आरएनए इन्टरफिरेन्स (RNAi) के द्वारा टमाटर के पर्ण कुंचन विषाणु (ToLCV) का दमन

Suppression of *Tomato leaf curl virus* (ToLCV) through RNA interference (RNAi)

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Suppression of *Tomato leaf curl virus* (ToLCV) through RNA interference (RNAi)

By

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CERTIFICATE

This is to certify that the thesis entitled "**Suppression of *Tomato leaf curl virus* (ToLCV) through RNA interference (RNAi)**", submitted to the Faculty of the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Biochemistry** by **Mr. Ramesh S.V.** embodies the results of *bonafide* work carried out by him under my supervision and guidance. No part of the thesis has been submitted by him for any other degree or diploma.

I further certify that any help or information received during the work on this thesis has been duly acknowledged.

Place : New Delhi

Date : 19th July, 2007

(Dr.Shelly Praveen)
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Advisory Committee

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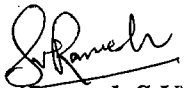

(Ramesh S.V)

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LIST OF ABBREVIATIONS

RNAi	RNA interference
ToLCV	*Tomato leaf curl virus*
ToLCD	Tomato Leaf Curl Disease
WTG	Whitefly Transmitted Geminivirus
Rep	Replication initiator (or) replicase protein
PDR	Pathogen Derived Resistance
CPMR	Coat Protein Mediated Resistance
MP	Movement Protein
siRNA	small interfering RNA
miRNA	micro RNA
VSP	Viral Suppressor Protein
T-*rep*	Truncated *rep*
IR-*rep*	Inverted Repeats *rep*
Ihp-*rep*	Intron spliced hairpin RNA *rep*
sh-*rep*	short hairpin RNA *rep*
hpRNA	hairpin RNA
TGS	Transcriptional Gene Silencing
PTGS	Post-Transcriptional Gene Silencing
DCL	Dicer-Like
HEN	HUA enhancer
HYL	Hyponastic Leaves
AGO	Argonaute
RISC	RNA Induced Silencing Complex
RITS	RNA Induced TGS Complex
amiRNA	artificial miRNA

1. INTRODUCTION

For many years, genomes and proteomes were regarded as the basis of life until the breakthrough at the end of the last century by Fire *et al.*, (1998) in *Caenorhabditis elegans,* with a crucial and pioneering contribution in the field of biology. It was, indeed, a revival of an early idea proposed by Jacob and Monod in 1961 in the form of hypothesis that RNA molecules are regulators of gene expression wherein they interact with operators and affect transcriptional and post transcriptional stages of gene expression. In the modern RNA world, many non-coding RNAs are gaining importance as they are delineated to be involving in gene regulation by sequence specific silencing in a phenomenon termed as RNA interference (RNAi). Since the demonstration of the RNA silencing and its important role in plant defence mechanism, against the invading viruses, many avenues got opened up to silence the viral gene and thereby achieving virus resistance. Over the years RNAi, as a tool in developing viral resistance in many of the commercially important plant species have displayed promising outcomes (Waterhouse *et al.*, 1998; Smith *et al.*, 2000; Poogin *et al.*, 2003). These studies raised our interest on RNAi as a viable strategy to counter the leaf curl disease menace in tomato, attributed to different species of leaf curl viruses. In the context of difficulty in managing the virus menace by cultural practices or chemical control of vector species and the non availability of the resistant genotypes, it is imperative that the modern genetic engineering approaches be applied for the effective management of the virus.

Tomato leaf curl virus, a whitefly transmitted geminivirus (WTG) belonging to genus Begomovirus of family Geminiviridae causes the devastating Tomato leaf curl disease (ToLCD) in tomato not only in India but also world wide. Geminiviruses that cause the disease, share only 70–90% homology in their genomes. In India alone five distinct geminiviruses have been reported to cause the disease (Varma and Malathi, 2003). The disease causes severe leaf curling, cupping of leaf lamina and overall stunting of growth in tomato (Fig. 5). The virus is characterized by the presence of single strand DNA (ssDNA) and possesses bipartite (DNA-A and DNA-B) or monopartite (equivalent to DNA-A) genome encapsidated within geminate particles (Fig.5). *Tomato leaf curl virus* possesses bipartite genome and the virus replicates in the host cell nuclei via double-stranded DNA intermediates using a rolling circle mechanism (Saunders *et al.*, 1991; Stenger *et al.*, 1991).

The viral genomic component of DNA-A is characterized with 6 ORFs, altogether in both the viral and complementary strand. The ORF AC1 encodes a replication initiator protein (Rep or replicase protein) essential for viral DNA replication in association with host DNA polymerase (Hanley-Bowdoin *et al.*, 2000). It is the only protein of viral origin that is indispensable for the virus to replicate inside the host cell via rolling circle replication mechanism. The protein possesses the DNA nicking-closing properties that initiate the process of virus replication (Jupin *et al.,* 1995; Laufs *et al.,* 1995). Previous studies in our laboratory have led to the characterization of the replication initiator protein gene of *Tomato leaf curl virus* (Sinha *et al.*, 2004; Praveen *et al.*, 2004). Molecular variability in the replicase gene of Indian isolates suggests 70-90 percent homology in the nucleotide sequence (Sinha *et al.,* 2004). Conserved core of the *replicase* gene, consisting of 330 nucleotides, has been identified (Praveen *et al* 2004). The functional domains of the protein were resolved, with DNA binding and oligomerisation domains essential for the functioning of the protein, falling in the conserved region of the protein (Dasgupta *et al.,* 2004).

Replication initiator protein of the virus, hence, is considered to be the most suitable target for RNAi based silencing. Replicase protein mediated resistance has already been successfully demonstrated against geminivirus in many instances (Asad *et al.,* 2003; Chellappan *et al.,* 2004(b); Yang *et al.,* 2004; Praveen *et al.,* 2005).Either full length *rep* gene or truncated *rep* sequences have already been deployed in conferring resistance to the leaf curl virus (Brunetti *et al.,* 2001; Chatterji *et al.,* 2001). With respect to the strategy of transgenic expression, initially, antisense RNA mediated silencing of the replication initiator protein was a preferred strategy in the development of virus resistant transgenics (Yang *et al.*, 2004; Praveen *et al.*, 2005). Simultaneous expression of sense and antisense RNA cognate to the viral replicase protein mRNA also rendered the tobacco plants resistance to *Cotton leaf curl virus* (Asad *et al.*, 2003). Later the strategies to combat the virus varied from direct protoplastic delivery of siRNAs to the constitutive expression of intron spliced hairpin RNA (hpRNA), culminating in the production of siRNAs that cleave target viral replication initiator protein mRNA (Vanitharani *et al.*, 2003; Fuentes *et al.*, 2006; Bonfim *et al.*, 2007).

Viruses are not passive in this ordeal as they are shown to exhibit the counter defense mechanism through viral suppressor proteins that alters the RNAi pathway and thereby evade the host defence mechanism (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998). Geminivirus is no exception as protein encoded by ORF AC4 has not been attributed with any function in viral DNA replication but its role in small RNA binding and as a pathogenicity determinant has been demonstrated (Krake *et al.*, 1998; Chellappan *et al.*, 2005). In two of the geminiviruses, *African cassava mosaic virus* - Cameroon strain [ACMV-Cam] and *Srilankan cassava mosaic virus* [SLCMV], AC4 ORF has been characterized as a suppressor of RNA silencing (Vanitharani *et al.*, 2004; Chellappan *et al.*, 2005). Characterization of ToLCV-AC-4 protein suggests its role in RNAi suppression and having probable secondary functions. Its close resemblance with other viral suppressors and presence of some amino acid residues from PAZ domain reflecting its probable role in binding of small RNAs. Severe developmental defects caused in *N. tabaccum* on its transgenic expression unequivocally demonstrated its probable role in binding of host micro RNA (kumari, 2006). Therefore it is imperative that any RNAi based transgenic approach requires the viral suppressor protein also to be taken care of in the development of trait stable transgenics.

With this view, the following objectives were formulated for the present investigation:

(i) *In silico* determination of the potent siRNAs from the *rep* gene of ToLCV and to generate truncated *rep* gene construct.

(ii) To develop RNAi inducing Inverted Repeats (IR) constructs.

(iii) To study the efficiency of the constructs through gene silencing assay.

2. REVIEW OF LITERATURE

RNA interference is an evolutionarily conserved, homology dependent gene silencing phenomenon found in all the eukaryotes. It operates through non-coding small RNA molecules, which recently gained wide spread attention, as molecular switches in complex gene regulatory networks. It involves dsRNA initiated sequence specific degradation of cognate RNA via 21 nt length effector molecules called small interfering RNAs (siRNAs), with the aid of cellular protein machinery. The journey from hypothetical non-coding RNAs towards its role in generation of virus resistant transgenics has been elucidated in a Timeline (Fig. 1).

Virus resistant transgenic plants

In order to develop virus resistant transgenic plants, the concept of Pathogen Derived Resistance (PDR) was proposed by Sanford and Johnston in 1985. It claims that the effective way to achieve resistance against plant viruses is to express the pathogen derived molecules in a dysfunctional form. Upon invasion of the viruses into the plant system, the modified dysfunctional molecule already expressing in plants, causes to inhibit the functional molecule of viruses, thereby inhibit the specific step in the life or infection cycle of the pathogen. The first successful demonstration of the resistance to the virus, based on the mechanism of PDR, was in tomato plants expressing *Tobacco mosaic virus* derived coat protein gene (Abel *et al.,* 1986). Such a model of resistance in plants expressing coat protein gene of virus is called as coat protein mediated resistance (CPMR.). CPMR has widely been used since then (Hemmenway *et al.*, 1988; Powell *et al.,* 1989; Osborn 1989; Nejidat and Beachy, 1990). The most successful story on CPMR is the revival of once ailing papaya industry by genetically modified virus resistant papaya variety Sunset, with coat protein gene of *Papaya ring spot virus* (PRSV).

The molecular mechanism behind the coat protein mediated resistance was initially thought to be protein mediated. In contrast to this, coat protein RNA of *Tobacco etch virus* (TEV) rather than coat protein itself was implicated in resistance (Lindbo and Dougherty, 1992). The phenomenon was referred to as RNA-mediated virus resistance, or RNA-mediated gene silencing.

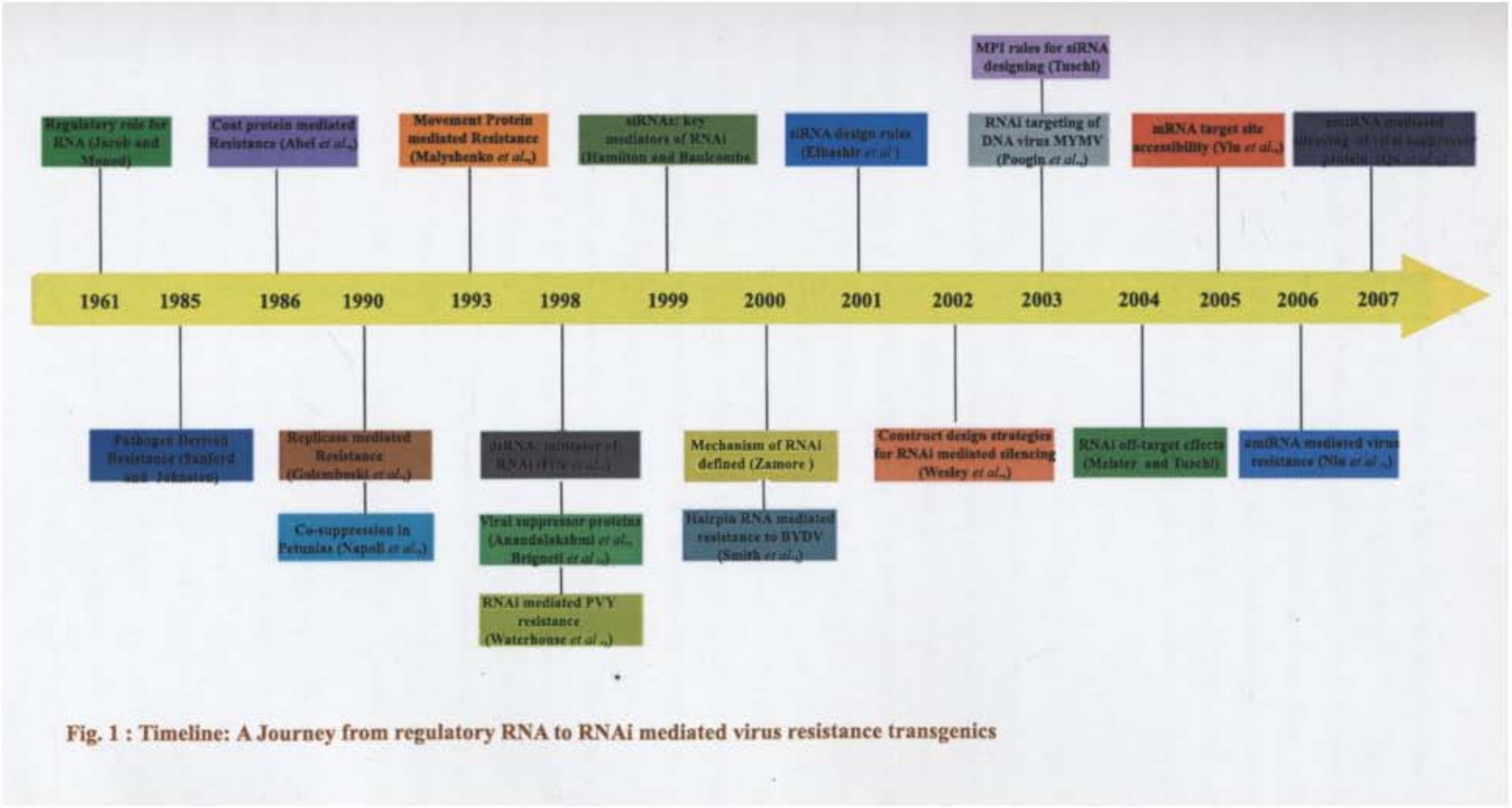


Fig. 1 : Timeline: A Journey from regulatory RNA to RNAi mediated virus resistance transgenics

Another viral gene employed in PDR is movement protein gene as movement of the viral particle is indispensable for its establishment in the plant system. In this system a dysfunctional movement protein of *Tobacco mosaic virus* (TMV) was expressed in tobacco and made to hinder the functioning of normal protein derived from viral infection (Lapidot *et al.,* 1993; Malyshenko *et al.,* 1993). It was proposed that the dysfunctional MP prevents the accumulation of the movement protein of the challenge virus in such a way that the MP of the challenge virus is unable to complete its function (Lapidot *et al.*, 1993).

Replication being an indispensable process in the viral infection cycle, numerous attempts were aimed at crippling the virus replication process by targeting replicase or rep protein. *Rep* mediated resistance was initially exploited in transgenic tobacco plants expressing putative Rep protein of *Tobacco mosaic virus* (TMV) (Golemboski *et al.,* 1990). Successful deployment of *Rep* gene in conferring virus resistance were made in group of unrelated viruses such as *Pea early browning virus, Potato virus Y* (PVY) and *Cucumber mosaic virus* (CMV) (MacFarlane and Davies, 1992; Audy *et al.*,1994; Hellwald and Palukaitis, 1995). The reports of *Rep* mediated resistance with reference to geminiviruses are many and the transgenic resistances via replication initiator protein are as follows. In an instance, antisense RNAs of Rep protein was expressed in *Nicotiana benthamiana* to interfere with the disease caused by *Tomato yellow leaf curl virus* (TYLCV) (Bendahmane and Gronenborn, 1997). Transgenic *N. benthamiana* plants expressing the mutated *AC1* gene, when infected with *African cassava mosaic virus* (ACMV), exhibited tolerance to infection and displayed delay in symptoms development (Sangare *et al.,* 1999). Truncated C1 gene of *Tomato yellow leaf curl Sardinia Virus* (TYLCSV), when potentially co expressed with the C4 protein, conferred resistance to the homologous virus (Brunetti *et al.,* 2001).

Transgenic tobacco was developed expressing sense and antisense RNAs of the replication initiator protein (AC1) among other genes of viral origin that conferred resistance to *Cotton leaf curl virus* (CLCV) (Asad *et al.,*2003). Integration of the *replicase* gene (AC1) from *African cassava mosaic virus* (ACMV) imparted resistance for homologous and two heterologous species of the cassava-infecting geminiviruses (Chellappan *et al.*, 2004b). Transgenic cassava plants were developed with increased ACMV resistance using improved antisense RNA technology by targeting the viral

mRNAs of Rep (AC1), TrAP (AC2) and REn (AC3). Transient expression of Rep protein, oligomerisation domain, was found to inhibit heterologous viral DNA accumulation by 70-86% in tobacco protoplasts and in *Nicotiana benthamiana* plants (Chatterji *et al.,* 2001). Transgenic tomato resistant to *Tomato yellow leaf curl virus* (TYLCV) was constructed using antisense *rep* gene construct of the virus (Yang *et al.,* 2004). Transgenic tomato resistant to tomato leaf curl disease (ToLCD) using *rep* gene in antisense orientation has been developed (Praveen *et al.,* 2005). In all the above studies it was also proved that the resistance was attributable to the post transcriptional stage and are RNA mediated.

