and mixed by vortexing, stored at -20C.
vii. Hybridization buffer	It was prepared by adding 0.25M sodium phosphate buffer (pH 7.2), 7% (w/v) SDS and 1mM EDTA.

6. Northern Hybridization

15% denaturing polyacrylamide gel (for 10 ml)	4.2 gm urea, 0.5 ml of 10X TBE, 3.75 ml of 40% (W/V) 19:1 Acylamide: bis acrylamide, 2.5 ml of water . Stirred at room temperature to dissolve urea. 70 µl of 10% ammonium per sulfate (APS) was added and 3.5 µl TEMED was poured immediately. The polymerization time was found around 30 min.
2X gel loading buffer	10 ml of deionized formamide, 200 µl of 0.5 M EDTA pH 8.0, 1mg Xylene cyanol FF and 1mg bromophenol blue. Stored upto 1 year at 4°C

PREPARATION OF COMMONLY USED STOCK SOLUTION

Calcium chloride (2.5M)

11g of CaCl2.6H_2O was dissolved in a final volume of 20ml of distilled H2O. The solution was sterilized by passing it through a 0.22 filter. It was then stored in 1ml aliquots at 4°C.

Deoxyribonucleoside triphosphates (dNTPs)

Each dNTP was dissolved in H_2O at an approximate concentration of 100mM. 0.05M Tris base and a micropipette was used to adjust the pH of each of the solutions to 7.0 (pH paper was used to check the pH). An aliquot of the neutralized dNTP was diluted appropriately and the optical density at the wavelengths as given below in the table was recorded. The actual concentration of each dNTP was calculated. The solutions were diluted with H_2O to a final concentration of 50mM dNTP. Each was stored separately at -80° C in small aliquots.

Base	**Wave length**	**Extinction Coefficient (E) ($M^{-1} cm^{-1}$)**
A	259	1.54 104
G	253	1.37 104
C	271	9.10 103
T	267	9.6 103

EDTA (0.5M, pH 8.0)

186.1g of disodium EDTA-2H_2O was added to 800ml of water. It was stirred vigorously on a magnetic stirrer and pH was adjusted to 8.0with NaOH (20g of NaOH pellets). It was dispensed into aliquots and sterilized by autoclaving.

NaOH (10N)

400g of NaOH pellets were added to 800ml of water, stirring continuously.The volume was adjusted to 1 litre with H_2O once the pellet got dissolved completely. The solution was stored in a plastic container at room temperature.

NaCl (Sodium Chloride, 5M)

292g of NaCl was dissolved in 800ml of H_2O. The volume was adjusted to 1litre with H_2O. It was dispensed into aliquots and then sterilized by autoclaving. The NaCl solution was stored at room temperature.

Potassium acetate (5M)

5M Potassium acetate- 60ml

Glacial acetic acid -11.5ml

H_2O- 28.5 ml

The resulting solution was 3M with respect to potassium and 5M with respect to acetate. The buffer was stored at room temperature.

SDS (10%w/v)

100g of electrophoresis-grade SDS was dissolved in 900ml of H_2O. It was heated to 68°C and stirred with magnetic stirrer to assist dissolution. The volume was adjusted to 1 litre with H_2O and stored at room temperature.

Sodium Acetate (3M, pH 5.2 and pH 7.0)

408.3g of sodium acetate, 3H_2O was dissolved into 800ml of H_2O. The pH was adjusted to 5.2 with glacial acetic acid. The volume was adjusted to 1 litre with H2O. It was later dispensed into aliquots and sterilized by autoclaving.

Tris HCl

121.1g of Tris base was dissolved in 800ml of H_2O. The pH was adjusted to desired value by adding concentration HCl, as mentioned below.

pH of HCl	Volume
7.4	70ml
7.6	60ml
8.0	42ml

The solution was allowed to cool at room temperature and the pH was adjusted. Then, volume of solution was adjusted to 1 litre with H_2O. This was then aliquoted and sterilized by autoclaving.

X-gal Solution (2% w/v)

A stock solution was prepared by dissolving X-gal in di-methyl formamide at a concentration of 20mg/ml solution. A glass or polypropylene tube was used. The tube containing the solution was wrapped in aluminium foil to prevent damage by light and was stored at -20°C. Sterilization of X-gal solution by filtration was not required.

Ammonium per sulfate (10%w/v)

1gm of ammonium per sulfate was dissolved in 10ml of H_2O and stored at 4ºC.