RNA interference (RNAi) - a plant defence

When the pathogen derived resistance in management of viral disease of plants gained momentum and wider applications are being embarked upon, the phenomenon and the mechanism behind the RNA mediated gene silencing got unraveled. RNA silencing in plants was born with a quirky observation made by two independent groups of workers (Napoli *et al.*, 1990 and Van der krol *et al.*, 1990) in petunias that an additional copy of chalcone synthase enzyme gene involved in anthocyanin synthesis does not increase the pigmentation of the flower. On the contrary it caused the suppression of the endogenous gene involved in pigment producing pathway. The phenomenon was termed as 'co-suppression' as both the transgene and homologous endogenous gene were silenced. Similar phenomenon was observed with filamentous fungi *Neurospora crassa* also and it was termed as 'quelling' (Romano and Macino, 1992). The trigger for this homology dependent gene silencing was later elegantly elucidated as dsRNA by Fire *et al.,* (1998) in *C. elegans*. Introduction of dsRNA in to *C.elegans*, led to the silencing of the corresponding endogenous gene in a sequence specific manner in a phenomenon called as RNA interference (RNAi). Since the discovery of the phenomenon in worms, it has been documented in wide array of living beings ranging from protozoans to mammals (Dalmay *et al.*, 2000; Tijsterman *et al.*, 2002). The silencing phenomenon results from either transcription inhibition [Transcriptional Gene Silencing (TGS) or from RNA degradation (Post-Transcriptional Gene Silencing (PTGS)] have been correlated with the accumulation of small interfering RNAs (siRNAs). TGS can be achieved by the siRNAs corresponding to the silenced

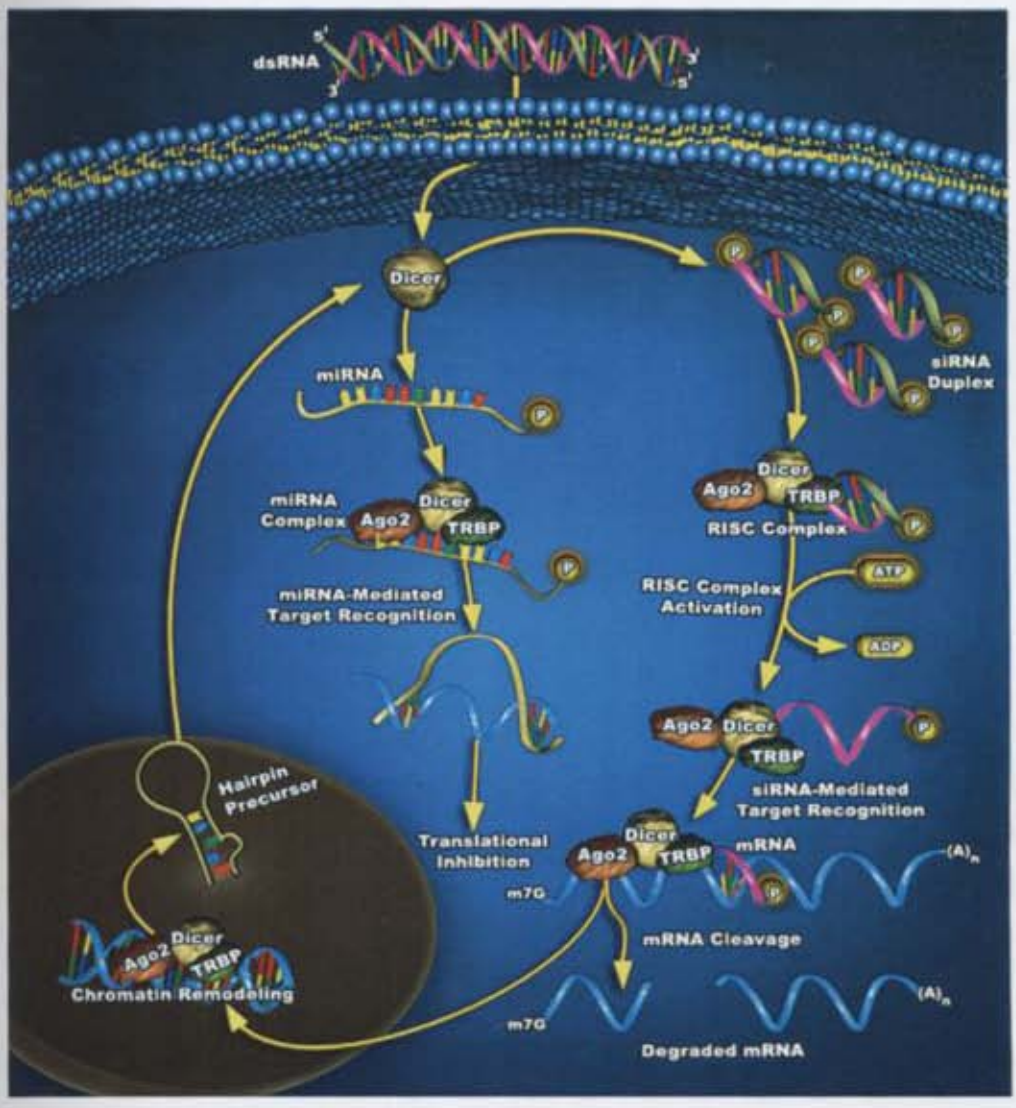


Fig. 2: Model of RNAi silencing
The scheme depicts enzymatic machinery involved in RNAi silencing pathway (Source : www.nature.com)

promoter regions and PTGS by the degradation of the mRNA (Hamilton and Baulcombe, 1999; Mette *et al.*, 2000). Dissection of biochemical pathway, leading to the silencing of the homologous gene, has shed light on the enzymatic machinery involved and their conserved nature in eukaryotes (Dalmay *et al.*, 2000; Fagard *et al.*, 2000; Bernstein *et al.*, 2001) (Fig. 2). The revelation that plant responds to the invading virus through the process of RNA interference as its defence measure, has prompted researchers to manipulate the endogenous silencing pathway to downregulate the expression of virus genes and thereby achieving virus resistance in plants (Lindbo *et al.*, 1993; Baulcombe, 1999). In this process both the viral RNA and viral derived transgene mRNA are degraded in a homology dependent manner, thus rendering the plant immune to the virus infection. Since then, RNA mediated silencing was explored for the generation of virus resistant plants and is considered a specific manifestation of the phenomenon of Post Transcriptional Gene Silencing (PTGS) operating in plants (Vazquez-Rovere *et al.*, 2002; Goldbach *et al.*, 2003).

The trigger for the biochemical process of virus resistance in plants is initiated by the expression of inverted repeats transgenes that is capable of generating self-complementary hairpin RNA cognate to viral RNAs (Smith *et al.*, 2000; Beclin *et al.*, 2002). It results in the production of dsRNA to be recognized by the RNAi machinery as an elicitor of silencing pathway. In plants the duplex RNAs generated so are processed by class of type III ribonuclease called as Dicer-like (DCL) enzymes (Bernstein *et al.*, 2001). Thus long dsRNAs originating from inverted repeats of transgenes are processed into siRNAs by the DCL-4 and DCL-3 giving rise to 2 types of siRNAs 21-22 nt and 24-26 nt respectively (Dunoyer *et al.*, 2005). The transgene derived siRNAs are methylated by HUA ENHANCER1 (HEN1) to avoid any degradation or poly-uridiylation in the system (Boutet *et al.*, 2003; Li *et al.*, 2005). DCL also interacts with HYPONASTIC LEAVES-1 (HYL-1), a double strand RNA binding protein, to produce mature siRNAs (Hiraguri *et al.*, 2005). Then one strand of the mature siRNA guides the sequence specific mRNA cleavage *i.e.*, cognate viral transcript, mediated by ARGONAUTE-1 (AGO-1) comprising enzymatic machinery called RNA Induced Silencing Complex (RISC) (Morel *et al.*, 2002; Baumberger and Baulcombe, 2005). siRNAs are also known to involve in directing DNA methylation by

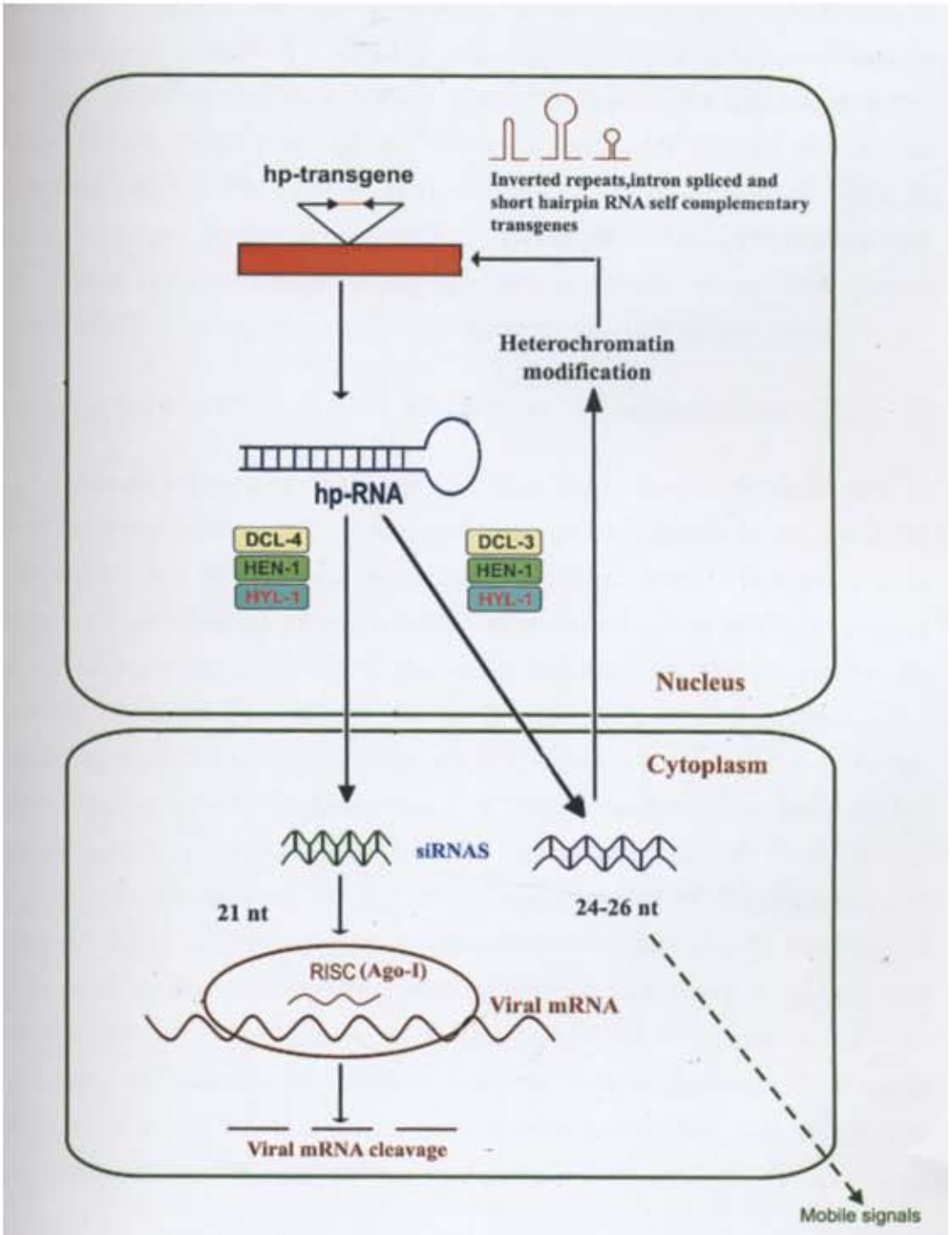


Fig. 3: Scheme depicting hairpin RNA (hpRNA) induced RNAi pathway targeting a viral mRNA

RNA Induced Transcriptional gene Silencing complex (RITS) and in systemic spread of the silencing signals (Tang *et al.*, 2003). The shorter class of 21-22 nt is implicated in mRNA degradation, longer one, 24-26 nt (heterochromatic siRNAs) are involved in directing DNA methylation and in systemic silencing. The entire mechanism is diagrammatically represented in figure.3. Although many other enzymatic complexes have been identified, including Nuclear RNA Polymerase IVa (NRPD1a), RNA-Dependent RNA Polymerases (RDR_x), Silencing Defective3 (SDE3), and Werner Exonuclease (WEX) (Dalmay *et al.*, 2001; Glazov *et al.*, 2003; Herr *et al.*, 2005) the steps at which these proteins act in the RNAi pathway are still not fully characterized. As a counter defence measure to this biochemical pathway viruses have evolved proteins called as viral suppressor proteins to evade the host defence mechanism.

Viral suppressor proteins of RNA interference - a counterdefence

Albeit the effective functioning of RNA silencing mechanism in plant system, to combat the virus infection, viruses are strong enough to establish in the system by suppressing the host defence mechanism. The suppressing activity is manifested in the form of increased virulence (Pruss *et al.*, 1997) or synergism among unrelated viruses in mixed infections (Shi *et al.*, 1997). The initial hint that viral derived proteins are involved in sabotaging the plant antiviral measure comes from the study, which revealed enhanced the virulence of unrelated viruses (CMV and TMV) on expression of Hc-Pro, a Potyviral encoded protein in plants (Pruss *et al.*, 1997). The direct involvement of viral suppressor proteins in interfering with the RNAi mechanism was demonstrated with Hc-Pro protein (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 2001). Different family of plant viruses possesses wide repertoire of proteins to effect this counter defense measure and are enumerated in Table.1, their mode of action in the RNAi pathway is depicted in Fig.4.The viral suppressor proteins do not share any sequence or functional homology with each another. They act at different steps of the RNAi silencing pathway and thus suggest their independent evolution to counteract the plants defence mechanism.

Geminiviruses are known to encode for three viral suppressor proteins *viz.*, AC2, AC4 and βC1. The family Geminiviridae does not undergo dsRNA phase in their replication cycle yet they encode proteins to suppress the RNAi silencing pathway. The

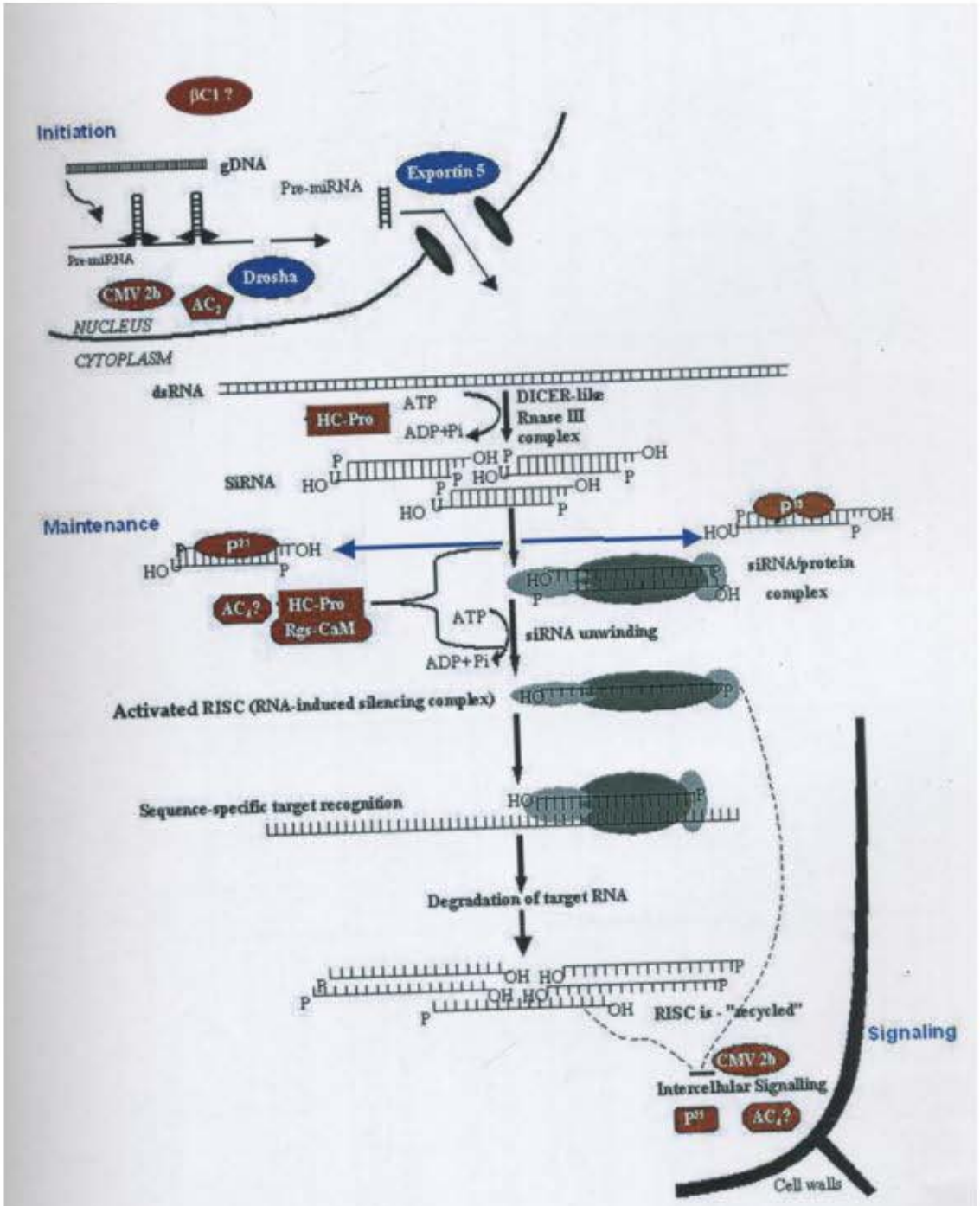


Fig. 4: Mechanism and point of action of plant viral suppressor proteins
Among geminiviral suppressors, AC2 acts in nucleus at initiation step, whereas AC4 and βC1 are likely to act at maintenance, signaling and initiation, respectively.
(Source: Praveen and Mangrauthia, 2006)

Table 1: Viral suppressor proteins – a viral counter defence

Virus genus	Virus	Suppressor protein	Suppressor activity	References
Positive strand RNA virus				
Aureusvirus	*Pothus latent virus*	P14	dsRNA binding	Merai *et al.*,(2005)
Carmovirus	*Turnip crinkle virus*	CP	Suppression of local silencing	Thomas *et al.*, (2003); Qu *et al.*, (2003)
Closterovirus	*Beet yellow virus*	P21	dsRNA binding	Reed *et al.*, (2003)
	Citrus tristeza virus	P20,P23,CP		Lu *et al.*, (2004)
	Grapevine leaf roll-associated virus-2	P24		
	Beet yellow stunt virus	P22		Reed *et al.*, (2003)
Crinivirus	*Sweet potato chlorotic stunt virus*	P22,RNase3	RNase III	Kreuze *et al.*, (2005)
Comovirus	*Cowpea mosaic virus*	Small CP		Voinnet *et al.*, (1999)
Cucumovirus	*Cucumber mosaic virus*	2b	dsRNA binding	Brignetti *et al* .,(1998); Lucy *et al.*, (2000)
	Tomato aspermy virus			
Furovirus	*Soil-borne wheat mosaic virus*	19K	Local and systemic silencing suppression	Dunoyer *et al.*, (2002)
Hordeivirus	*Barley stripe mosaic virus*	γb		Yelina *et al.*, (2002)
Pecluvirus	*Peanut clump virus*	P15	Protein interaction	Dunoyer *et al.*, 2002
Polerovirus	*Beet western yellow virus*	P0		Pfeffer *et al.*, (2002)
Potexvirus	*Potato virus X*	P25	Intracellular signaling	Voinnet *et al.*, (2000)
Potyvirus	*Tobacco etch virus*	Hc-Pro	Prevents accumulation of siRNAs, siRNA binding activity	Brignetti *et al.*, (1998); Anandalakshmi *et al.*, (1998)
	Potato virus Y			
	Turnip mosaic virus			

Sobemovirus	*Rice yellow mottle virus*	P1		Voinnet *et al.*, (1999)
Tobamovirus	*Tobacco mosaic virus*	P130		Voinnet *et al.*, (1999); Ding *et al.*, (2004)
	Tomato mosaic virus		Local silencing suppression	Kubota *et al.*, (2003)
Tobravirus	*Tobacco rattle virus*	16K		
Tombusvirus	*Tomato bushy stunt virus*	P 19	dsRNA binding	Voinnet *et al.*, (1999); Lakatos *et al* ., (2004)
	Cymbidium ring spot virus			Silvahy *et al.*, (2002)
Tymovirus	*Turnip yellow mosaic virus*	P69	Local silencing suppression	
Vitivirus	*Grapevine virus A*	P10		
Negative strand RNA virus in plants				
Tenuivirus	*Rice hoja blanca virus*	NS3		
Tospovirus	*Tomato spotted wilt virus*	NSs		Bucher *et al.*, (2003)
Double stranded RNA virus				
Phytoreovirus	*Rice dwarf virus*	Pns 10		Cao *et al.*, (2005)
DNA viruses				
Begomovirus	*Tomato leaf curl virus*	C2	DNA binding, nuclear localization, modulation of endogenous proteins.	Voinnet *et al.*, (1999); Van wezel, (2002)
	TYLCCV-Y10 β	βC1		
	African cassava mosaic virus (KE)	AC2		Vanitharani *et al.*, (2004)
	EACMV,ICMV,TGMV			
	Mungbean yellow mosaic virus			
	African cassava mosaic virus (CM)	AC4	miRNA binding	Chellappan *et al.*, (2005)
Curtovirus	*Beet curly top virus*	L2		Wang *et al.*, (2003)

recent study revealed that geminiviruses like *Tomato yellow leaf curl virus* (TYLCV), *African Cassava mosaic virus*- Cameroon (ACMV-CM), *Sri Lankan cassava mosaic virus* (SLCMV) and *East African cassava mosaic virus*-Cameroon(EACMV-CM) and *Indian Cassava mosaic virus* (ICMV) are potential targets of RNAi (Chellappan *et al.,* 2004a). The dsRNA phase in the viral replication cycle may be due to the action of host RNA dependent RNA polymerase (RDR) on aberrant read through transcripts or over expressed viral transcripts. Since genome possesses a bi-directional promoter, a characteristic feature of this family of virus, overlapping transcripts from opposite polarities also contribute to the dsRNA phase in its life cycle (Townsend *et al.*, 1985).