Yeast Transformation Solutions

The following stock solutions are necessary in order to prepare the yeast transformation solutions outlined below:

10× Lithium Acetate (LiAc)

A stock solution of 1 M LiAc (Sigma Catalog #L 6883) was prepared in double distilled water. pH to 7.5 was adjust with dilute acetic acid. Finally, the solution was autoclave and stored at room temperature

50% (w/v) PEG 3350

50 g of PEG (Sigma Catalog #P 3640, average molecular weight: 3350) was dissolved in dH_2O and the volume was made upto 100 ml. Finally, the solution was filter sterilized or autoclave and stored at room temperature

10× TE buffer

10× TE buffer was prepared by adding 100 mM Tris-HCl (pH 7.5) and 10 mM EDTA (pH 8.0) solutions which was then autoclaved and stored at room temperature.

TE–LiAc–PEG Solution (1× TE buffer, 1× LiAc, 40% (w/v) PEG 3350)

TE–LiAc–PEG Solution was prepared by adding 1 ml of 10× TE buffer and 1 ml of 10× LiAc in 8 ml of 50% (w/v) PEG 3350.

TE–LiAc Solution (1× TE buffer and 1× LiAc)

A solution of TE–LiAc was prepared by adding 1 ml of 10× TE buffer and 1 ml of 10× LiAc solutions in 8 ml of sterile dH2O.

1× TE Buffer

1X TE buffer was prepared by adding 1 ml of 10× TE buffer in 9 ml of dH2O

Two-Hybrid Vector System Media and Reagents

YPAD Agar (per Liter)

20 g of Difco peptone, 10 g of yeast extract and 15–20 g of agar were added to 960ml of deionized H_2O and the pH was adjusted to 5.8. 40 mg of adenine sulfate was added into it. The final solution was autoclaved and cooled to 55°C. 2% (v/v) glucose was added to the solution which was prepared by adding 40 ml of a 50% stock solution, filter sterilized or autoclaved separately.

YPAD Broth (per Liter)

20 g of Difco® peptone and 10 g of yeast extract were added to 960ml of deionized H_2O and the pH was adjusted to 5.8. 40 mg of adenine sulfate was added into it. The final solution was autoclaved and cooled to 55°C. 2% (v/v) glucose was added to the solution which was prepared by adding 40 ml of a 50% stock solution, filter sterilized or autoclaved separately.

Synthetic Minimal Medium

SD Agar (per Liter)

6.7 g of Difco yeast nitrogen base (Difco Catalog #0919-15-3) without amino acids and 15–20 g of agar were added to 860 ml deionized H_2O and the pH was adjusted to 5.8. The solution was then autoclaved and cooled to 55°C. 100 ml of the appropriate 10X dropout solution and 40 ml of a 50% stock solution of glucose (which was filter sterilized or autoclaved separately) were added and finally poured into 100 – 150 mm petri dishes.

SD Medium (per Liter)

6.7 g of Difco yeast nitrogen base without amino acids was added to 860 ml deionized H_2O and the pH was adjusted to 5.8. The solution was then autoclaved and cooled to 55°C. 100 ml of the appropriate 10× dropout solution and 40 ml of a 50% stock solution of glucose (which was filter sterilized or autoclaved separately) were then added into it.

Chemical for Chromatin immunoprecipitation (ChIP)
Cross-linking buffer 0.4 M sucrose
10 mM Tris–HCl pH 8, 1 mM PMSF,1 mM EDTA and 1% formaldehyde.

Nuclei isolation buffer
0.25 M sucrose, 15 mM PIPES pH 6.8, 5 mM MgCl2 ,60mMKCl,15mMNaCl,1mMCaCl2 ,0.9%TritonX-100, 1mMPMSF,2mgml^{-}1 pepstatin A and 2mgml^{-}1 aprotinin.

Nuclei lysis buffer
50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM, EDTA, 1% SDS, 0.1% sodium deoxycholate and 1% Triton X-100, 1mgml^{-}1 pepstatin A and 1mgml^{-}1 aprotinin.

Elution buffer
0.5% SDS and 0.1 M NaHCO3.m. Low salt wash buffer 150 mM NaCl, 20 mM Tris–HCl pH 8, 0.2% SDS, 0.5% Triton X-100 and 2 mM EDTA.