Among the three characterized suppressors of this family of virus, AC2 was the first protein to be studied and its role in suppressing the RNA silencing had been unequivocally established. It is a 15 KDa protein alternatively known as C2, AL2, or TrAP (Transcriptional activator protein). Voinnet *et al.,* (1999) demonstrated that ACMV AC2 possesses the silencing suppressor activity employing PVX vector in a GFP expressing transgenic *N. benthamiana* plants. This pioneer work in suppressors of geminiviruses led to identification of similar effects of AC2 with other host virus interactions also. It s role as suppressor has been established in *Tomato yellow leaf curl virus* also (Van wezel, 2002; Dong *et al.,* 2003,). It was also established that AC2 acts in the host nucleus by modification of the host DNA and through its transcriptional activation activity. The study on the transcriptome profiling upon expression of AC2 protein of ACMV and MYMV in the protoplasts of *Arabidopsis* revealed that more than 30 important genes are up regulated. One notable instance is WEL1 (Werner Exonuclease like -1) whose role in silencing suppressor had been established (Trinks *et al.,* 2005).AC2 of *Tomato golden mosaic virus* (TGMV) has been implicated in transcription-independent silencing suppression also. It acts indirectly by inhibiting host ADK (Adenosine Kinase) which is needed to sustain methyl cycle involved in maintaining RNAi silencing (Wang *et al.,* 2005).Thus evidence suggest that the suppressor protein participate in an indirect suppression mechanism involving metabolic inhibition of siRNA directed transmethylation, which could interfere with epigenetic modification of the viral genome (Bisaro, 2006).

AC4 is an embedded ORF present with in the Rep coding sequences but in a different reading frame. Vanitharani *et al.,* (2004) have investigated the AC4 protein of cassava infecting geminiviruses for their role in suppression of RNA silencing. An *Agrobacterium* leaf infiltration assay using GFP transgenic *N. benthamiana* revealed that AC4 of ACMV-[CM] and SLCMV, the recovery type viruses, have the capacity to suppress the induced RNA silencing phenomenon. By contrast, the AC2 of EACMCV and ICMV, the non-recovery type viruses, were identified as suppressors of induced RNA silencing. Transgenic expression of *African cassava mosaic virus*-Cameroon Strain (ACMV-CM)-encoded AC4 protein was correlated with decreased accumulation of host miRNAs and increased development abnormalities in *Arabidopsis*. Also downregulation of miRNA correlated with an up-regulation of target mRNA level (Chellapan *et al.,* 2005). The results provided direct evidence that AC4 protein binds preferentially to single strand miRNA. Thus it acts at a step downstream to unwinding of small RNA duplex before being loaded on to RISC complex to effect silencing function. AC4 is also implicated in effecting synergism in aggravating the symptoms. The synergistic effect between ACMV-[CM] and EACMCV is mediated by the differential and complementary RNAi suppression ability of the AC4 and AC2 of ACMV-[CM] and EACMCV, respectively, in a temporal and spatial manner (Vanitharani *et al.*, 2004).

DNA β is a satellite DNA associated with some of the monopartite geminiviruses in manifesting the typical symptoms associated with viral infection on plants (Briddon *et al.,* 2001: Mansoor *et al.,* 2003). *Nicotiana benthamiana* plants expressing GFP, βC1 protein of *Tomato yellow leaf curl china virus* –Y10 (TYLCCV) could reverse the silencing effect already set in (Cui *et al.,* 2005). Although it is similar to AC2 in its size, DNA binding properties, and nuclear localization, it might target a different step in the RNA silencing mechanism because the developmental defects associated with its expression are entirely different (Bisaro, 2006).

Among other plant viral suppressor proteins, Hc-Pro of potyvirus is a first known suppressor of plant viral origin and it was shown that the silenced state of the reporter gene GUS was restored upon expression of Hc-Pro in those plants thus effecting silencing suppressor function (Anandalakshmi *et al.*, 1998; Brigneti *et al.,* 1998). Hc-Pro interacts with host rgs-CAM (regulator of gene silencing-calmodulin-

like protein) protein, an endogenous regulator of RNA silencing, and enhances its activity (Anandalakshmi *et al.*, 2000). A recent report unveils that Hc-Pro among other viral suppressors inhibit the RNA silencing pathway by binding to siRNAs (Lakatos *et al.*, 2006). *Cucumber mosaic virus* (CMV) 2b, another suppressor protein of importance, was shown to affect the RNA silencing pathway differently as it cannot suppress RNA silencing in tissues where RNA silencing is already established. However, it was shown to be able to prevent the initiation of RNA silencing in newly emerging tissue (Beclin *et al.*, 1998; Brigneti *et al.*, 1998). Thus it might be involved in inhibiting the systemic spreading of the silencing signal. It is also known to get localized to the host nuclei thereby suppressing RNA silencing at nucleus.

Another most important plant viral suppressor is P-19 of Tombusviruses. It was shown binding, preferentially to RNA duplexes of 21 nt length with two nt 3'overhangs. It does not bind or do so ineffectively with ssRNAs, or dsRNAs of longer length (Silhavy *et al.*, 2002). The resolved crystal structure of the protein has finally established it s role in small RNA binding (Vargason *et al.*, 2003). It was found that P19 activity prevents siRNAs from incorporating into RNA silencing effectors such as RISC (Lakatos *et al.*, 2004). Viral suppressor proteins are the area of concern for RNAi mediated gene silencing hence are to considered while designing any effective trait stable transgenic technology based on RNAi phenomenon.

RNAi mediated resistance for plant RNA viruses

RNAi technology for generating virus resistance in plants was first demonstrated in RNA virus, *Potato virus Y* (PVY) where in complete immunity to the virus was reported in potato plants on simultaneous expression of both the sense and antisense transcripts of the viral helper-component proteinase (*HC-Pro*) gene (Waterhouse *et al.*, 1998). It paved way for deploying RNAi mediated resistance against many of the plant RNA viruses, since then, as enumerated in Table 2. In barley, *Barley yellow dwarf virus* derived hairpin RNAs confers immunity to the virus in an effective manner (Wang *et al.*, 2000). *N. benthamiana* plants infiltrated with cultures of *Agrobacterium* carrying a hairpin RNA construct derived from the 54-kDa region of *Pepper mild mottle virus* (PMMoV) were resistant to subsequent infection

Table 2: RNAi mediated resistance against plant RNA viruses

	Virus	Region targeted	System used	References
1	Potato virus Y (PVY)	HC-Pro	Potato	Waterhouse *et al.*, (1998)
2	*Barley yellow dwarf virus* (BYDV)	Hc-pro	Barley	Wang *et al.*, (2000)
3	*Pepper mild mottle virus* (PMMoV)	54KDa arbitrary region	*N. benthamiana*	Tenllado and D´ıaz-Ru´ız, (2001)
4	*Cucumber mosaic virus* (CMV)	Arbitrary sequence	N. tobacco	Kalantidis *et al.*,(2002)
5	*Tobacco etch virus* (TEV)	Arbitrary sequences	*N.tobaccum*	Tenllado *et al* ., (2003, a)
6	*Alfalfa mosaic virus* (AMV)	Arbitrary sequences	*N. tobaccum*	Tenllado *et al.*, (2003,b)
7	*Potato virus Y (PVY)*	Coat protein	Potato	Missiou *et al.*, (2004).
8	*Plum pox virus* (PPV)	Hc-Pro	*N. benthamiana*	Nicola-Negri *et al* ., (2005)
9	*Citrus tristeza virus* (CTV)	Coat protein, p20,p23,3'UTR	*N. benthamiana*	Roy *et al* ., (2006)
10	*Beet necrotic yellow vein virus* (BNYVV)	Replicase gene	Sugarbeet	Lennofors *et al* .,(2006)
11	*Soybean dwarf virus* (SbDV)	Coat protein	Soybean	Tougou *et al.*, (2006)
12	*Tobacco mosaic virus* (TMV)	Replication associated protein	*N. tobaccum*	Zhao *et al.*,(2006)
13	*Cucumber green mottle mosaic virus* (CGMMV	Coat protein	*N. benthamiana*	Kamachi *et al.*, (2007)

by PMMoV. At an interval of 3 or more days between agroinfiltration with PMMoV hairpin RNA and virus inoculation, plants were protected against virus infection, as indicated by the absence of PMMoV RNA in both the inoculated and the upper leaf tissue In contrast, *Agrobacterium*-mediated expression of constructs containing inverted repeats (head-to-tail) PMMoV 54-kDa sequences in the sense or antisense orientations were ineffective as initiators of RNAi, and disease symptoms were displayed in upper leaves (Tenllado and D´ıaz-Ru´ız, 2001).

Transgenic tobacco lines were generated with transgene derived from *Cucumber mosaic virus* (CMV) and was capable of producing intramolecular dsRNA. When the transgenic plants were challenge inoculated with CMV, three different types of plants were obtained viz., susceptible, recovered plants, and symptomless resistant plants. The resistance was correlated with the expression of short RNAs from viral derived sequences (Kalantidis *et al.,* 2002). Potato plants were engineered to express self-complementary RNAs corresponding to conserved region of the coat protein gene of *Potato Virus Y* (PVY). The transgenic plants were detected for the presence of small interfering RNAs homologous to coat protein gene of PVY. Transgenic lines producing siRNAs and were highly resistant to three strains of PVY, each belonging to three different subtypes of the virus PVY^{N}, PVY^{O} and PVY^{NTN}. Thus a single inverted repeats construct was able to attain resistant for three different strains of the virus (Missiou *et al.*, 2004). Hairpin RNA mediated resistance was successfully achieved against *Plum pox virus* (PPV) employing the constructs designed to produce silencing effect against *P1* and *Hc-Pro* genes of the virus. In the transient leaf disc assay 38 out of 40 transgenic plants were found to be resistant to the virus. The study thus aims to silence the expression of viral suppressor protein Hc-Pro of PPV (Nicola-Negri *et al.*, 2006). *Nicotiana benthamiana* plants transformed with constructs coding for self-complementarity transcripts targeting the capsid protein (CP), p20, p23, 3'UTR regions from *Citrus tristeza virus* (CTV) genome were resistant to the virus. The resistant lines were analyzed for the expression of transgene specific small interfering RNAs and were found to be positive for siRNAs. Thus it was proved that the resistance in these plants was mediated by RNAi silencing mechanism (Roy *et al.,* 2006).

In the rescue of sugar beet cultivation from 'rhizomania' a disease caused by *Beet necrotic yellow vein virus* (BNYVV) transgenic resistance seemed to be the only option. To this effect transgenic sugarbeet plants were generated expressing inverted repeats of 400bp from *replicase* gene from the virus. Upon challenge inoculation with virus spreading vector species *Polymyxa betae,* the transgenic lines exhibited resistance even under high inoculation pressure (Lennefors *et al.,* 2006). Soybean transgenic lines were generated on transforming with the constructs to produce inverted repeats RNA targeting the *coat protein* gene of *Soybean dwarf virus* (SbDV) into soybean somatic embryos. The resistance to the virus was evaluated till T2 stage and the viral coat protein specific siRNAs were detected in the transgenic resistant lines (Tougou *et al.,* 2006). Besides stable integration of transgene and silencing of viral gene, *Agrobacterium* mediated transient short hairpin RNA expression could also inhibit *Tobacco mosaic virus* (TMV) in *Nicotiana tobaccum* plants. The target gene in the study was 126 KDa *replication associated protein* gene of TMV. The silencing was very specific and the unrelated CMV viral expression was not silenced when assayed for (Zhao *et al.,* 2006). In an yet another instance in *Nicotiana benthamiana, Cucumber green mottle mosaic virus* (CGMMV) resistance was obtained by expressing the inverted repeats RNA targeting the coat protein gene of the virus under constitutive expression control of 35 S promoter (Kamachi *et al.,* 2007).

RNAi mediated resistance for geminiviruses

Geminiviruses being a major threat to world agriculture, breeding resistant crops against these DNA viruses is one of the major challenges faced by plant virologists and biotechnologists. Since the conceptual development of pathogen derived reistance, much progress has been achieved to protect plants against DNA virus infections.The natural recovery of *African cassava mosaic virus* –Cameroon (ACMV-CM) infection, a geminivirus and the correlation of viral derived small interfering RNAs with the recovery has prompted the researchers to look for RNAi or PTGS mediated resistance for these group of viruses (Chellappan *et al.,* 2004 a). Applicability of RNAi as a viable approach for attaining resistance against ssDNA viruses in general, Geminiviruses in particular has been demonstrated in many of the studies as enumerated in Table 3.

Table 3: RNAi mediated resistance against Geminiviruses

S.No.	Virus	Region targeted	System used	References
1	*Mungbean Yellow mosaic virus-vigna* (MYMV-vig).	Bi-directional promoter sequences	*Vigna mungo* (Blackgram)	Pooggin *et al.*, (2003)
2.	*African cassava mosaic virus*–Cameroon (ACMV-CM)	Replication associated protein	*Nicotiana tobaccum* protoplasts	Vanitharani *et al.*, (2003)
3	*African cassava mosaic virus*(ACMV)	Replication associated protein	Cassava	Chellappan *et al.*, (2004b)
4	*Tomato yellow leaf curl Sardinia virus* (TYLCSV)	Replication initiator protein	Tomato	Noris *et al* ., (2004)
5	*Tomato yellow leaf curl virus* (TYLCV)	Replication initiator protein	Tomato	Fuentes *et al* ., (2006)
6	*African cassava mosaic virus* (ACMV)	Bi directional promoter	Cassava	Vanderschuren *et al.*, (2007)
7	*Bean golden mosaic virus* (BGMV)	Replication initiator protein	Common bean	Bonfim *et al* ., (2007)

A review by Vanderschuren *et al.*, (2007), documents the most recent transgene-based approaches that have been developed to achieve durable geminivirus resistance. The initial successful attempt in RNAi targeting of DNA virus was reported against the *Mungbean Yellow mosaic virus-vigna* (MYMV-vig). Pooggin and co workers in 2003 have demonstrated that intron spliced hairpin RNA targeting of the intergenic region or bi directional promoter of this geminivirus resulted in resistance to this virus. Although the promoter region does not ordinarily get transcribed but does so due to read through transcripts which is a common phenomenon found in geminivirus infection. Thus the work unequivocally proved that RNAi can be an effective strategy in combating DNA virus infections in plants. Short interfering RNAs targeting replication associated protein mRNA of *African cassava mosaic virus* –Cameroon (ACMV-CM) were developed. Upon transfection of these siRNAs into protoplasts isolated from N*icotiana tobaccum* and co infection with the virus resulted in 91% reduced accumulation of mRNA corresponding to the replication associated protein of the virus. It was also reported that the silencing effect was found with genomic DNA of the virus also which accounts for 66% of the reduced accumulation in the cultured protoplasts cells (Vanitharani *et al.,* 2003). Similarly, expression of siRNAs specific for replication associated protein of *African cassava mosaic virus* in cassava plants led to the inhibition of the non- homologous virus in the plants. Upon challenge inoculation with the virus, it was deduced that the virus DNA accumulation reduced up to 98 % when compared with the controls. In this study the resistance was imparted even against non-homologous CMD-causing geminiviruses, *East African cassava mosaic Cameroon virus* (EACMCV) and *Sri Lankan cassava mosaic virus* (SLCMV) as all the four transgenic lines displayed enhanced resistance to both viruses compared with control (Chellappan *et al.,*2004 b).

In yet another first report of intron spliced hairpin RNA mediated silencing of *Tomato yellow leaf curl virus* (TYLCV), tomato plants, were transformed with an intron–hairpin genetic construction to induce RNAi mediated silencing against the early TYLCV *rep* gene (C1). Intron–hairpin RNA produced, transcribes into 726 nt of the 3' end of the TYLCV C1 as the arms of the hairpin, and the castor bean catalase intron between the arms. The extreme resistance to the virus by the transgenic plants was ascertained with dot blot hybridization and it was found that plant exhibited no

TYLCV DNA presence even after 60 days post challenge inoculation with virus (Fuentes et *al.,* 2006).The bi directional promoter of the *African cassava mosaic virus* was targeted with intron spliced hairpin RNA construct designed to express dsRNA cognate to the promoter sequence of the virus in cassava plants. Transgenic cassava lines showed drastically reduced symptoms, associated with the normal virus infection, on challenge inoculation with the virus (Vanderschuren *et al.,* 2007). The concept of RNAi is again explored to manage the *Bean golden mosaic virus* in the common bean plants. The intron spliced hairpin RNA designed was directed against Replication initiator protein mRNA of the virus. The transgenic common bean plants exhibited resistance even under high pressure of 300 viruliferous whiteflies on challenge inoculation (Bonfim et *al.*, 2007).

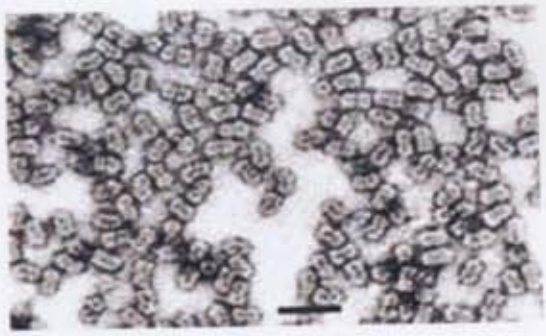
Although the results suggests the applicability of the RNAi based viral gene silencing a study in *Tomato yellow leaf curl Sardinia virus* has revealed that breakdown of silencing could be a possibility. RNA mediated silencing of the *rep* – mRNA of the TYLCSV, on either simultaneous expression of sense and antisense RNA or sense RNAs in multiple copies, could not be sustained. With the high pressure of the viral inoculum on whitefly mediated transmission of the virus the resistance through RNA silencing could be overcome (Noris *et al.,* 2004).

Tomato leaf curl virus – a geminivirus

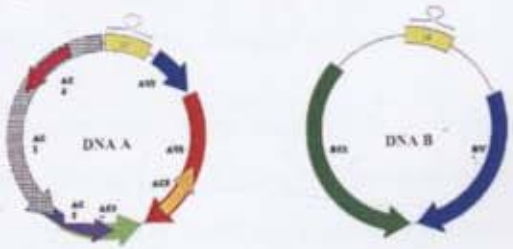
One of the most important geminivirus *Tomato leaf curl virus* (ToLCV), belongs to genus Begomovirus of family Geminiviridae. It causes severe leaf curling, cupping of leaf lamina and overall stunting of growth in tomato (*Solanum lycopersicum*) leading to severe yield and ensuing economic losses (Fig.5) (Rataul and Brar, 1989, Dougherty *et al.*, 1994).The virus is transmitted by whitefly B*emisia tabaci.* Leaf curl virus is characterized by the presence of single-stranded DNA genome and possesses bipartite (DNA-A and DNA-B) or monopartite (equivalent to DNA-A) genome encapsidated within geminate particles. Besides, study on their geographical distribution, has revealed that the begomoviruses affecting tomato in northern India are bipartite and those affecting tomato in southern India are monopartite. These two groups of viruses are quite distinct in their biological activity and genomic organizations. Irrespective of its genomic nature virus is replicated in the

(a)

(b)



(c)



(d)

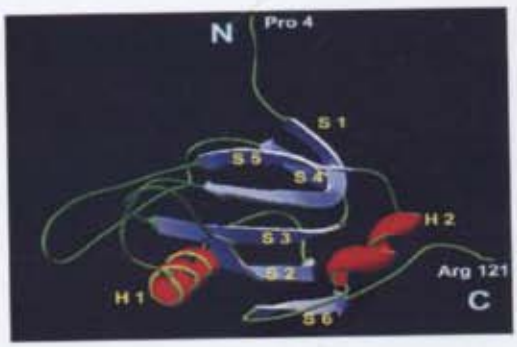


Fig. 5:(a) : Infected tomato showing leaf curl symptoms
(b) : *Tomato leaf curl virus* particles under EM (Bar =100 nM)
(c) : Genome organisation of the virus
(d) : Predicted structure of replication initiator protein
(Source: Dasgupta *et al.*, 2004)

host nuclei via double-stranded DNA intermediates using a rolling circle mechanism (Saunders *et al.*, 1991; Stenger *et al.*, 1991).

Tomato leaf curl virus possesses a bipartite genome, both the components of the genome are essential for causing infection. DNA-A is characterized with 6 ORFs, AC1 encodes a replication-associated protein (Rep) essential for viral DNA replication in association with host DNA polymerase(Hanley-Bowdoin *et al* .,2000); AC2 encodes a transcription activator protein (TrAP) (Sunter and Bisaro ,1992); AC3 encodes a replication enhancer protein (REn)(Sunter *et al.,* 1990); AC4 in ToLCV has not been attributed with any function in viral DNA replication rather its role in small RNA binding and altering host small RNA metabolism have been demonstrated in related geminiviruses also (Krake *et al.*,1998;Chellappan *et al.,* 2005). AV1 and AV2 encode coat protein and pre-coat protein, respectively (Padidam *et al.*, 1996); DNA-B has BV1 and BC1 genes that encode a nuclear-shuttle protein (NSP) and movement protein (MP), respectively (Sanderfoot and Lazarowitz, 1995).Owing to the difficulty in managing the virus menace by cultural practices or chemical control of vector species and the non availability of the resistant genotypes, it is imperative that the modern genetic engineering approaches are being applied for the effective management of the virus.

Construct design strategies for RNAi mediated resistance

In plants variety of approaches have been taken to express double stranded RNAs cognate to viral transcripts so as to initiate the process of viral gene silencing. Initially this was achieved by separately expressing sense and antisense genes of viral origin in plants and bringing them under a single genetic background by crossing. These studies have also revealed that double stranded RNA is a potential elicitor of RNAi than the sense and antisense form of genes separately (Waterhouse *et al.,* 1998). In plants, engineered inverted repeats transgenes derived from viruses, were made to express RNAs capable of duplex formation thereby conferring resistance to the homologous invading viruses (Smith *et al.*, 2000; Wang *et al.*, 2000; Kalantidis *et al.*, 2002). On comparing the silencing ability of the hairpin RNA mediated suppression and antisense RNA mediated it was concluded that the former accounts for 90-100% increase in gene silencing as against the latter (Wesley *et al.,* 2001;

Helliwel and Waterhouse, 2003: Miki and Shimamoto, 2004). Different types of construct design strategies were studied for their relative efficiency. The four different forms of hairpin RNA mediated silencing, viz., hairpin RNA, intron spliced hairpin RNA (Ihp RNA). Ihp RNA overhang, and IhpRNA spacer, evaluated for resistance against *Potato virus Y* (PVY). Among these four constructs intron spliced hairpin RNA was found to be superior as 90% of the plants carrying the construct exhibited viral gene silencing (Wesley *et al.,* 2002). Based on these studies many generic intron spliced hairpin vectors (pHANNIBAL or pKANNIBAL etc.,) have been generated for the purpose of RNAi. The strategy of employing short-hairpin RNA (sh-RNA) vectors or constructs to achieve silencing of the genes of viral and other endogenous origin have been widely explored successfully (Lu *et al.*, 2004). With the RNAi attaining greater attention in recent years in management of viral diseases of plants, the recent technological innovation added in this direction is artificial microRNA (amiRNA) mediated silencing of the viral gene expression in plants.

Another evolving approach in RNA based gene silencing is utilizing the intrinsic property of host gene regulation i.e., microRNA. This innovative approach deploys manipulated host miRNA pathway in achieving virus resistance .The findings that the 21 nt of endogenous microRNA can be altered without disturbing its biogenesis and maturation have raised interests in employing microRNA as a tool to silence transcripts of interest (Guo *et al.,* 2005). Employing the *Arabidopsis thaliana* micro RNA backbones Alvarez *et al.,* (2006) and Schwab *et al.,* (2006) have proved the applicability of the technology in silencing the endogenous transcript(s). In the field of virus resistance, pre-miR159a of *Arabidopsis* was modified to generate artificial pre-miRNAs^{159} (pre-amiRNAs^{159}) containing sequences complementary to genomes of two plant viruses, *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV). Transgenic lines carrying 35S-pre-amiRNA^{159} showed specific resistance to either TYMV or TuMV, depending on the expression of the cognate amiRNA. Moreover, transgenic plants that expressed both amiRNAs were resistant to both viruses and the virus resistance trait is heritable through at least 3 generations (Niu *et al.*, 2006).

In yet another instance, expression of an artificial miRNA, targeting sequences encoding the silencing suppressor 2b of *Cucumber mosaic virus* (CMV), has

efficiently inhibited 2b gene in transient expression assays and confers transgenic tobacco plants with effective resistance to CMV infection (Qu *et al.,* 2007). The technology appears superior in the context where siRNA mediated silencing is nullified when the temperature falls below 15° C (Szittya *et al.*, 2003; Kameda *et al.*, 2004) as artificial microRNA mediated resistance works even at a low temperature (Niu *et al.*, 2006).

In silico designing of potent siRNAs for RNAi

Irrespective of the method of production of siRNAs, the initial and foremost important step in achieving RNAi mediated silencing is designing potent siRNA i.e., to choose the potent siRNA target site. A siRNA being a sub string of the target mRNA not all sub string of siRNAs in a given mRNA is potent (Holen *et al.,* 2002). Elbashir and co-workers (2001) have formulated that a potent siRNA is characterized by the 3' overhangs of UU di nucleotides. Many other stipulations for the potent siRNAs are GC content of 30-50% is found to be more active than siRNAs with higher GC content. Stretches of more than four T's or A's in the target sequence are generally to be avoided. Selection of a target cDNA region 50-100 nucleotides downstream of the start codon, avoidance of 5' or 3' untranslated regions and high G-rich areas are some of the parameters (Elbashir *et al.*, 2001). Further to solve the problem, Tuschl *et al.,* (2003) came out with set of principles called as Max-Planck-Institute (MPI) principles for designing effective siRNAs (2003, http://www.rockefeller.edu/labheads/tuschl/sirna.html).Thermodynamic properties of the siRNAs also play a role in deciding the out come of the siRNA mediated gene silencing. An asymmetry with less stable 5' end of the antisense strand compared with 5' end of the sense strand was noticed in highly functional siRNAs (Khvorova *et al.,* 2003, Schwarz *et al.,* 2003).

The differential thermodynamic stability of the two termini of an RNA duplex is an important factor in deciding which one of the two strands gets incorporated in to the RNA induced silencing complex (RISC). Besides this, a specific base preferences at certain locations of the siRNA duplex have also been noted (Reynolds *et al.,* 2004). A multiple number of design algorithms have been developed for selecting effective siRNAs from a given target sequences (Gong and Ferrell, 2004; Henschel *et al.,*

2004; Reynolds *et al.,* 2004; Snove *et al.*,2004). The algorithms have in built screening system for elimination of ineffective siRNAs. The improved design algorithms, developed by academic groups and commercial vendors, also perform *in silico* screening to eliminate candidate siRNAs with near perfect complementarity with any unintended genes of the genome background whose backdrop the designed siRNAs are to be utilized. For plant biologists computational modeling is still a bottle neck due to non availability of data base, hence designing can only be done keeping three complete genomes like *Oryza*, *Arabidopsis*, and *Populus*. Although most of the miRNA genes deciphered in Arabidopsis are found to be conserved in all the 3 sequenced species, still designing siRNA for effective silencing in plants is a challenging task. The list of web based design algorithms available free are enumerated in Table 4. On comparison of these design algorithms, Yiu *et al.,* (2005) proposed additional parameters like target site accessibility and increased stringency with the design algorithms for effective filtering of siRNAs.

Although the aforementioned design rules ensures potent siRNAs are effectively picked for the silencing, many a times the results are not corroborative with the findings. Thus other criteria for achieving effective RNAi have been evaluated. One such parameter is target site accessibility i.e., structure of target site may affect the effectiveness of a siRNA (Kretschmer-Kazemi and Sczakiel, 2003). Later it was concluded that local mRNA structure is responsible for differential efficiency of the siRNAs in silencing process (Luo and Chang, 2004; Schubert *et al.,* 2005). Of late algorithms have incorporated a parameter to look into the secondary structures of the target mRNA that is deduced employing algorithms like Zucker'sMFOLD (Zuker, 2003) and another RNAfold program from the Vienna package 1.4 (Hofacker and Stadler, 2005) etc. An in built or stand alone BLAST search are incorporated in the design algorithms to ensure that the potent siRNA does not go off-target by silencing unintended transcripts available in the system. Nevertheless, it is clear that all of these features still do not provide an exhaustive description of the determinants of siRNA potency. We can therefore expect additional factors to be identified that contribute to the activity of siRNAs. The effectiveness of such predictions can be validated, only when the experiments and studies conducted *in vivo* yield predicted results.

Table 4: List of the web based design algorithms available for potent siRNA designing (Adapted from Kurreck, 2006)

S.No.	Source	URL
1	Dharmacon	www.dharmacon.com/sidesign
2	Hannon Lab	katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=siRNA
3	Integrated DNA Technologies	scitools.idtdna.com/RNAi
4	Sonnhammer Lab	sisearch.cgb.ki.se
5	Qiagen	http://www1.qiagen.com
6	Invitrogen rnaidesigner	.invitrogen.com/sirna
7	Whitehead Institute	http://Jura.wi.mit.edu/siRNAext
8	siRNA target finder (Genscript)	http://www.genscript.com/ssl-bin/app/rnai
9	Sfold Algorithm	http://sfold.wadsworth.org/index.pl
10	Jack linns	http://www.sinc.sunysb.edu/Stu/shilin/rnai.html
11	MWG-Biotech	http://www.mwg-biotech.com/html/s
12	DEQOR scionics	http://cluster-1.mpi-cbg.de/Deqor/deqor.html

Off-target silencing

RNAi mediated silencing by siRNAs is based on sequence recognition, so targeting a gene by RNAi can give rise to the silencing of another gene with close sequence similarity. This phenomenon is referred to as off-target silencing or cross-reaction and can occur through mRNA degradation or through translational repression (Meister and Tuschl, 2004). Initially the non specific nature of siRNAs was attributed to the inherent sequence and thermodynamic properties of siRNAs. With the improvement in the siRNA design algorithms the problems arising out of these factors have been solved to some extent. Nevertheless the off target effects of the siRNAs are still prevalent in the RNAi mediated silencing. Off target effects are first characterized as a change in 2-3 fold expression levels of mRNAs in a system upon transfection with a siRNA. The expression profiling is unique for a siRNA suggesting that the phenomenon is sequence specific (Jackson *et al.,* 2003). Studies also revealed that a 6 or 7 consecutive matches between the siRNA guide strand and off- target RNA in the context of matching sequence within the unintended mRNA are some of the important parameters that influence the off target mRNA silencing (Lin *et al,* 2005; Birmingham *et al,* 2006).The number and position of mismatches between the siRNA strands and a cross-hybridizing gene affects the likelihood of off target silencing (Du *et al.*, 2005; Holen *et al.*, 2005).

Minimization of off target effects

As siRNA specificity is a sequence dependent process, local alignment algorithms (BLAST and Smith-Waterman) are relied on to weed out the off target generating candidate siRNAs *in silico.* However design algorithms are not effective in culling out the potential off target inducing siRNAs (Birmingham *et al.*, 2006). The next way is to reduce the concentration of the siRNAs to minimum so as to achieve only the on target gene silencing. But the strategy does not work as the minimum concentration, at which siRNAs exhibit reduced off targeting effects, on target silencing also reduced concomitantly (Brown and Samarsky, 2006).Off-target effects resulting from the passenger siRNA strand can potentially be eliminated by modifications that prevent its incorporation into the RISC. For example, chemically modified siRNAs and locked nucleic acids have been used to prevent passenger siRNA strand mediated RNA cleavage etc. (Elmen *et al.,* 2005; Judge *et al.*, 2006).

3. MATERIALS AND METHODS

Molecular biological procedures outlined in Sambrook and Russell, 2000 were followed. Details of commonly used protocols as modified are given in Appendix-II and the composition of buffers and reagents are provided in Appendix-III.

DNA sequence Data

The study aims at RNAi based silencing of replication initiator protein (or replicase protein or AC1 protein) of *Tomato leaf curl virus* (ToLCV). Hence the reference nucleotide sequence of the protein gene was taken from public database NCBI (gi|21930131|gb|AF524893.1| *Tomato leaf curl virus* replicase associated protein (AC1) gene) (Dasgupta *et al.*, 2004).

***In silico* designing of potent siRNAs**

Web based siRNA design tools were used in determining the potent siRNAs of replication initiator protein gene. Here the importance was given for the siRNA inducing capability of Truncated rep (T-*rep*) region, a sub region of replicase protein gene. T-*rep* sub region was formulated comprising of conserved core of replication initiator protein gene and an embedded ORF AC4 encoding for putative geminiviral suppressor protein of RNA silencing. The design algorithms employed and their URL are given below:

S.No.	Design algorithm	URL
1.	DEQOR	http://cluster-1.mpi-cbg.de/Deqor/deqor.html
2.	siRNA target finder: Genscript	http://www.genscript.com/ssl-bin/app/rnai
3.	MWG siRNA design tool	http://ecom.mwgdna.com/cgi/sirna_design.cgi
4.	siRNA at whitehead	http://jura.wi.mit.edu/bioc/siRNAext
5.	Jack Lin's siRNA sequence finder	http://www.sinc.sunysb.edu/Stu/shilin/rnai.html

The rational parameters set out for siRNA designing with the design tools are as follows:(Besides the standard siRNA design rules employed by algorithms are provided in the Appendix-I)

siRNA should possess:

GC content of 30-60%

No stretch of G or C or A or T residues

No U residue at 3' end

No Single nucleotide polymorphism (SNP) sites.

19 mer arm and 2 nt overhangs.

Start with AA and end with TT nucleotides (only in Jack Lin's siRNA sequence finder)

No cross-silencing with transcripts of *Arabidopsis, Oryza, Zea mays* and Human (as the case may be)

In silico secondary structure prediction

Replication initiator protein gene was given as input query in the MWG siRNA design tool to predict the secondary structure of the replication initiator protein mRNA the target for RNAi mediated silencing. The design tool employs the RNAfold program, from the Vienna package 1.4 (Hofacker and Stadler, 2005)

Strategies designed for RNAi silencing

Four different RNAi based strategies were designed to silence the replication initiator protein gene with the concomitant silencing of AC4. Antisense Truncated *rep* (T-*rep*), designed encompasses the 479 nt length (129 to 607) of *replicase* gene. Besides antisense T-*rep*, 60 nucleotides (254-213 nucleotides of *replicase* gene) and 80 nucleotides (254-339 nucleotides of *replicase* gene) from this T-*rep* region were also chosen based on the *in silico* predictions for self complementary inverted repeats (IR) and intron spliced hairpin RNA (IhpRNA) construct formation to encode arms of the hairpins respectively. A region comprising of 21 nucleotides (254-274 nucleotides of replicase gene) of potent siRNA based on DEQOR design algorithm prediction was also

selected for a short hairpin RNA (shRNA) construct formation. In Intron spliced hairpin RNA construct (Ihp RNA), the intronic region of 85bp (synthetic plant intron) (Poogin *et al.*, 2003) to connect the arms of the hairpin is chosen. Whereas in short hairpin RNA construct the octa nucleotide loop was designed between the repeats to express it as hairpin RNA.

Generation of antisense T-*rep* construct

The detailed protocol for the generation of the RNAi inducing constructs is given in the Appendix-II. A truncated *rep* sequence of 479 nucleotides (129–607 of *rep* gene) was amplified using specific primers (5'CATCAAGATCTGTGGAGAGAGC 3' and 5'CGTCGATTGGGTCT CGTCTA 3') from using ToLCV infected plant DNA as template. The PCR fragment was cloned in the pGEM–T–EASY vector and confirmed by sequencing. T-*rep* was further sub cloned in the pUC 118 vector under the transcription control of the *Cauliflower mosaic virus* 35 S promoter and 35S terminator at the *Not*I site. The cassette comprised of the 35S promoter, T-*rep*, and terminator (1078 bp) was released from the pUC 118 clone using *Bam*HI and *Hin*d III, then sub cloned in pCAMBIA 2301, a binary vector at the respective sites.

Generation of RNAi inducing inverted repeats constructs

Inverted repeats (IR-*rep*) construct

DNA sequences homologous to 60 bases of viral genome of ToLCNDV consisting of 18 nucleotides from the AC4 region and 42 nucleotides from the conserved region were selected. Inverted repeats of 120 bp were synthesized in vitro using *Taq* polymerase and appropriately designed oligonucleotides. The sequence spans the viral genome from 254–313 (Acc. No. AF 524893), and consequently, it includes a potent siRNA-inducing sequence:

CACATTTCCATCCGAACATTC(→sense)
TCGTGTAAAGGTAGGCTTGTA(←antisense).

It was cloned in the pUC 118 vector between and under the transcription control of the *Cauliflower mosaic virus* 35 S promoter and 35S terminator. The cassette comprised of the promoter, the inverted repeat, and the terminator (720 bp) was released from the pUC 118 clone using *Eco*RI and *Hin*d III then sub cloned in pCAMBIA 2301, a binary vector at the respective sites.

Intron spliced hairpin RNA (Ihp RNA-*rep*) construct

DNA sequences homologous to 80 bases of viral genome consisting of 18 nucleotides from the AC4 region, and 62 nucleotides from the conserved core of the AC1 gene were selected. Inverted repeats of 250 bp was synthesized in vitro as described earlier with an intron (Pooggin *et al.*, 2003) of 85 bp placed between the repeats. The sequence spans the viral genome from 254–339 (Acc. No. AF 524893) and consequently, it includes a potent siRNA-inducing sequence as mentioned previously. It was cloned in the pUC 118 vector between and under the transcription control of the *Cauliflower mosaic virus* 35 S promoter and 35S terminator. The cassette comprising of the promoter, the inverted repeat, and the terminator (850 bp) was released from the pUC 118 clone using *Eco*RI and *Hin*dIII, then sub cloned in pCAMBIA 2301, a binary vector at the respective sites.

Short hairpin RNA (sh RNA-*rep*) construct

A DNA sequence of 21 bases homologous to potent siRNA in the viral genome (254–274) was selected for synthesis of short inverted repeats with an eight nucleotide sequence for loop formation. It was cloned in the pUC118 vector between and under the transcription control of the *Cauliflower mosaic virus* 35 S promoter and 35S terminator. The cassette comprising of the promoter, the inverted repeat, and the terminator (650 bp) was released from the pUC 118 clone using *Eco*RI and *Hin*dIII, then sub cloned in pCAMBIA 2301, a binary vector at the respective sites.

Mobilization of constructs into *Agrobacterium*

The recombinant binary vector pCAMBIA 2301 carrying T-*rep*, IR-*rep*, Ihp-*rep* and sh-*rep* were mobilized into *Agrobacterium tumefaciens* strain LBA 4404 by freeze and thaw method (Hofgen and Willmitzer, 1988) as described in Appendix II.

Tomato transformation (*Solanum lycopersicum*)

Type of explants

For obtaining stable transgenic plants leaf discs excised from one week old tomato plants of cv Pusa Early Dwarf (PED) were used. The leaf disc method is advantangeous as it does not require treatment of any phenolic compounds like acetosyringone for inducing *Agrobacterium* infection and T-DNA integration.

Surface sterilization of explants

The leaves collected from one week old tomato plants were surface sterilized with 0.1% $HgCl_2$ for 5 minutes and washed with sterile double distilled water to remove any adhering sterilizing agents.