High salt wash buffer
500 mM NaCl, 20 mM Tris–HCl pH 8, 0.2% SDS, 0.5% Triton X-100 and 2 mM EDTA.mCRITICALPrepare fresh and keep at 41C.LiCl wash buffer 0.25 M LiCl, 1% sodium deoxycholate, 10 mM Tris–HCl pH8, 1% NP-40 and 1 mM EDTA.TE buffer 1 mM EDTA and 10 mM Tris–HCl pH 8.

Pepstatin A
Prepare a 1 mg ml^{-}1stock solution in methanol.

Aprotinin
Prepare 1 mg ml $^{-}$1stock solution in water.

Western Blotting Chemicals
Blocking buffer
Blocking Buffer PBSSM
1% non-fat dried milk
0.05 M phosphate
0.64 M NaCl
pH 7.4
0.1% TWEEN-20

Primary and Secondary Antibody Incubation buffer
1X PBS + 0.1% Tween20 + 1% blocking buffer

Washing Buffer
1X PBS+ 0.1% Tween20

Diaminobenzidine (DAB)
0.05% DAB in PBS

Hydrogen Peroxide
H2O2 (30%) in water

Total Plant Histone Isolation buffer

Nucleus Isolation buffer
20 mM Tris-HCl (pH 7.5), 10 mM KCl, 10 mM MgCl2, 6% sucrose, 0.6% Triton X-100, 0.05% β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF)

Nuclei Lysis and histone extraction buffer
20 mM Tris-HCl (pH 7.5), 10 mM KCl, 10 mM MgCl2, 6% sucrose, 0.6% Triton X-100, 0.05% β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF)