Preparation of explants

After surface sterilization explants were blot dried using autoclaved 3 mm filter paper discs and cut into small pieces with the help of scalpel. Such leaf discs were used as explants. Before *Agrobacterium* infection, leaf discs were allowed to incubate on the regeneration medium for 2 days at 25° C temperature in tissue culture room. Proper care was taken while placing leaf discs on the medium so that dorsal surface was always in contact with the medium.

Co-cultivation of leaf discs with *Agrobacterium*

Agrobacterium containing constructs were grown in Luria broth (LB) with kanamycin (50µg/ml) and streptomycin (100 µg/ml) at 28° C and 200 rpm overnight. 50

ml of overnight grown agro-culture was taken in centrifuge tubes and pelleted at 4° C temperature, 6000 rpm for 10 minutes. Approximately 20 ml autoclaved MS liquid (MS salt without agar) along with 200 µl of agroculture were taken and the leaf discs was transferred into it and kept for 20 minutes with occasional slow shaking of the plate for proper infection. The leaf discs were then transferred in another plate for drying using sterile blotting paper.

Re-transfer of co-cultivated leaf discs to regeneration medium

After blot drying the leaf discs were transferred into regeneration medium for two days with complete darkness for proper transfer of T-DNA into plant genome.

Selection and regeneration of plantlets

After 48 hours of co-cultivation the leaf discs were transferred into selection medium for callus formation. Further, they were transferred to shooting medium for shoot development. Once shootlets were formed, they were transferred into rooting medium for complete plantlet regeneration.

Molecular analysis of transformants

PCR detection

Total DNA isolated from the transformed plants were used as templates for detection of transgene integration by performing PCR. The primers employed were 35 S P-T which detects the entire cassette of all the transgene constructs (as described in Appendix II).

Copy number analysis of transgenes

Total DNAs extracted from transformed plants, after restriction digestion with *Bam*HI, were electrophoresed in 1.2% agarose gel. DNA fragments were transferred to nitrocellulose membrane (NCM, Milipore) employing the procedure developed by Southern (Southern, 1975). The gel was immersed in alkaline denaturing solution (1.5M

NaCl, 0.5N NaOH) for 30 min followed by soaking in neutralizing solution (1M Tris-HCl, pH 7.4, 1.5M NaCl) for 30 min and rinsed with distilled water, later the gel was transferred to transfer tank containing transfer buffer (10X SSC) and placed in inverted position on the glass plate over the pre-soaked Whatman paper wig, and the surrounding portion of gel was covered with polythene sheet. Over the gel, pre-soaked nylon membrane and 3 pieces of wet Whatman paper No. 3 was kept. Then about 40 coarse filter paper folds were placed over the Whatman paper followed by 0.5-1 kg weight at the top of the filter papers. The capillary transfer of DNA was allowed for 18-20 hours. At the end of transfer, NCM was marked at the well region and was rinsed in 2X SSC to remove traces of agarose sticking to the membrane. Then the DNA on the membrane was fixed by baking at 80°C for 2 h. The NCM was used for hybridization with radio labeled probe.

Prehybridization

- The baked NCM blots were kept in hybridization cylinders.
- Prehybridization solution was added at the rate of 0.2 ml/sq cm.
- The cylinders were then incubated at 65°C for 4h in hybridization oven with gentle rotation.

Probe preparation (Random primer labeling of DNA)

The random primer labeling reaction was done in 50 µl reaction mixture. 100 ng of DNA template (sh-*rep*) and 1µl of random primer was added into an microfuge tube containing 34.5 µl of nuclease free H_2O and boiled for 2 min and immediately kept on ice. 5 µl of 10 X labelling buffer, 2 µl dNTPs without dCTP, 2.5 µl DTT, 2 µl BSA, 1 µl [α-^{32}P] dCTP (10 µCi/µl, 3000 Ci/mmol) and 1µl Klenow enzyme were added to the microfuge tube and incubated at 37°C for 1-2 h. The labeled probe was stored at -20°C till further use.

Hybridization

The double stranded α-^{32}P labelled DNA probe was denatured in a boiling water bath for 5 min. The denatured probe was added to pre-hybridization solution at 0.5 x 10^6 cpm/ml concentration. It was allowed to hybridize for 18h at 65^0C in hybridization oven with gentle rotation.

Autoradiography

The hybridization solution was discarded and the membrane was washed 3 times with 2 X SSC, 0.1 percent SDS at 65^0C, each time with duration of 15 min. The washed membrane was dried on a paper towel, then placed within fold of cling film, placed in a lead cassette and exposed to X-ray film (KODAK) for 18h at -70^0C. Autoradiograph was then developed as per X-ray films manufacturer's instructions.

Gene silencing studies

Tomato plants infected with the *Tomato leaf curl virus* were maintained in a glasshouse condition through whitefly transmission. For gene silencing assays, calli derived from young symptomatic leaves were regenerated and co cultivated separately with *Agrobacterium* carrying the constructs (McCormick, 1991). As a control non infected leaf explants and infected leaf explants were regenerated without co-cultivation of *Agrobacaterium.* Explants were incubated in Petri plates on callusing medium containing MS salts, vitamins, 3% sucrose, 0.2 mg/L NAA, 1 mg/L BAP, 50 mg/L kanamycin (in the case of co cultivated explants), and 200 mg/L cefotaxime (pH 5.8). The culture conditions were 25°C for a 16-hour photoperiod. They were later transferred to shooting and rooting medium, respectively (MS salts, 2.5 mg/L BAP, 0.5 mg/L IBA; half MS, 0.2 mg/L NAA, respectively). Kanamycin (50–100 mg/ L) was used for selection of transgenic tomato plants. The rooted explants were transplanted into small pots containing sterilized mixture (soil:peat:vermiculite, 1:1:1) for establishment. The transformed as well as the control plants were shifted to large earthen pots in the greenhouse before being assayed for the presence of virus. The plants were observed for

the development of leaf curl symptoms from the shooting stage onward and the presence/absence of virus was substantiated by PCR analysis using specific primers

(Forward primer: 5'-TTGGATCCATGGCGAAGCGACCA-3' and
Reverse primer: 5'-AAGAGCTCTTAATTTGTGACCGA- 3') for the coat protein gene (AV1; AY390957) of ToLCV.

Sequence alignment

The nucleotide sequence data of short hairpin RNA derived siRNA and recently reported put-microRNA of tomato (Pilcher *et al.*, 2007) was analyzed with BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999). The same was used for any other sequence alignment functions during the study until mentioned otherwise.

4. RESULTS

RNA interference (RNAi) is a double-stranded RNA (dsRNA)-induced gene-silencing phenomenon that is conserved among various organisms. Because of its high specificity and efficacy, it has been widely used as an efficient tool to downregulate the expression of a gene. Its application in the development of virus resistant transgenics is gaining momentum and numerous successful attempts have been made in that direction. The present study sought to achieve the RNAi mediated resistance against *Tomato leaf curl virus* infecting tomato plants. The target sequence for the RNA mediated silencing was replication initiator protein or replicase protein encoded by ORF AC1 of the viral complementary sense DNA. It is an indispensable protein for the virus replication to occur in the host cellular system in association with host DNA polymerase (Jupin *et al.*, 1995; Choi and Stenger, 1996; Hanley-bowdoin *et al.*, 2000). The protein possesses the DNA nicking and joining properties, essential for the viral DNA replication.

The ORF encoding replicase protein (AC1) is characterized by the presence of 330 nucleotides as a conserved core among the various isolates of the viruses causing the leaf curl disease in tomato (Dasgupta *et al.,* 2004). An embedded small ORF, AC4 although not attributed with any function, but its role in small RNA binding have been demonstrated in related geminiviruses (Chellappan *et al.,* 2005). Its role in small RNA binding has been revealed in *Nicotiana benthamiana* expressing AC4 (Kumari, 2006). Hence the ORF AC4 is considered as a putative viral suppressor protein (Chellappan *et al.*, 2005). Therefore any RNAi based resistance approach for the leaf curl virus resistance will be more effective with the concomitant silencing of AC4 for trait stable virus resistant transgenics.

Replicase sequences for RNAi based silencing

A sub region of replication initiator protein, named as truncated *rep* (T-*rep*), was identified as a suitable source of transgene for RNA mediated silencing of the virus (Fig.6).

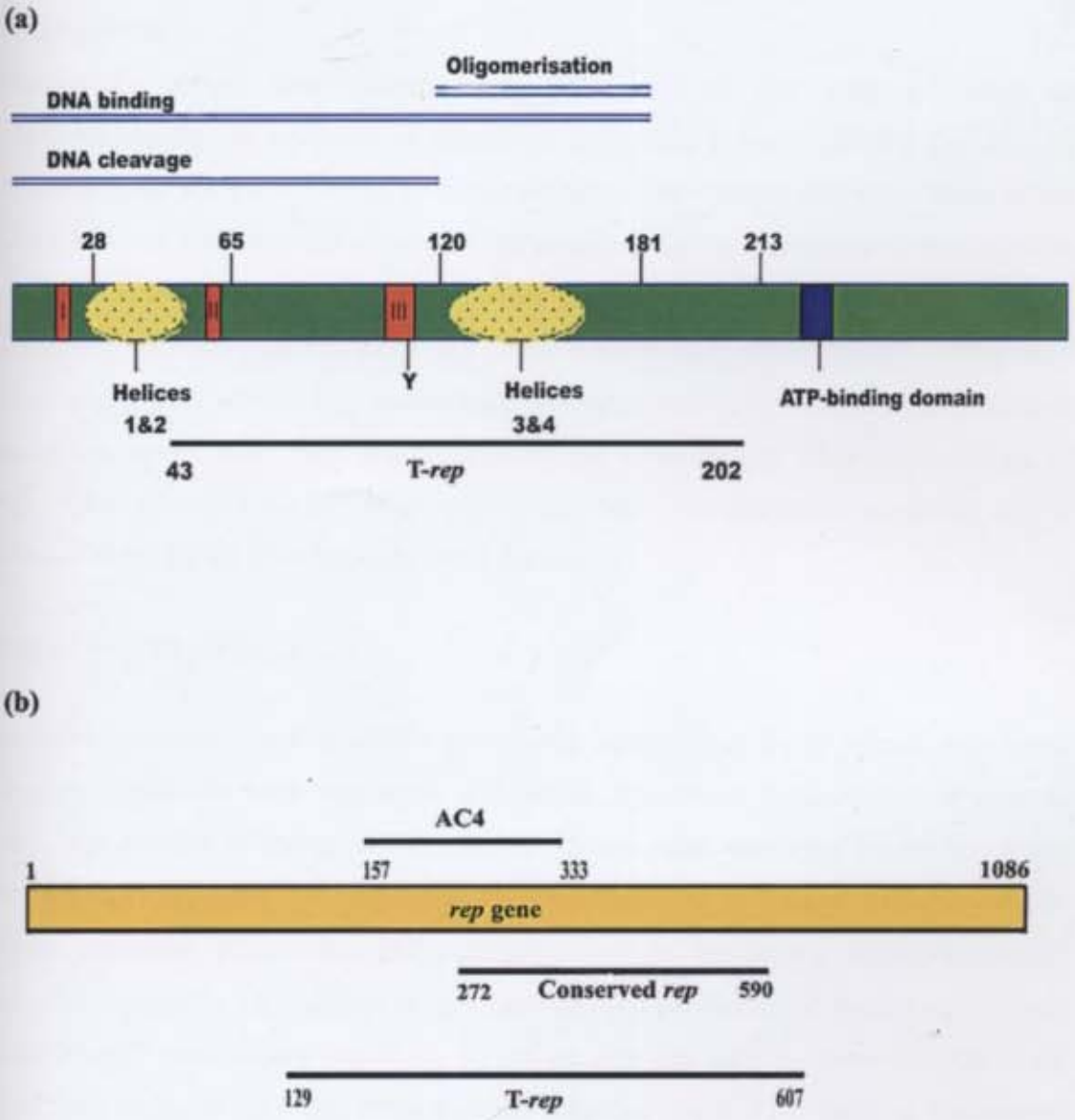


Fig. 6: Replication initiator protein and gene depicting Truncated *rep* (T-*rep*) region

(a): Functional domains and motifs (I,II and III) of replication initiator protein with T-*rep* targeting DNA binding, cleavage and oligomerization domains including active site Tyr-103

(b): *rep* gene showing conserved core region and embedded ORF AC4, encoding putative viral suppressor protein, as target for T-*rep* region

The T-*rep* region is characterized as to possess

a) A conserved core of 318 nucleotides stretch coding for conserved amino acids of the protein
b) Nucleotide region that encodes for motif III of the protein, which is characterized by the presence of conserved aminoacid residue Tyr-103 (Y-103), an active site for the protein in its catalytic activity. The domain also contributes to the DNA binding and cleavage activity of the protein which is indispensable for the virus to establish in the intracellular system of the plants.
c) An embedded ORF, AC4 of ToLCV a putative RNAi suppressor protein. With a view to concomitantly silence this putative suppressor protein, target nucleotide sequences encoding for the same were also included in the T-*rep* region. Thus the identified T-*rep* region stretches for 479 nt in length (129 -607 co-ordinates in *rep* gene), and is characterized by all the aforementioned features.

Designing of Potent siRNAs

In order to identify, potent siRNA generating capability of T-*rep* region, web based siRNA design algorithms were employed with entire replication initiator protein gene as input query. The number of potent siRNAs and the design rules employed by the concerned algorithms are in Fig (7) and were analyzed for their thermodynamic properties, nucleotide composition, antisense preference, polynucleotide feature depending upon the design algorithms (See Appendix-I for siRNA design rules and out put results of design algorithms). An in-built BLAST search was necessary to ensure that the siRNAs have no significant homologies with endogenous genes other than the intended target. This filtering was carried out to minimize the off-target effects of siRNAs with the genome or transcripts database of *Arabidopsis* in DEQOR, siRNA target finder, Jack Linns design tools. Besides *Arabidopsis*, DEQOR package also analyzed the off-target effects of siRNAs against the genomes of *Oryza* and *Zea mays*. To ascertain any remotest possibility of these siRNAs acting off-targets against the transcripts of human beings, the ultimate consumer of the genetically modified product, the BLAST homology search was carried out with design tools DEQOR,

(a)

Design algorithms	Potent siRNAs in *rep* gene	siRNAs in T-*rep* region	Off- targets checked against	Design rules	Features
DEQOR	10	2	*Arabidopsis*, *Oryza*, *Zea mays*, human	Own set of rules	Suitable for plant RNAi
siRNA target finder (Genscript)	10	5	*Arabidopsis*	Combination of Tuschl, Reynolds, Ui-Tei	-
siRNA white head	5	3	Human	Combined Tuschl and Reynolds, adjusted	Considers thermodynamic properties of siRNAs
Jack linns	10	7	*Arabidopsis*	Tuschl	Shows off- targets with *Arabidopsis* genome
MWG siRNA design tool	16	5	Human	Reynolds	Analyses target accessibility for siRNAs

(b)

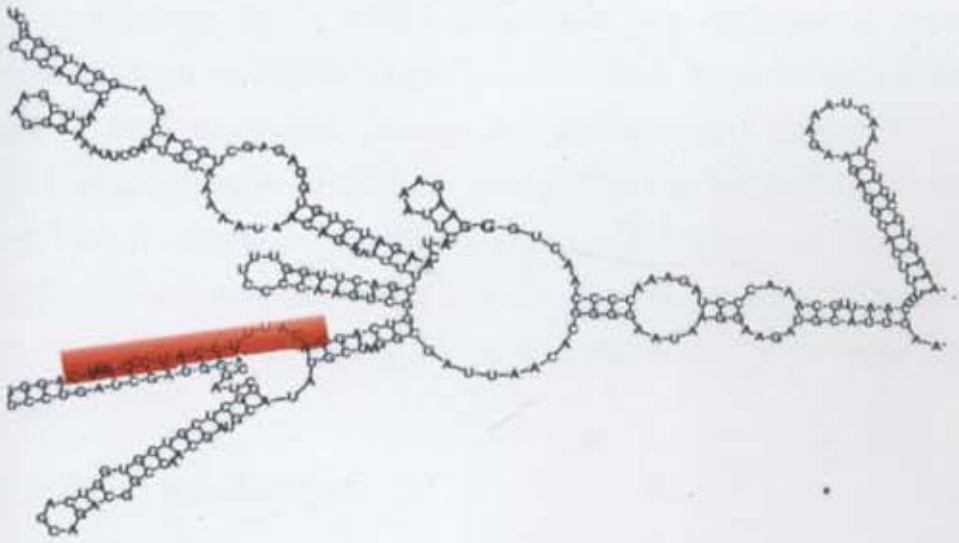


Fig. 7: *In silico* designing of potent siRNAs from replication initiator protein gene
(a): Out put of siRNA design algorithms elucidated with their characteristic features
(b): Accessibility of replicase protein mRNA for potent siRNA binding and cleavage
Potent siRNA binding target site is highlighted in red.

siRNA white head, MWG siRNA design tool. The siRNAs were thus filtered in various strata to find suitable one for RNAi based silencing.

Based on these results a potent siRNA inducing target region was identified in T-*rep* region. The potent siRNA of 21 nucleotides length running from nucleotide position 254-294 in the replication initiator protein gene was chosen for further RNAi inducing constructs formation.

CACATTTCCATCCGAACATTC(→sense)
TCGTGTAAAGGTAGGCTTGTA(← antisense).

This siRNA possesses favourable GC content of 42.9 %, antisense preference, and exhibits no polynucleotide feature. It not only targets the conserved core region of the replication initiator protein, but also the putative viral suppressor protein encoded by overlapping ORF AC4.

Viral gene target accessibility

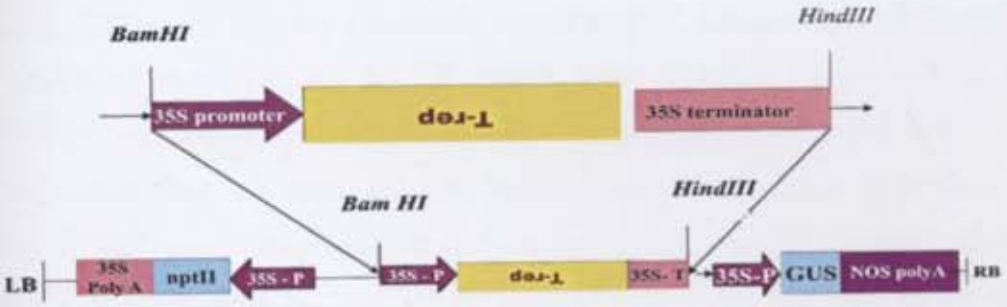
The design algorithm MWG siRNA design tool was employed to predict the secondary structure of target replication initiator protein mRNA. It was carried out using the RNA fold program from the Vienna package 1.4. (Hofacker and Stadler, 2005). The candidate siRNA obtained from the DEQOR design algorithm was analyzed for it's ability to access the target mRNA without any hindrance. It was deduced that the siRNA has free access to the replication initiator protein mRNA as is evident from the Fig.7. The target site exhibited, open region with a high number of unpaired nucleotides in the target replicase mRNA of the virus.