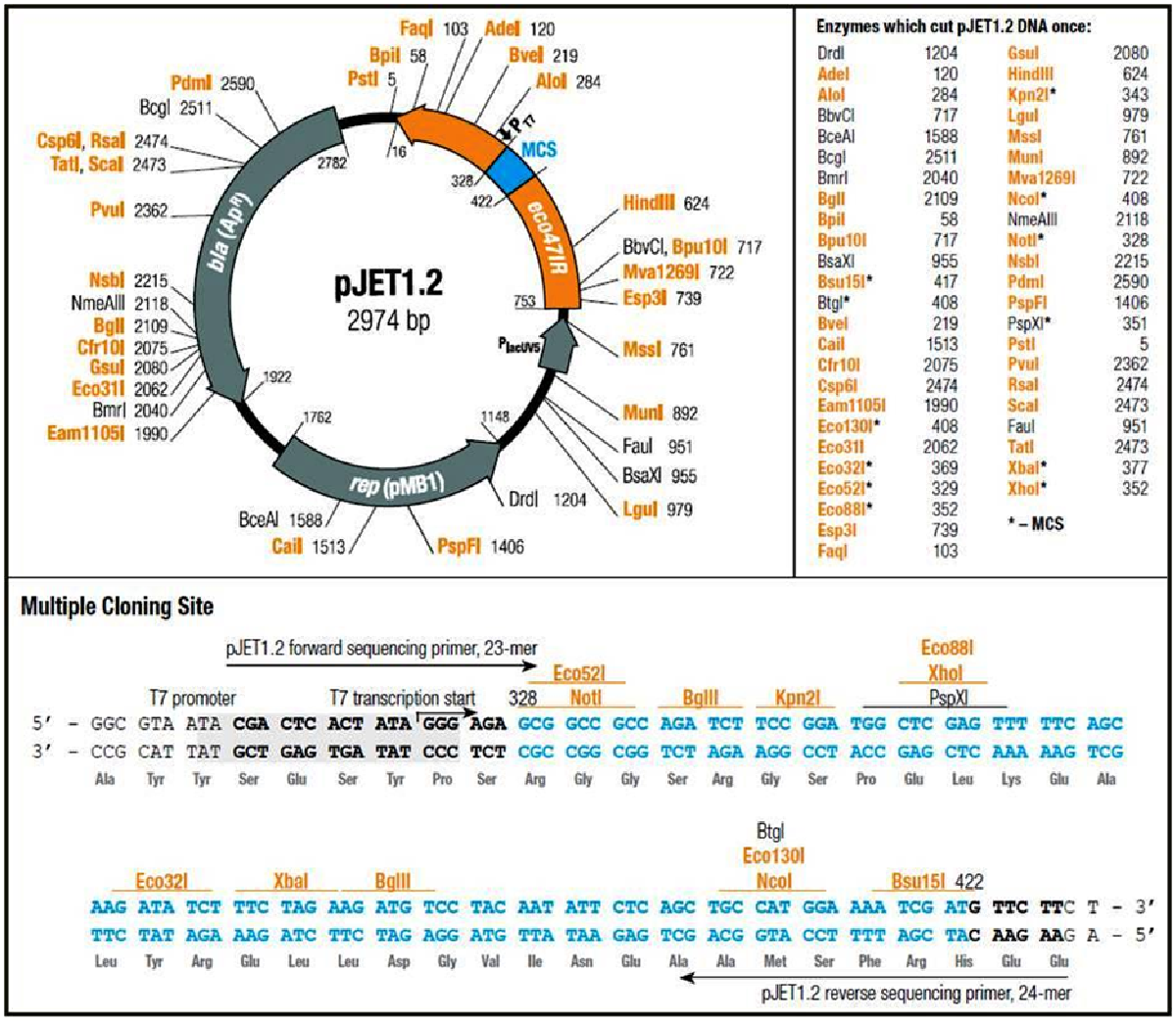

Figure.1 pJET1.2 vector map showing the position of multiple cloning sites, T7 promoter, replication origin and ampicillin resistance gene (bla). The vector contains a lethal restriction enzyme gene that is disrupted by ligation of a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids are able to form colonies

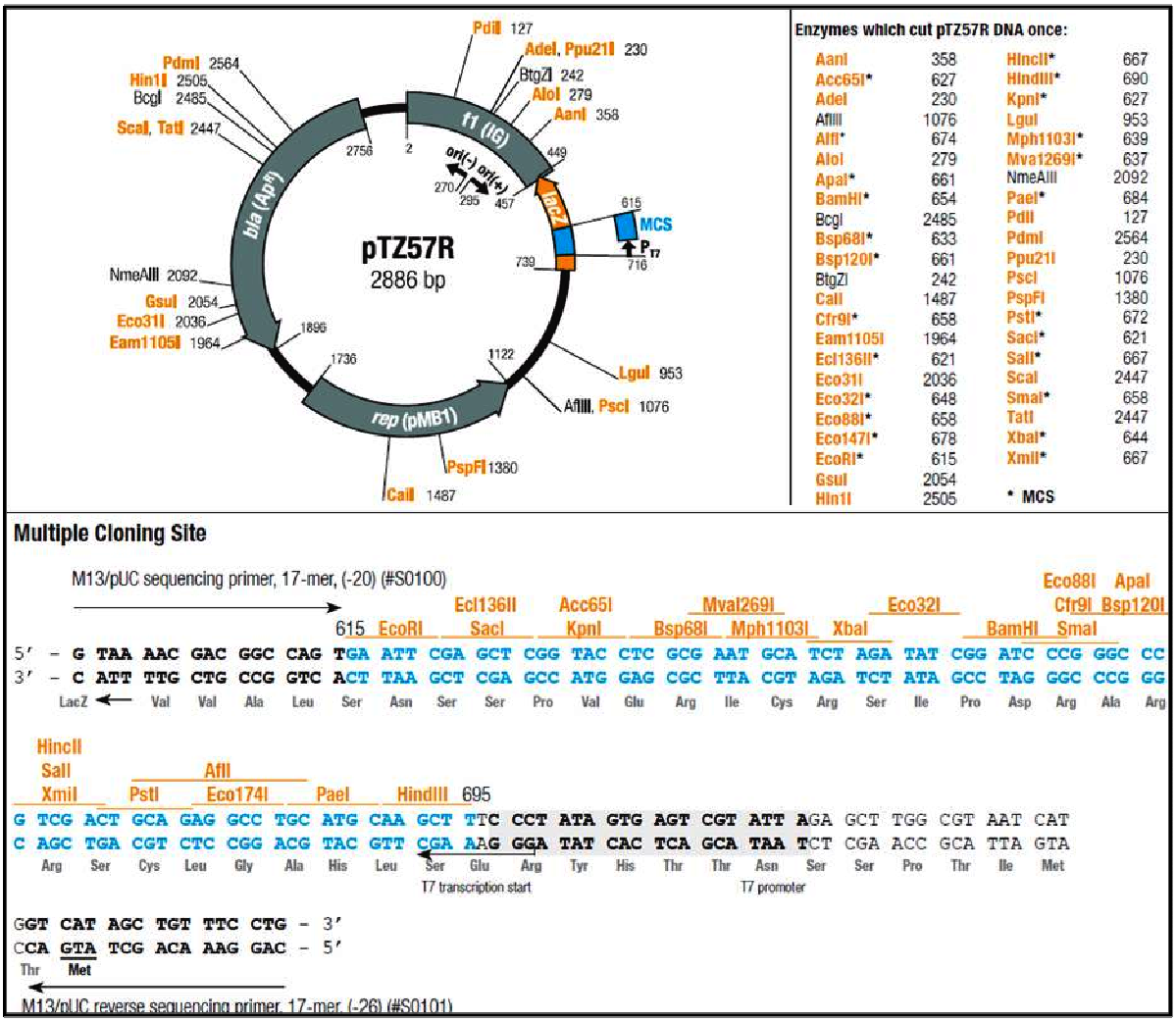

Figure.2 pTZ57R/T vector map showing the position of multiple cloning sites, LacZ gene, T7 promoter, replication origin and ampicillin resistance gene (bla).

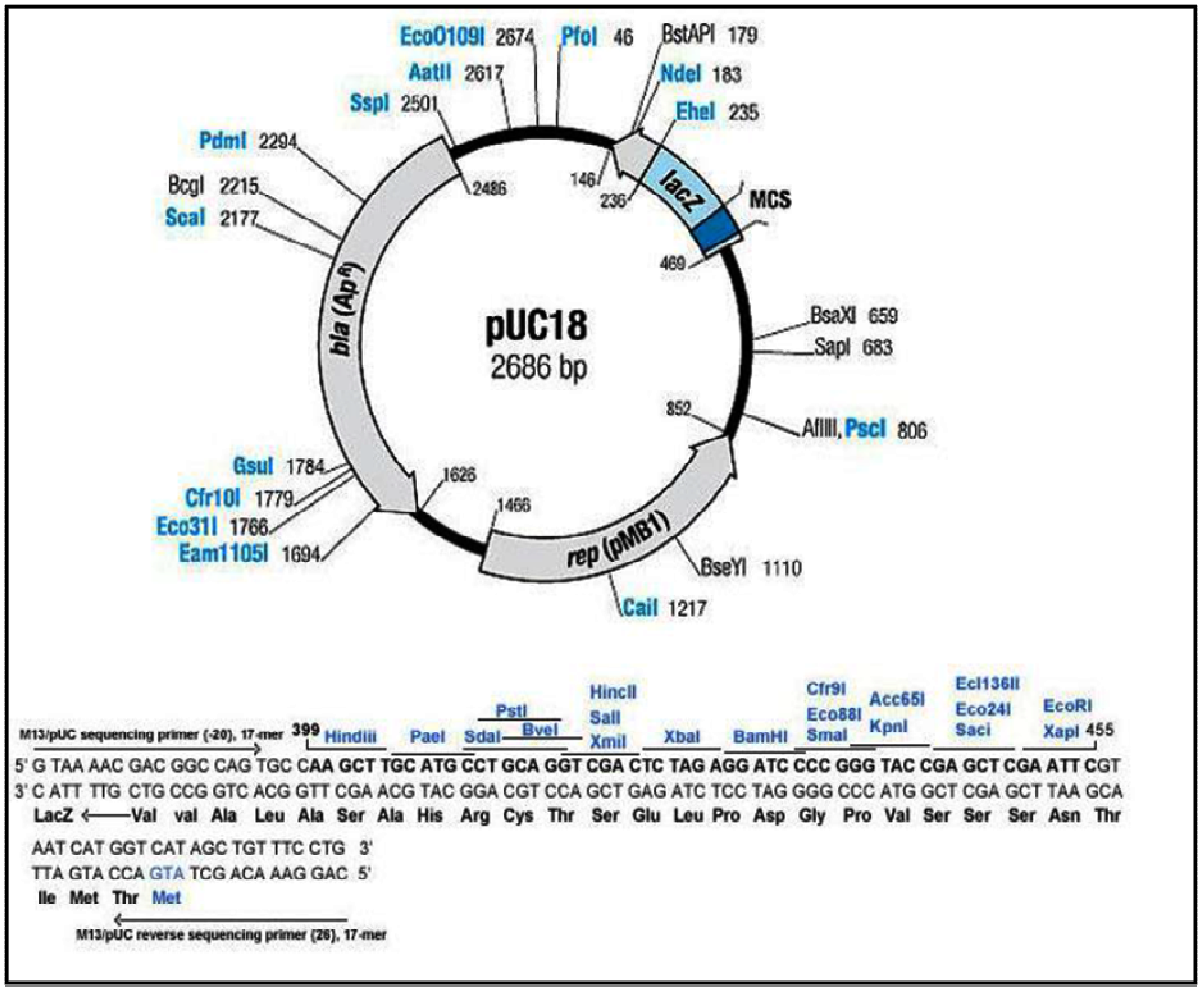

Figure.3 pUC18 vector map showing the position of multiple cloning sites, LacZ promoter, replication origin and ampicillin resistance gene (bla).