Construction of RNAi based vectors

Antisense T-*rep* construct

An amplicon of 479 bp corresponding to T-*rep* region was obtained with DNA template of ToLCV infected plant while the healthy plant did not show any amplification indicating the viral origin of the amplicon. The PCR purified amplicon (479 bp) was then cloned in pGEM-T Easy vector. Successful clones of the T-*rep* insert in the pGEM-T Easy

(a)



(b)

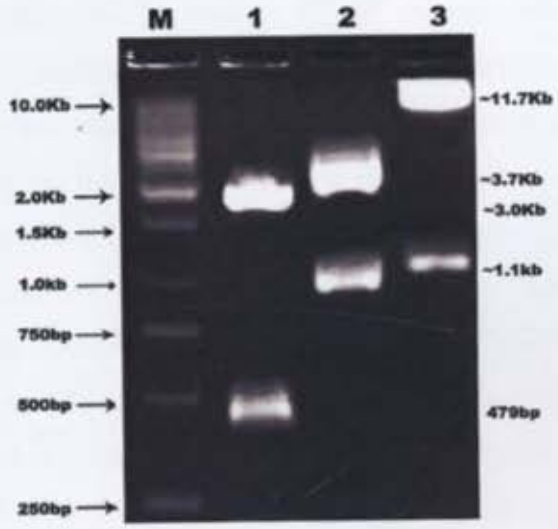


Fig.8: Generation of antisense T-*rep* construct

(a): Schematic representation of antisense T-*rep* construct

(b): Release of T-*rep* from the respective plasmid vectors

Lane M:1 Kb Molecular size marker, Lane1: pGEM-T- T-*rep* (*Not* I restricted)
Lane 2 : pUC-118-35S-P-T-T-*rep*, Lane 3: pCAMBIA 2301-T-*rep*
(*Bam*HI and *Hin*dIII digested)

vectors was identified by colour screening on indicator plates containing ampicillin, X-gal and IPTG, as the vector contains the ampicillin resistance marker gene and the insert interrupts the coding sequence of β-galactosidase thus producing white colonies upon overnight incubation at 37°C temperature. Around eighty white colonies were found in X-gal, IPTG, and ampicillin plate. They were streaked on master plates separately and ten representative colonies among them were screened for the presence of insert of 479 bp by performing colony PCR using specific primers described earlier. On the basis of the colony PCR results, pGEMT-T-*rep* clone no. 4,6,7,9 and 10 were found to be positive for the presence of 479 bp T-*rep* insert. Among them, clone no. 6 (pGEMT –T-*rep*-6 clone) was picked from the master plate and subjected for restriction enzyme digestion to confirm the presence of the T-*rep* insert. Electrophoresis of the restriction enzyme digested mixture found that pGEMT-T-*rep*-6-clone released 479 bp insert [Fig 8(b)], the expected length of T-*rep*.

The gel purified fragment containing the T-*rep* insert released from the pGEMT- T-*rep*-6 clone was cloned in the pUC118 vector. Fifteen colonies were randomly picked for colony PCR from ampicillin plates as the vector carries ampicillin resistance marker gene. Out of these colonies only two colonies were found to be positive showing an amplicon of 479 bp. Plasmid DNA from the positive colony designated as pUC118-T-*rep*-12 was restricted with *Not* 1 enzyme to release the 479bp. It was again reconfirmed by restriction with *Bam*H1 and *Hin*dIII enzymes, as the positive clone released the whole cassette (CaMV 35S Promoter + T-*rep* gene + CaMV 35S terminator), as one fragment of ~ 1.1 kb [Fig.8 (b)]. The presence as well as orientation of the T-rep gene in the above clone was confirmed by sequencing. The released ~ 1.1 kb fragment carrying the cassette was gel eluted and sub-cloned in binary vector pCAMBIA 2301 which was linearised by double digestion with *Bam*HI and *Hin*dIII enzymes. The 12 colonies obtained in kanamycin selection plate were then screened for recombinants by colony PCR using specific primers. Only one colony (pCAMBIA 2301-T-*rep*-8) was positive for the presence of T-*rep* insert as shown by the amplicon of 479bp. Plasmid DNA from positive colony was then restricted with *Bam*HI and *Hin*dIII to confirm the release of a cassette. The positive colony (pCAMBIA 2301-T-*rep*-8) released ~ 1.1 kb fragment on restriction [Fig 8(b)].

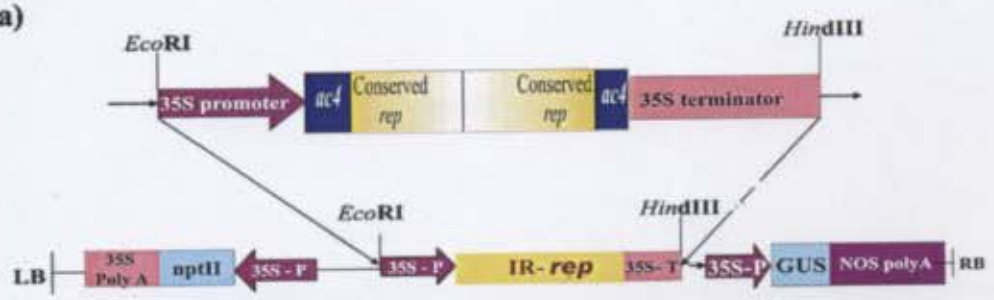
Inverted repeats constructs

Three different types of RNAi inducing inverted repeats constructs viz., Inverted repeats (IR-*rep*), intron spliced hairpin RNA (Ihp-*rep*) and short-hairpin RNA (sh-*rep*) were developed as explained earlier in materials and methods. The *in vitro* recombinant pUC 118 plasmids carrying inverted repeats *rep* (IR-*rep*) sequences (pUC118-IR-*rep*), Ihp-*rep* sequences (pUC 118-Ihp-*rep*) and sh-*rep* sequences (pUC 118 sh-*rep*) were confirmed for the cloning of the insert with restriction enzymes *Eco*RI and *Hin*d III. Double digestion of these recombinant plasmids resulted in the release of ~720bp cassette (35S promoter: inverted repeats-*rep*: 35S terminator), 850 bp cassette (35 S promoter: Intron spliced hairpin RNA-*rep*: 35 S terminator) and 650bp cassette (35 S promoter: short hairpin RNA-*rep*: 35 S terminator) respectively (Fig 9-11(b)). The released inserts were gel eluted and cloned individually in binary vector pCAMBIA 2301 that was suitably restricted with enzymes *Eco*RI and *Hin*d III for cloning. The recombinant clones were screened on kanamycin containing plates. Out of 12, 15 and 5 colonies obtained in kanamycin plates for IR-*rep*, Ihp-*rep* and sh-*rep* cloning respectively 3, 6 and 2 colonies were positive for the presence of respective sequences, when subjected to colony PCR using primers specific for 35 S promoter and terminator sequences flanking the inverted repeats sequences. Among them one each resultant positive pCAMBIA 2301 plasmid (pCAMBIA 2301-IR-*rep*-3), (pCAMBIA 2301-Ihp-*rep*-4) and (pCAMBIA 2301- sh-*rep*- 1) were confirmed for the cloning of respective sequences upon restriction with enzymes *Eco*RI and *Hin*d III. The positive clones exhibited the release of the whole cassette (CaMV 35S Promoter + inverted repeats sequences + CaMV 35S terminator), as one fragment of ~ 720bp, 850bp and 650bp respectively for IR-*rep*, Ihp-*rep* and sh-*rep* respectively (the expected sizes) [Fig. 9-11(b)]

Mobilization of constructs into *Agrobacterium*

Recombinant binary plasmids carrying all the four different kinds of gene constructs individually were mobilized into *Agrobacterium tumefaciens* strain LBA 4404 by freeze and thaw method. Number of colonies obtained on kanamycin and streptomycin selection plate were 7,13,4 and 6 respectively for (pCAMBIA 2301-T-*rep*-6) (pCAMBIA 2301-IR-*rep*-3), (pCAMBIA 2301-Ihp-*rep*-4) and (pCAMBIA2301- sh-*rep*- 1) plasmids. Upon screening by

(a)



(b)

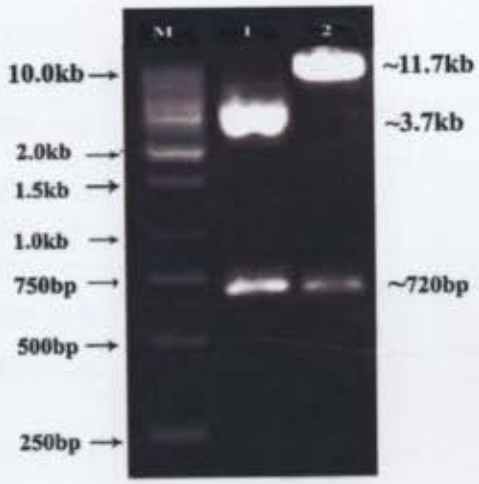


Fig. 9: Generation of inverted repeats-*rep* (IR-*rep*) construct

(a): Schematic representation of IR-*rep* construct

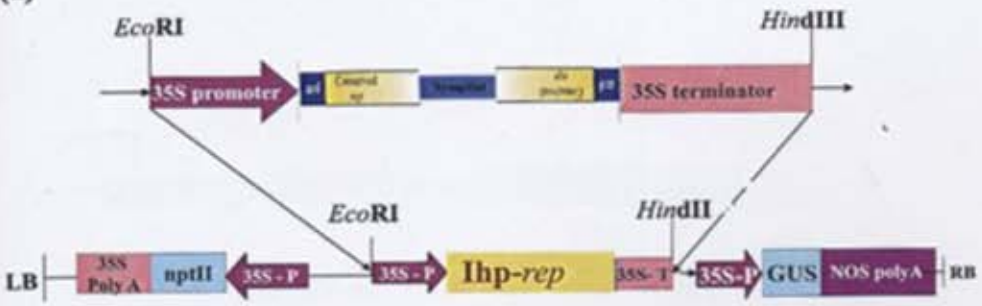
(b): Release of inverted repeats-*rep* construct from the respective vectors on double digestion with *Eco*RI and *Hin*dIII

Lane M:1Kb Molecular size Marker

Lane 1:pUC-118-P-T-35S-IR-*rep*

Lane 2:pCAMBIA 2301-IR-*rep*

(a)



(b)

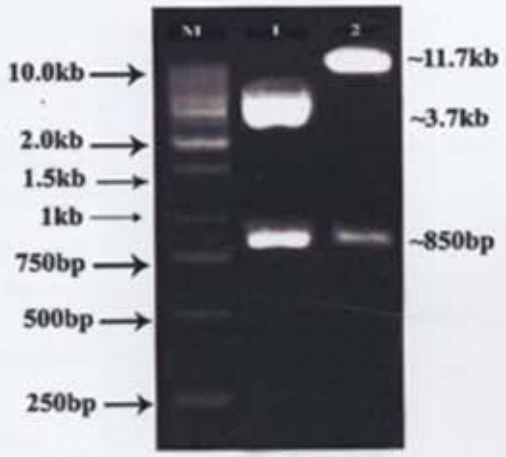


Fig. 10: Generation of Intron spliced hairpin RNA-*rep* (Ihp-*rep*) construct

(a): Schematic representation of Ihp-*rep* construct

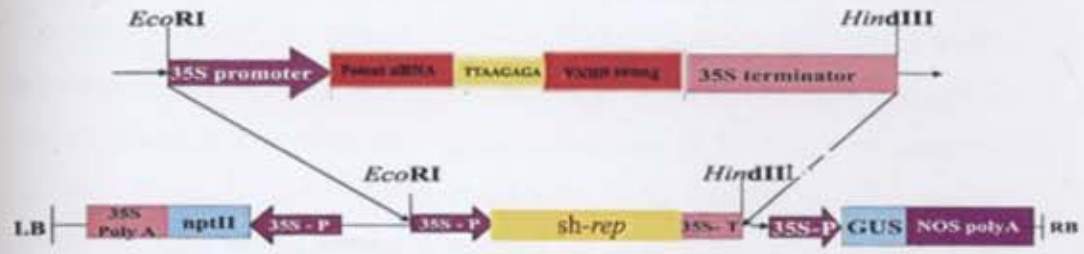
(b): Release of Ihp-*rep* construct from the respective vectors on double digestion with *Eco*RI and *Hin*dIII

Lane M:1Kb Molecular size Marker

Lane 1: pUC-118-35S-P-T-Ihp-*rep*

Lane 2: pCAMBIA 2301-Ihp-*rep*

(a)



(b)

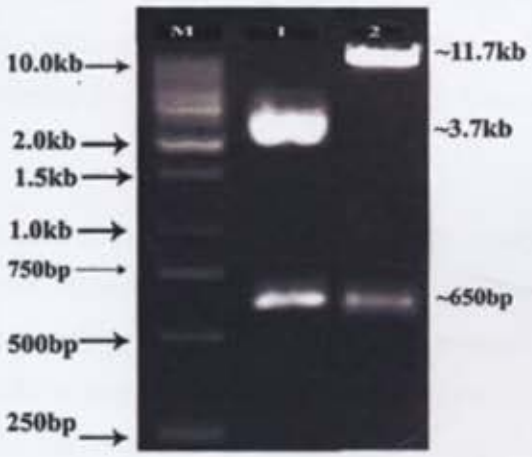


Fig. 11: Generation of short hairpin RNA-*rep* (sh-*rep*) construct

(a) : Schematic representation of sh-*rep* construct

(b) : Release of the sh-*rep* construct from the respective vectors on restriction with *Eco*RI and *Hin*dIII

Lane M: 1Kb Molecular size Marker

Lane 1: pUC-118-35S-P-T-sh-*rep*

Lane 2: pCAMBIA 2301-sh-*rep*

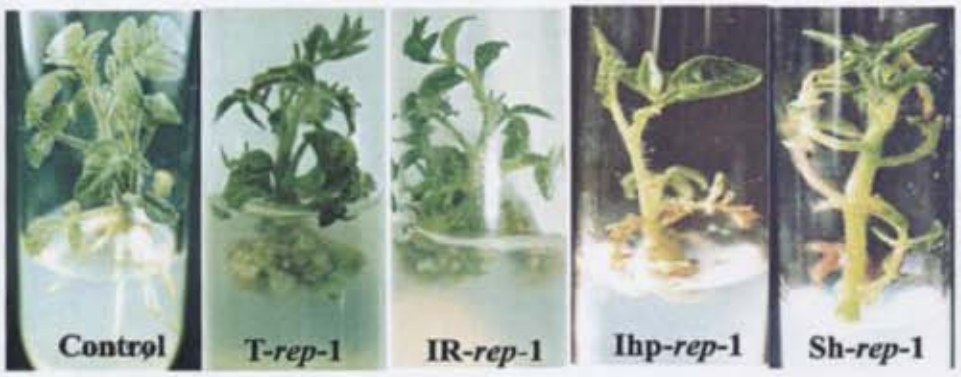
colony PCR 25, 37, 24, and 23 *Agrobacterium* colonies were positive for the presence of antisense T-*rep*, IR-*rep*, Ihp-*rep* and sh-*rep* containing binary plasmids, respectively by showing the respective size amplicons.

Tomato transformation

A total of 100 cotyledonary leaf explants of *Solanum lycopersicum* (*cv.* Pusa Early Dwarf) per transformation were co-cultivated with *Agrobacterium* carrying the construct. Also 20 cotyledonary leaf explants were kept for regeneration as a control. On Kanamycin plates 65, 73, 82 and 37 co-cultivated explants were selected leading to the formation of calli, ready for organogenesis with T-*rep*, inverted repeats (IR-*rep*), Intron spliced hairpin RNA (Ihp-*rep*) and short hairpin RNA (sh-*rep*) constructs respectively. Shoot induction was observed after 10-15 days on shooting medium with kanamycin followed by rooting for another 10-15 days in rooting medium. Thus the number of plantlets regenerated completely with all the four constructs differed widely (Fig.12). Transformation of intron spliced hairpin RNA construct (Ihp-*rep*) led to regeneration of 79 shootlets as against mere 30 shootlets regenerated with short hairpin RNA (sh-*rep*) construct. Both T-*rep* and inverted repeats (IR-*rep*) constructs providing 60 and 65 shootlets respectively. Thus on complete plant regeneration the constructs behaved differentially in all the stages of transformation as elucidated in the Table 5.

The confirmation of transgene integration in each transformation event was carried out with PCR detection of transgenes in the transformed plants. PCR based detection was carried out with primers specific for 35 S promoter and 35S terminator so that the entire cassette is amplified (Fig12.b) It was found that 60 plantlets were detected positive for the intron spliced hairpin RNA (Ihp-*rep*) transgene integration. Whereas only 13 plantlets were found to have the integration of short hairpin RNA (sh-*rep*) construct as against 55 and 45 with the transformation of T-*rep* and inverted repeats (IR-*rep*) constructs (Table 5). Thus transformation efficiency of the different constructs varied, with Ihp-*rep* providing 60% transformation efficiency as against only 13 % efficiency attained with sh-*rep* transformation. T-*rep* and IR-*rep* exhibited a transformation efficiency of 55 and 45 % respectively.

(a)



(b)

(c)

Fig. 12: Tomato transformtion of RNAi constructs

(a): Transformants along with control plants

(b): PCR confirmation of transgene integration using 35S-P-T primers

(c): Southern blot analysis of transformants using sh-rep sequences as probe

Table 5: Transformation efficiency of different RNAi constructs at various stages of transformation

Transformation stage	RNAi constructs			
	Truncated *rep*	**Inverted repeats-*rep***	**Intron spliced hairpin RNA-*rep***	**Short hairpin RNA -*rep***
No. of cotyledonary leaves per transformation	100	100	100	100
No. of shoots selected on kanamycin medium	60	65	79	30
No. of plantlets confirmed by PCR	55	45	60	13
Transformation efficiency	55%	45%	60%	13%

Copy number analysis of transgenes

To confirm the integration of the transgene in tomato plants and to analyze the copy number of the transgenes, Southern blotting was carried out with sh-*rep* sequences as labeled probe as it is present embedded in all the four different type of RNAi constructs. Seven independent transgenic events (T-*rep* 1 - 7) were confirmed to be positive for T-*rep* constructs by PCR Southern analysis. The genomic Southern of one of the representative transgenic event showed the presence of 2 copies of transgene. In case of intron spliced hairpin RNA (Ihp-*rep*), Inverted repeats (IR-*rep*) and short hairpin RNA (sh-*rep*) constructs, five (Ihp-*rep* 1 - 5), four (IR-*rep* 1 - 4) and four (sh-*rep* 1-4) transgenic events were detected in PCR Southern analysis. Representative transgenic event from each of the constructs showed the presence of 2 copies on transformation with IR-*rep* and a single copy of transgenic event each in Ihp-*rep* and sh-*rep* constructs respectively (Fig.12.C).