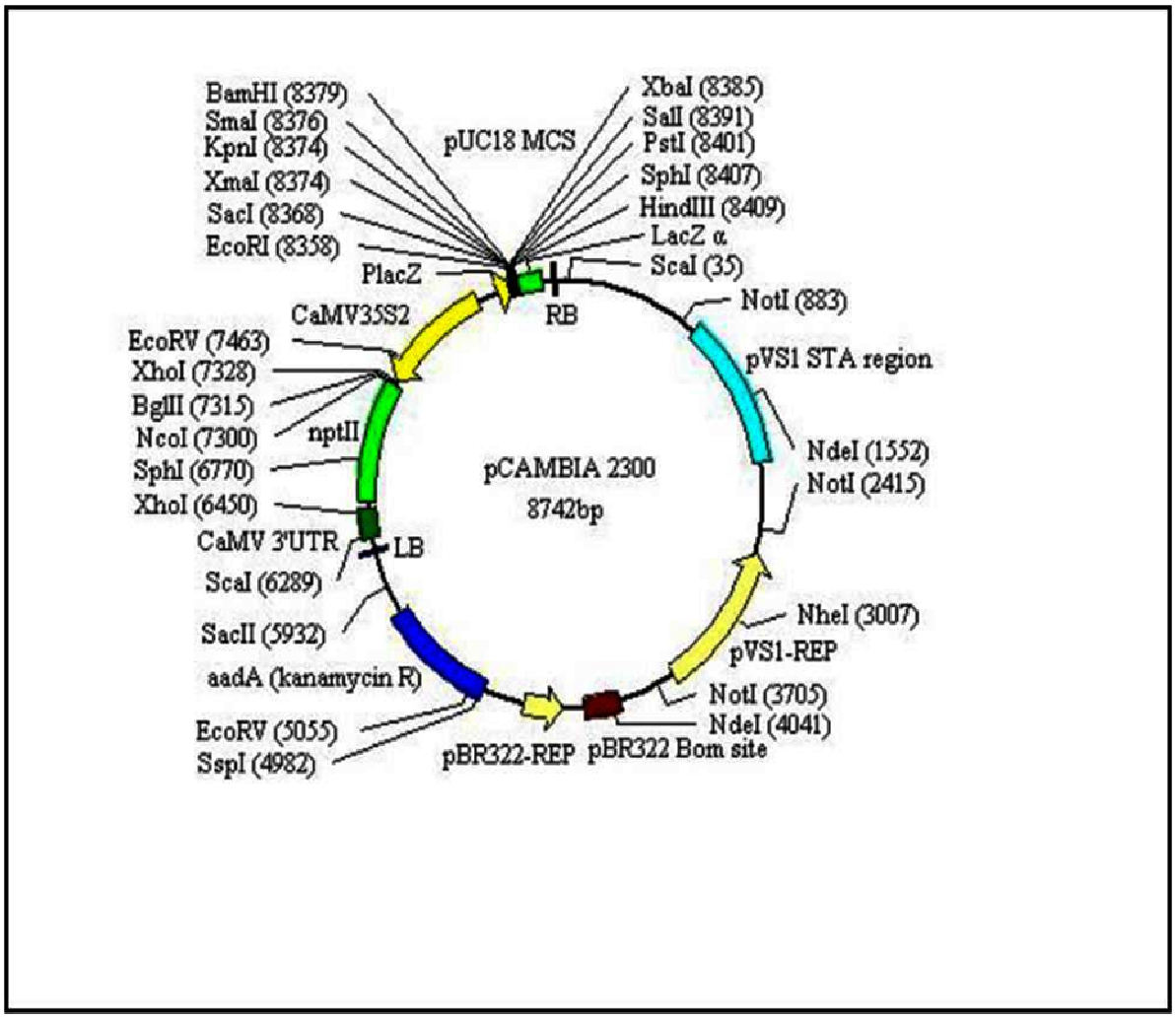

Figure.4 Schematic diagram of binary vector pCAMBIA2300 showing position of MCS, NPTII gene, left and right borders and pBR322 replication origin site.

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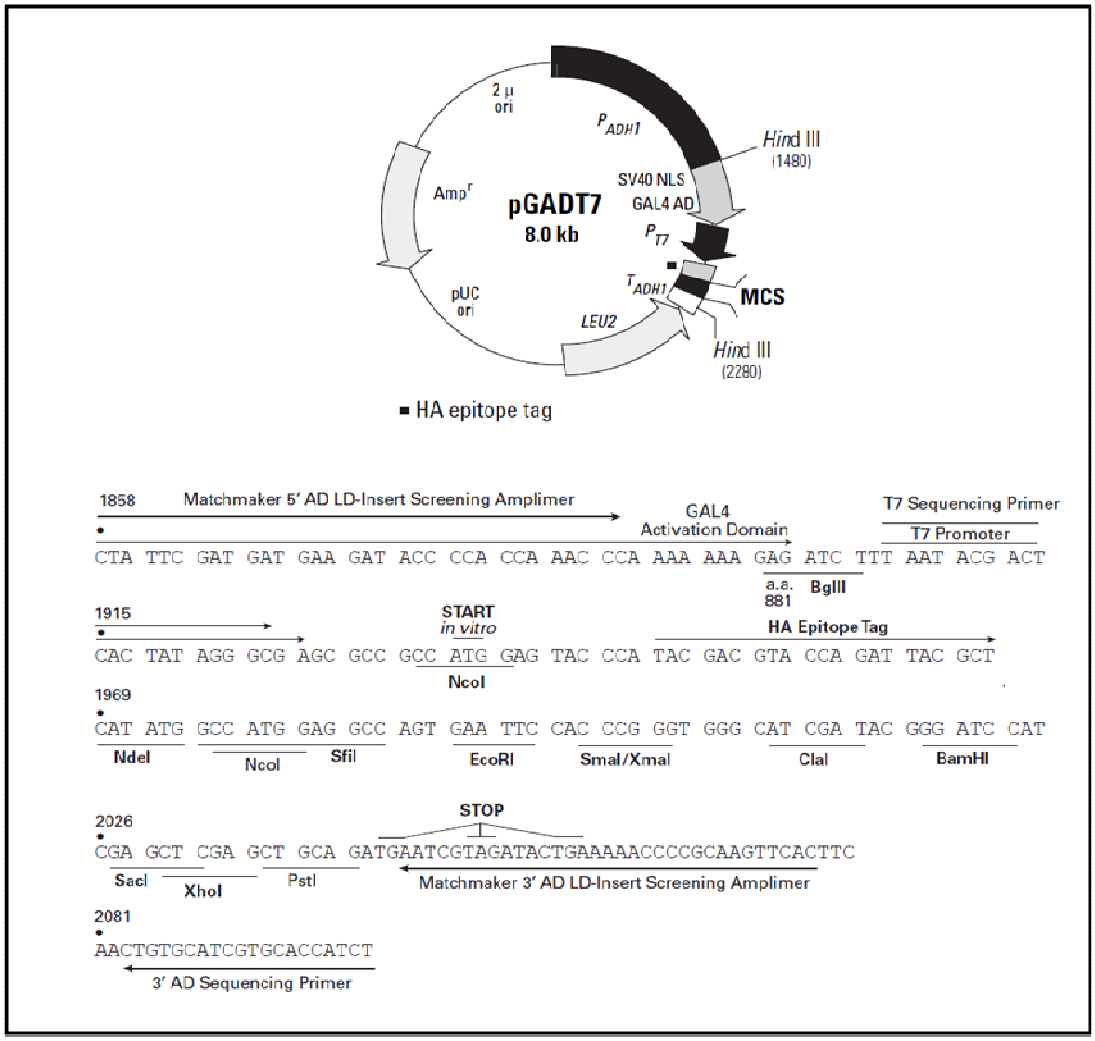

Figure 6. pGADT7 AD vector showing multiple cloning sites , full-length S. cerevisiae ADH1 (PADH1) promoter, GAL4 AD (GAL4 activation domain with SV40 Nuclear Localization Signal [NLS]), T7 RNA polymerase promoter, HA Tag (hemagglutinin epitope tag), S. cerevisiae ADH1 Terminator (TADH1) , LEU2 coding sequences, pUC ori (pUC replication origin), Amp^r gene and 2 µ ori (Yeast 2 µ replication origin).