Gene silencing Studies

To study the efficacy of constructs in silencing *Tomato leaf curl virus* genome, all these four constructs were mobilized in a healthy and ToLCV-infected tomato through Agrobacterium-mediated transformation. Calli derived from young *Tomato leaf curl virus* infected leaves of tomato were regenerated and co-cultivated separately with *Agrobacterium* carrying the constructs. Non-transformed infected and healthy leave explants were maintained as controls. Transformed and non transformed plants were observed for the development of leaf curl symptoms from the shooting stage onward. The gene silencing was substantiated by PCR analysis using specific primers (Forward primer: 5' TTGGATCCATGGCGAAGCGACCA 3'and Reverse primer 5'AAGAGCTCTTAATTTGTGACCGA 3' for the coat protein gene of (AV1; AY390957) of ToLCV. 38 out of 40 ToLCV-infected explants, when transformed with each of antisense T-*rep*, IR-*rep* , and Ihp-*rep* gene constructs were found to be fully recovered from the virus infection, while the non-transformed (regenerated from the ToLCV-infected leaf) plants showed the typical leaf curl symptom, poor growth, and poor root formation.(Fig13.b). Recovery of infected plants from virus infection was supported at the molecular level by the PCR-based analysis of the presence or absence of the viral coat protein gene. The suppression of the viral genome was

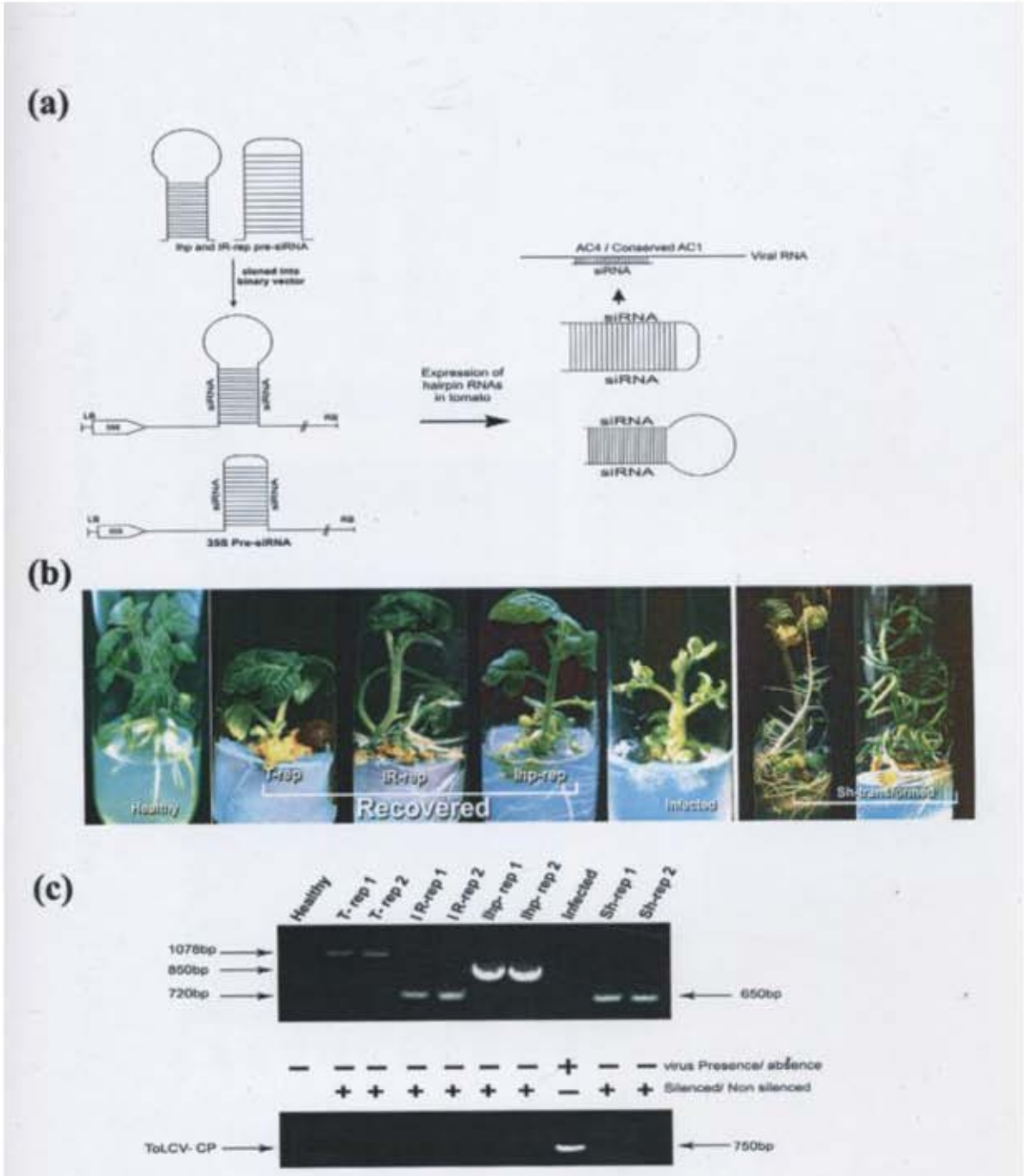
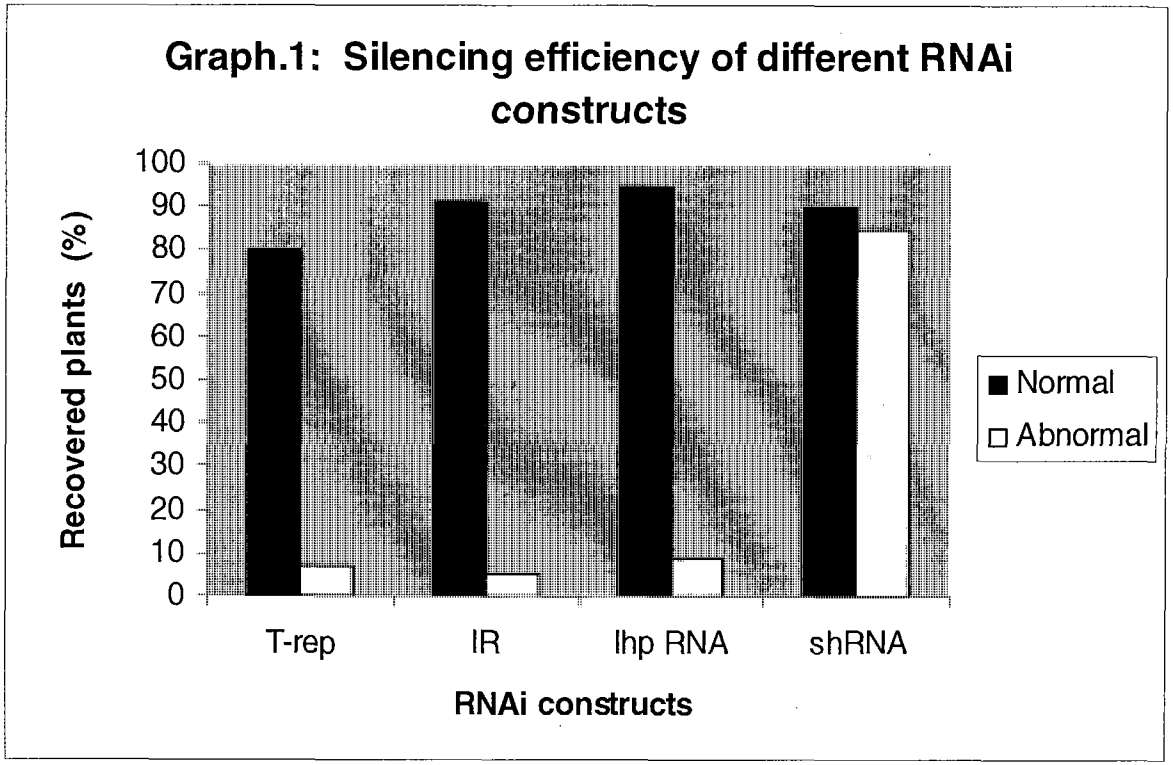


Fig.13: Gene silencing studies with RNAi constructs

(a) :Ihp-*rep* and IR-*rep* pre-siRNA derived siRNA targeting viral Replicase mRNA

(b) :Tomato expressing RNAi constructs showed recovery from ToLCV infection

(c) :Viral gene silencing was ascertained by the presence or absence of ToLCV coat protein gene through PCR using gene-specific primers.


Graph.1: Silencing efficiency of different RNAi constructs
Recovered plants (%)
100
90
80
70
60
50
40
30
20
10
0
T-rep
IR
Ihp RNA
shRNA
RNAi constructs
Normal
Abnormal

confirmed by the non amplification of the coat protein gene in plants transformed with *Agrobacterium* containing the constructs against the presence of the coat protein gene of ToLCV in non transformed regenerated plants (Fig.13c). The percentage recovery in different RNAi constructs varied from 80 to 95% of transformed plants. Upon transformation with Ihp-*rep* construct 95% of plants exhibited recovery from typical leaf curl symptoms. In case of T-*rep* and IR-*rep* constructs 80 and 91 % of plants manifested recovery from viral infection where as 90 %of sh-*rep* transformed plants showed recovery phenotype and corroborated with the non amplification of ToLCV coat protein gene. The transformed plants also exhibited severe phenotypic anomalies but the extent of severity varied with constructs (Graph. 1).

Developmental aberrations in transformants

Phenotypic abnormalities were observed with the transformation of all the RNAi constructs in tomato. In case of Ihp-*rep* transformation 5% of plants showed phenotypic abnormalities like needle shaped leaves, radial rooting pattern etc. Whereas with T-*rep* and IR-*rep* constructs 7 and 5% of the transformed plants exhibited developmental anomalies like needle shaped leaves, chloroplastic roots etc. The peculiar case, is with the transformation of sh-*rep* construct, 85% among them were phenotypically abnormal right through callusing, shooting, rooting and hardening stage of transformation. The anomalies include poor growth, from callusing to rooting stages and developed abnormalities ranging from aberrant needle shaped leaf to agravitropic roots (Graph.1). Early apical apoptosis, decreased apical dominance, uneven leaf shape/ curvature, agravitropic roots, decreased lateral rooting, shortened petiole, reduced stature and aberrant leaf shape were some of the phenotypic anomalies associated with sh-*rep* expression in tomato plants (Fig. 15 b).

5. DISCUSSION

RNA interference (RNAi) refers to the process of sequence-specific regulation of gene expression triggered by double-stranded RNA (dsRNA) (Fire *et al.*, 1998) and the silencing mechanisms are conserved in almost all the eukaryotes. It operates through small, non-coding RNA molecules of 21- 26 nt length single strand RNA (ssRNA) through various RNA silencing systems sharing overlapping enzyme complexes. Generally in the biological systems small RNAs interfere with gene regulation, chromatin modification and defence against viruses. Viruses manipulate host molecular mechanisms to prosper in the hostile cellular environment. The interactions between host defense (through endogenous small RNAs) and viral counter defense (through viral micro RNAs and suppressor proteins) offer an interesting insight into the natural evolutionary processes. Recently generated data on molecular mechanisms, on regulations of small RNAs and their interference by viruses have helped in understanding the basis of resistance and to develop strategies for transgenic resistance. Impressive results have been obtained against various plant viruses using RNA interference as a tool to target viral genes in the tissue culture models. The present investigation sought to achieve the same for *Tomato leaf curl virus* which belongs to genus of Begomovirus of family Geminiviridae, transmitted by whitefly.

RNAi and *Tomato leaf curl virus*

Tomato leaf curl virus causes a devastating, Tomato leaf curl disease (ToLCD), leading to severe economic losses (Rataul and Brar, 1989, Dougherty *et al.*, 1994). Due to the lack of natural source of resistance and the chemical control of vector species is troublesome, the role of transgenic resistance to the disease is sought. To explore this possibility of transgenic resistance against *Tomato leaf curl virus* work was mooted based on the principle of RNAi, for targeting well characterized replication initiator protein of the *Tomato leaf curl virus* (Dasgupta *et al.*, 2004; Sinha *et al.*, 2004).

Silencing of replicase protein is a viable option to achieve the resistance against virus, as this is the only protein of viral origin indispensable for its replication

inside the plant cell (Jupin *et al.*, 1995; Choi and Stenger1996). Replication initiator protein or replicase protein was chosen as viral target as it was demonstrated that *rep* mediated resistance is highly successful for attaining virus resistance transgenic plants against geminiviruses in general and *Tomato leaf curl viruses* in particular (Asad *et al.*, 2003; Chellppan *et al.*, 2004a ;Yang *et al.*,2004; Praveen *et al.*, 2005).

Although the mechanism of RNA silencing seems to work for establishing virus resistance, viruses are able to evade this silencing phenomenon effectively through repertoire of proteins called as viral suppressor proteins (Voinnet *et al.*, 1999; Bisaro, 2006). *Tomato leaf curl virus* is no exception, it encodes for a putative viral suppressor protein, AC4 which was characterized to function as a small RNA regulator (kumari 2006). Hence any RNAi mediated resistance against *Tomato leaf curl virus* may not be successful until the viral suppressor encoding ORF, AC4 is given due consideration while designing strategies to silence the virus. Fortunately enough, ORF AC4 is a small, embedded ORF within the replicase protein coding ORF but with different reading frame (Vanitharani *et al.*, 2004). Hence a single transgenic sequence can very well be deployed targeting both the viral proteins in a homology dependent RNAi based silencing system.

To confer transgenic resistance in this study, full length *replicase* gene was not considered rather a sub-region of the *replicase* gene called as truncated *rep* (T-*rep*) of 479 nt was employed as transgene (Fig1). The conclusion to arrive at T-*rep* region as a source of transgene is based on a reason, that the region is highly conserved among various species of leaf curl viruses causing ToLCD not only in India but also worldwide (Praveen *et al.*, 2004). Sequence homology between the transgene derived transcript and viral transcript is an important factor for obtaining successful RNA based silencing. Any sequence homology less than 90% does not confer resistance to the invading viruses (Lindbo *et al.*, 1993 and Dougherty *et al.*, 1994). Hence conserved region of 330 nucleotides is a suitable source of transgene that may provide stable and broader RNA mediated transgenic resistance against the geminiviruses causing tomato leaf curl disease in India (Praveen *et al.*, 2004). Besides this T-*rep* sequence also encompasses entire ORF of AC4, which encodes for putative viral suppressor protein so that suppressor protein is also concomitantly silenced. Moreover the T-*rep* region also includes the nucleotides that encode active site of the protein Tyr-103. Moreover the recombination and trans-encapsidation, or

complementation effect (with mutant strains of viruses) and such risks associated with the full length gene expression are averted with such non-translatable form of transgene (Hull 1998; Aaziz and Tepfer, 1999). The resistance sought to achieve is through RNA mediated process and it does not necessitate the production of active protein component hence the strategy is a boon for virus resistant transgenics as the risks associated with the biosafety issues are minimized in RNAi based transgenics.

Similar reports of deploying truncated *rep* sequences to confer virus resistance in geminiviruses are many. In an instance of silencing of *Tomato yellow leaf curl Sardinia Virus* (TYLCSV), truncated *rep*, when co expressed with the C4 protein, conferred resistance to the homologous virus (Brunetti *et al.,* 1997). Expression of oligomerisation domain of rep protein alone was found to inhibit heterologous viral DNA accumulation as shown by Chatterji *et al.,* in 2001. In yet another first report of hairpin RNA mediated silencing of *Tomato yellow leaf curl virus* (TYLCV), tomato plants were transformed with an intron–hairpin genetic construction to induce RNAi mediated silencing against the early TYLCV *rep* gene (C1) targeting 3' end of the rep protein. The extreme resistance to the virus by the transgenic plants was reported on challenge inoculation with virus (Fuentes *et al.,* 2006). The concept of RNAi was again explored to silence *Bean golden mosaic virus* by targeting the rep protein of the virus effectively (Bonfim *et al.*, 2007).

A given transgene derived mRNA manipulated to work through RNAi silencing mechanism produces dsRNA which further gets processed via RNAi machinery to form siRNAs of 21 nucleotides length, the ultimate effector molecules of the silencing pathway (Elbashir *et al.*, 2001). Since every such sub-string of an mRNA is not a potent siRNA (Holen *et al.*, 2002), T-*rep* was analyzed for its potent siRNA producing capability the resultant siRNAs were analyzed for their cross silencing ability. Efficient designing of potent siRNAs alone does not ensure the successful RNA based silencing because the accessibility of the target mRNA for the silencing activity of siRNA is also an important criterion (Yiu *et al.*, 2005). The secondary structure of replicase protein mRNA predicted by MWG siRNA design tool reveals that the potent siRNA predicted by DEQOR has the requisite accessible target site in the mRNA. The target site exhibited, open region with a high number of unpaired nucleotides, and is preferred for high potency as it is correlated with low negative local free energy. The presence of a large number of unpaired nucleotides

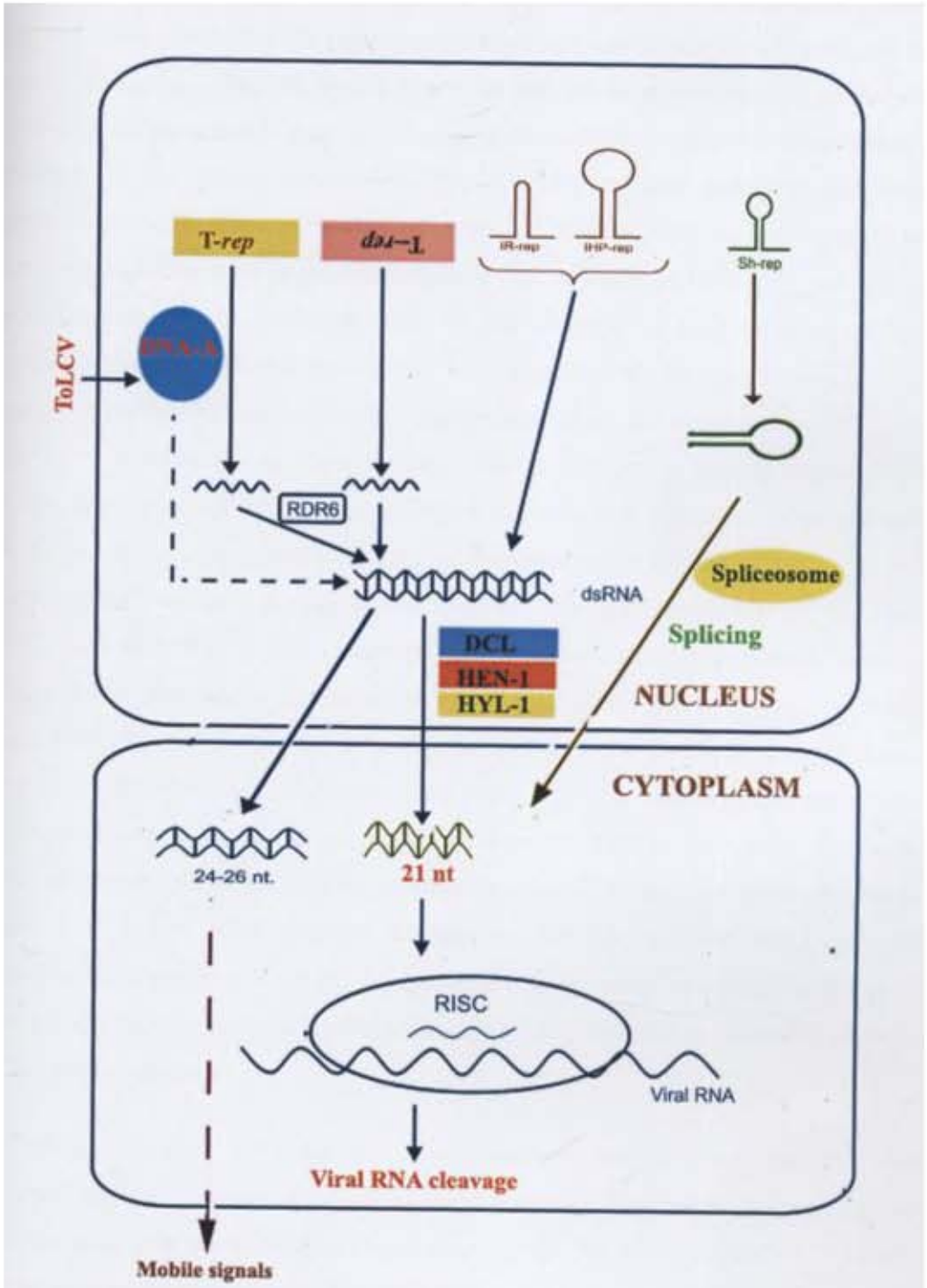


Fig. 14 : The schematic representation of *in vivo* processing of transgene derived transcripts and their mode of action

Leaf explants were used for transformation of tomato with all these four RNAi inducing constructs. The method of plantlet regeneration with leaf explant is a widely used one as the success of the plant regeneration and transformation efficiency in this method is higher. The wounded tissue in the plant transformation protocol is preferred as it ensures the greater callusing potential and thereby the transformation efficiency of the plants regenerated. The RNAi constructs exhibited differential transformation efficiency with all the stages of tomato plant transformation. The transformation efficiency of the tomato plants also depends on various factors like the *Agrobacterium tumefaciens* strain used in the process, its concentration used for transformation, the nutrient source and its concentration, the concentration of the phenolic compounds, vitamins concentratrion employed for the growth of the plant tissue in the medium (Cortina and Culianez-Macia, 2004). The three RNAi constructs (IR-*rep*, Ihp-*rep* and T-*rep*) exhibited high transformation efficiency of the 45-60%. The sh-*rep* constructs showed diminished transformation efficiency of 13 % only which can be explained owing to the very low survival percentage of the plants transformed with the sh-*rep* construct. Plants transformed with sh-*rep* construct showed severe phenotypic abnormalities hence their survival rate is also low. It may be due to the ill and unintended effects associated with the constitutive expression of the potent siRNA sequences (Meister and Tuschl, 2004). Although the same potent siRNA encoding sequence is present in all three constructs other than sh-*rep*, the extent of phenotypic abnormalities exhibited by them on transformation into tomato plants is very low. One possible explanation for this may be the constitutive expression of a potent siRNA in sh-*rep* construct, increases its concentration in the plant system thereby performing the off-target activity besides the intended silencing of viral gene expression.