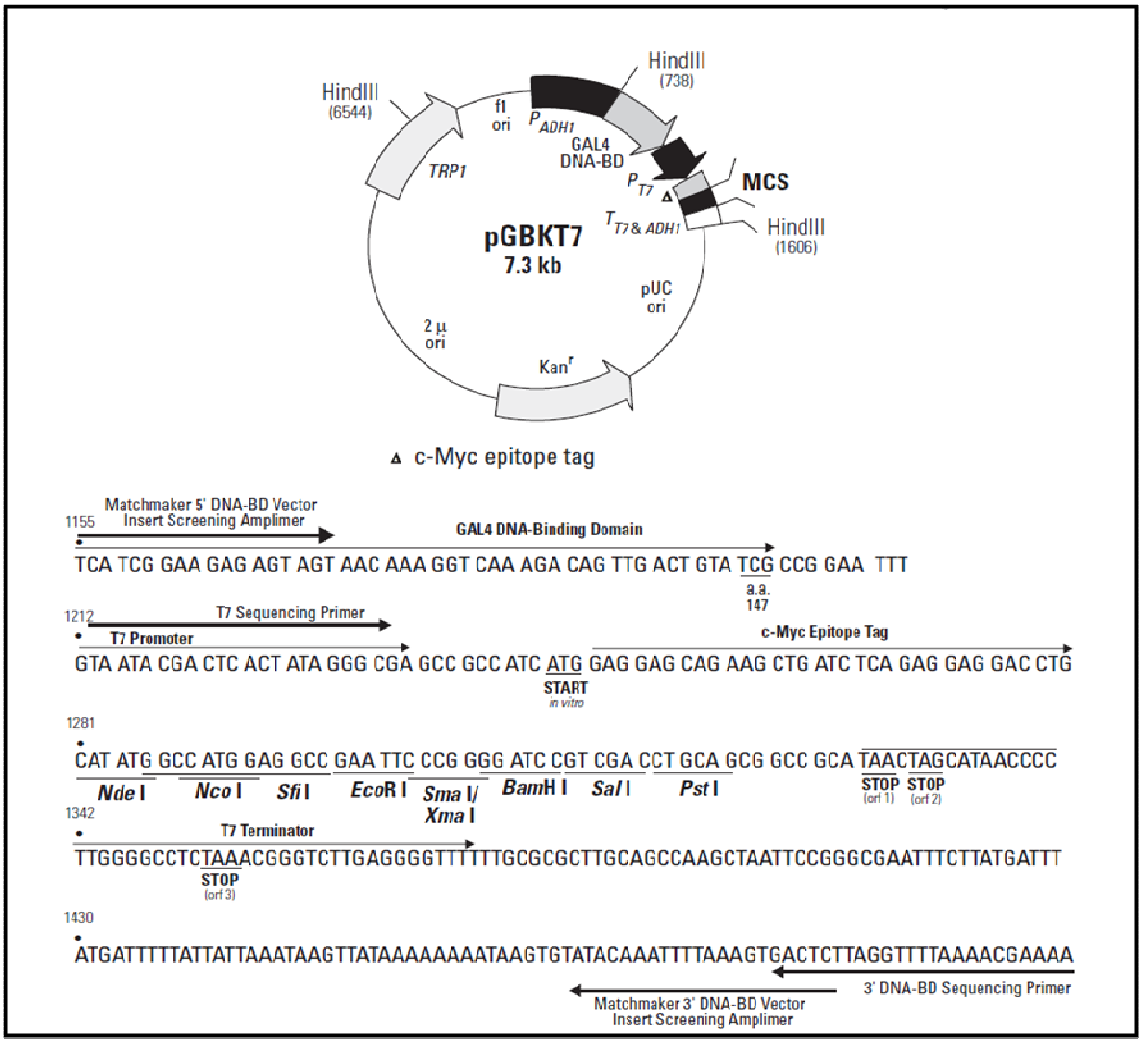

Figure 7. pGBKT7 is the DNA-BD Vector showing position of unique restriction sites (MCS) in frame with the 3' end of the GAL4 DNA-BD for constructing fusion proteins with a bait protein. The bait protein is also expressed as a fusion to a c-Myc epitope tag. The T7 promoter is used for in vitro transcription and translation of the epitope tagged fusion protein. [Note that the DNA-BD is not expressed during the in vitro transcription and translation reactions.]