The constructs were not only transformed in tomato plants but also were employed in demonstration of the RNAi based silencing. The results of the study were in consonance with the presumed mode of action for the RNAi constructs. Although all the four constructs were efficient in silencing the viral gene expression, the percentage of recovery in different RNAi constructs varied from 80% to 95%. Among those four, Ihp-*rep* transformed plants exhibited highest recovery of 95%. Studies have demonstrated the superiority of intron spliced hairpin RNA construct design strategy over any other type of RNAi constructs (Smith *et al.*, 2000; Wesley *et al.*,

2001; Fuentes *et al.*, 2006). Similar results of successful silencing of the geminiviral replication associated protein employing RNAi as a tool have been documented (Vanitharani *et al.,* 2003; Chellappan *et al.,* 2004; Praveen *et al.,* 2005; Fuentes *et al* ., 2006). The differential silencing in terms of percentage recovery might also be attributed to the construct efficacy or positional effect of the transgene integration etc.

The successful demonstration of the viral gene silencing was due to the fact that the strategy designed and consequently the constructs generated were aimed at silencing of viral suppressor proteins also. Thus viral counter defense strategy too was crippled by way of RNAi constructs targeting transcripts of such proteins. Silencing of viral suppressor proteins would ensure trait stable transgenics working in the principle of RNAi as reported in various studies (Waterhouse *et al.,* 1998; Nicola-Negri *et al.*, 2005; Qu *et al.*, 2007).

Off-target silencing of host genes

The study was conceptually set out to explore the possibility of employing RNAi as an effective tool in silencing the ToLCV genome. An interesting observation was made in the course of the study as phenotypic abnormalities were associated with the tomato transformants, which is having more pronounced effect in sh-*rep* expressing plants. The nucleotide sequences of siRNA derived from sh-*rep* construct and tomato specific put-miRNA3 (Pilcher *et al.,* 2007) were then analyzed for any sequence homology or complementarity between them. The study revealed that, 12 out of 21 nucleotides of sh-*rep* derived siRNA showed complementarity with mature miRNA sequence of put-miRNA 3 (Fig.15) .The complementarity exhibited between siRNA and put miRNA3 might have resulted in probable down regulation of put-miRNA 3 transcript /or target transcripts of the put miRNA3. The altered microRNA mediated gene regulation might be the primary reason for the developmental anomalies associated with expression of sh-*rep* construct in tomato. Micro RNAs (miRNAs) are key regulators of gene expression in eukaryotes in a sequence specific manner and each miRNA generally governs the expression of a family of transcription factors so that large number of genes is controlled by a single miRNA (Bartel, 2004).The transcription factors associated with the developmental anomalies were deduced (Table.6).Hence it was indirectly deduced that put-miR-3 may be a key

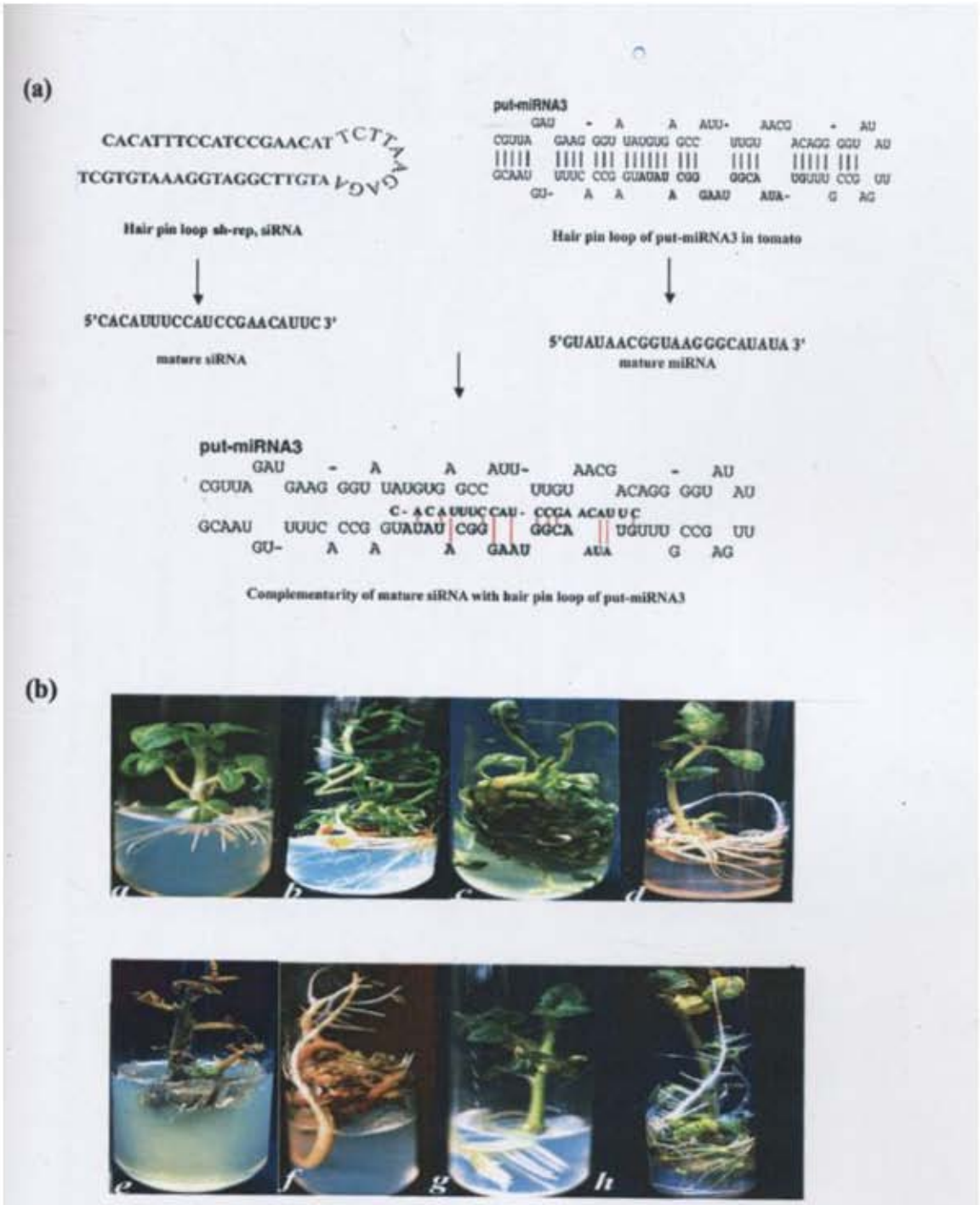


Fig. 15 : Developmental anomalies in tomato transformants

(a): sh-*rep* derived siRNA cross reacting with put-miRNA 3 of tomato (adapted from Pilcher *et al.*, 2007)

(b): Phenotypic aberrations associated with expression of sh-*rep* construct

a: Healthy control; b : needle shaped leaves ; c: stunted growth; d: absence of apical dominance; e: apical apoptosis;f: agravitropic root; g: absence of root hairs and lateral root ; h : pigmented roots.

Table 6: miRNAs and their downstream transcriptional factors governing the phenotypic anomalies observed in transformants

S. No.	miRNA	Gene family	Consequences of altered expression	References
1	miR156	SPL transcription factors	Decreased apical dominance	Schwab *et al.*, (2005)
2	miR319	TLP transcription factors	Uneven leaf shape curvature	Palatnik *et al.*, (2003)
3.	miR160	ARF transcription factors	Agravitropic roots with disorganized root caps	Wang *et al.*, (2005)
4	miR164	NAC domain transcription factors	Reduced lateral rooting	Guo *et al.*, (2005)
5	miR159	MYB 33	Shortened petiole and reduced stature	Millar and Gubler (2005)
6	miR164	CUC-1/CUC-2	Aberrant leaf shape	Laufs *et al.*,(2004) Mallory *et al.*, (2004)
7.	Put-miR3	?	Early apical apoptosis, decreased apical dominance, uneven leaf shape/ curvature, agravitropic roots, decreased lateral rooting, shortened petiole, reduced stature and aberrant leaf shape	Pilcher *et al.*,(2007)

regulator of the abovementioned transcripts families or host of transcripts thereby effecting the phenotypic anomalies.

Based on these observations, a model on the phenotypic expressions can be proposed, to be governed by transcripts or transcriptional factors in unified framework, which describe the transcriptional regulation by different miR genes (Chen and Rajewsky, 2007). It is proposed that newly identified miR (put-miR 3) may be a key regulator in controlling the transcripts/ transcriptional factors of gene families involved in leaf shape/ formation, root development (gravitropism, differentiation into lateral roots, non-pigmentation), apical dominance (apical bud differentiation) with an overlapping control by miR156, miR159, miR160 and miR164. Besides, the two novel phenotypic aberrations viz. early apical apoptosis and pigmentation in the roots can possibly be attributed to transcripts or transcriptional factors regulated by put-miR 3. Further experimental evidence is needed to prove the role of the put-miR3 in controlling the transcripts or transcript families. Thus the study not only provides insight into the effective designing of the siRNA but also in deciphering the probable role of the put- miRNAs functioning in the plant system.

Once such discrepancies arising out of off-target effects are weeded out short hairpin RNA (shRNA) strategy, owing to its small construct length, would be a boon to stack multiple viral derived sequences in a single construct to target multiple viruses belonging to diverse groups infecting a single host. The idea has been given a try and was shown successfully that efficient, simultaneous targeting of four different tospoviruses can be achieved by using a single small transgene based on the production of minimal sized chimaeric cassettes (Bucher *et al.,* 2006). Another follow up that can be carried forward is artificial microRNA mediated gene silencing aimed at achieving virus resistance through miRNA mediated pathway of RNAi silencing mechanism. Reports of breakdown of siRNA mediated gene silencing when plants are exposed to temperature conditions less than 15° C (Szittya *et al.*, 2003; Kameda *et al.*, 2004) led to explore the alternative method of attaining virus resistance, which works in a temperature insensitive manner (Niu *et al.,* 2006) . The resistance discussed in this thesis also needs to be worked for temperature insensitiveness. Any discrepancy arising out with the interplay of the temperature in siRNA mediated gene silencing has to be circumvented through artificial microRNA mediated resistance.

6. SUMMARY

RNA interference (RNAi) is an evolutionarily conserved, homology dependent gene silencing mechanism found in all the eukaryotes. Besides being an area of intense, upfront basic research, the process is gaining importance in various technological applications with virus resistant transgenics being one among them. Given the sequence specificity with which the phenomenon works and its role in plant defence and viral counter defence it is conceivable that RNAi will play major role in the development of virus resistant genotypes. To explore the possibility of transgenic resistance to the *Tomato leaf curl virus*, an important constraint in tomato production, through the principle of RNA interference (RNAi) the study was constituted. Owing to non-availability of natural resistant genotypes and the hazards associated with chemical control of vector species, transgenic resistance to the disease is considered a viable option. Among various protein coding ORFs of the virus genome, Replication initiator protein or replicase protein (*rep*) was considered to be suitable target for the RNA based silencing as it has been widely and successfully deployed for the resistance against the Begomovirus in general and *Tomato leaf curl virus* in particular. Therefore the present study had its objectives as:
(i) *In silico* determination of the potent siRNAs from the *rep* gene of ToLCV and to generate truncated *rep* gene construct; (ii) To develop RNAi inducing Inverted Repeat (IR) constructs; and (iii) To study the efficiency of the constructs through gene silencing assays.

The following are the major findings of present study.

- A sub-region of *replication initiator protein* gene termed as truncated *rep* (T-*rep*) was identified as a potent source of viral derived transgene for the RNA based silencing. T-*rep* spans a stretch of 479nt length sequence of the replication initiator protein gene (*rep*) and consequently encompasses, conserved core region of the *rep* gene besides including the nucleotide sequence encoding for putative viral suppressor protein AC4.
- *In silico* analysis of *rep* gene with the web based siRNA design algorithms revealed that the region is capable of generating 2-7 potent siRNAs. A potent siRNA sequence of 21 nt (including a two nucleotide overhang) length falling in conserved *rep* and AC4 encoding region was picked up from the results of DEQOR, a siRNA design algorithm. The potent siRNA possesses favourable

GC content of 42.9%, antisense preference and no polynucleotide feature. Assessment of the accessibility of the replication initiator protein mRNA for the potent siRNA binding and cleaving activity, revealed that the target site exhibited open region with high number of unpaired nucleotides thus favourable for potent siRNA accessibility.

- With that potent siRNA sequence as nucleus, four different RNAi inducing constructs viz., [a] antisense Truncated *rep* (T-*rep*) [b]Inverted repeats–*rep* (IR-*rep*) [c] Intron spliced hairpin RNA–*rep* (Ihp-*rep*) and [d] short hairpin RNA–*rep* (sh-*rep*) were designed to silence the replication initiator protein of the virus. The RNAi constructs showed differential behaviour for the transformation efficiency in all the stages of the transformation. Ihp-*rep* showing highest transformation efficiency of 60% followed by T-*rep* and IR-*rep* exhibiting 55% and 45% of efficiency respectively. The lowest transformation efficiency of only 13% was recorded on transformation with sh-*rep* construct.

- Transformation of the tomato with ToLCV infected leaf as explant resulted in regeneration of the plants with recovery phenotype. The molecular evidence for the silencing of the virus was ascertained with the absence of the PCR amplification of the viral coat protein gene. The differential behaviour of the constructs was noticed with respect to the percentage of plants showing recovery from viral infection owing to their different mode of processing presumed to occur *in vivo*. Upon transformation of the Ihp-*rep* construct 95% of the plants exhibited recovery phenotype whereas in other RNAi constructs of T-*rep*, sh-*rep* and IR-*rep* 80, 90 and 91 % of the plants respectively manifested recovery from the symptoms.

- As an interesting addition to this study, tomato transformants showed phenotypic anomalies like early apical apoptosis, decreased apical dominance, uneven leaf shape/ curvature, agravitropic roots, decreased lateral rooting, shortened petiole, reduced stature and aberrant leaf shape. It was attributed to the off target silencing of siRNA derived from sh-*rep* on tomato specific put-miRNA-3, providing insights into the probable role of the put-miRNA-3 *in vivo*.

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ABSTRACT

Four different plasmid vector-based siRNA generation strategies were employed to silence *Tomato leaf curl virus* (ToLCV). RNA interference (RNAi) using short interfering RNAs (siRNAs) has been widely explored for the suppression of intracellular viral target mRNAs. Replication initiator protein gene (encoded by ORF AC-1) of ToLCV with the concomitant silencing of AC4 gene (an embedded ORF with in AC1) was demonstrated through various RNAi constructs. The RNAi target sequence of 479 nt length termed as Truncated *rep* (T-*rep*) comprising the conserved regions in *replication initiator protein* gene with an overlapping sequence of the AC4 was chosen as a viral derived transgene source. *In silico* analysis of the entire *rep* gene employing the siRNA design algorithms revealed that the T-*rep* region is capable of generating 2 to 7 potent siRNAs. The study on the mRNA target site accessibility indicated that it exhibited open region with high number of unpaired nucleotides thus favourable for potent siRNA

binding and cleavage. Different RNAi mediated strategies like antisense Truncated *rep* (T-*rep*), self-complementary inverted repeats-*rep* (IR-*rep*), intron- spliced hairpin RNA-*rep* (Ihp-*rep*), and short hairpin RNA-*rep* (sh-*rep*) were deployed for efficient and predictable silencing of the virus in tomato plants.

The RNAi constructs showed differential behaviour for the transformation efficiency in all the stages of the tomato transformation. Ihp-*rep* showed highest transformation efficiency of 60% followed by T-*rep* and IR-*rep* exhibiting 55% and 45% of efficiency respectively. The lowest transformation efficiency of only 13% was recorded on transformation with sh-*rep* construct. The constructs also differed in their efficiency to silence the viral genome. Upon transformation with the Ihp-*rep* construct 95% of the plants exhibited recovery phenotype, whereas in other RNAi constructs T-*rep*, sh-*rep* and IR-*rep,* 80, 90 and 91 % of the plants manifested recovery from the viral symptoms respectively. These strategies imply that ToLCV *rep*-driven RNAi, targeting AC4 and conserved viral sequences, provides a promising approach to suppress ToLCV infection at the broad spectrum level and in the development of trait stable transgenics. During the course of study, an interesting observation on phenotypic anomalies like early apical apoptosis, decreased apical dominance, uneven leaf shape/ curvature, agravitropic roots, decreased lateral rooting, shortened petiole, reduced stature and aberrant leaf shape reflects off-target silencing effects in sh-*rep* transformants. These off target effects provided insights on the transcripts regulated by newly identified put-miR 3 of tomato.

आरएनए इन्टरफिरेन्स (RNAi) के द्वारा टमाटर के पर्ण कुंचन विषाणु (ToLCV) का दमन

सारांश

अन्त: कोशिका विषाक्त लक्ष्य mRNAs के दमन के लिए, शार्ट इन्टरफिरिंग RNAs (siRNA) का प्रयोग करते हुए RNA इन्टरफिरेन्स (RNAi) का व्यापक रूप से अन्वेषण किया गया। चार विभिन्न प्लाज़्मिड वाहक-आधारित siRNA जनरेशन रणनीतियों का उपयोग AC4 जीन की कन्कोमिटेन्ट साइलेन्सिंग (AC1 में ORF के प्रवेश) का उपयोग टमाटर के पर्ण कुंचन विषाणु (ToLCV) का दमन (साइलेन्स) करने हेतु किया गया। AC4 को एक प्यूटेरिन विषाण्विक दमन कारी प्रोटीन माना गया है। विषाण्विक पराजीवी स्रोत के रूप में AC4 जीन को एक ओवरलेपिंग क्रम के साथ रिप्लिकेशन इंजीनिएटर प्रोटीन में संरक्षित क्षेत्र के रूप में चुना गया जिसमें RNAi टारगेट क्रम होते हैं जिनकी लम्बाई 479 nt होती है और जिन्हें ट्रन्केटेड *rep* (T-*rep*) कहा जाता है। siRNA डिज़ाइन एल्गोरिद्म्स को नियोजित करके सम्पूर्ण *rep* जीन के *इनसिलिको* विश्लेषण से पता चला कि T-*rep* रीजन, 2 से 7 पोटेन्ट siRNAs उत्पन्न करने में सक्षम है। mRNA टारगेट साइट अभिराम्यता से संकेत मिलता है कि यह अयुग्मी न्यूक्लिओटाइड्स की उच्च संख्या के खुले रीजन को प्रदर्शित करता है जो पोटेन्ट siRNA बंधनकारी (बाइन्डिंग) और विदरण (क्लीवेज़) के लिए अनुकूल है। टमाटर के पौधों में विषाणु के पूर्वानुमान करने योग्य और कारगर दमन के लिए, विभिन्न RNAi माध्यम वाली रणनीतियाँ जैसे एन्टीसेन्स ट्रन्केटेट *rep* (T- *rep*), स्व-पूरक अन्वर्टेट रिपीट्स-*rep* (IR-*rep*), इन्ट्रॉन-स्प्लिस्ड हेयरपिन RNA-*rep* (Ihp-*rep*) और शॉर्ट हेयरपिन shRNA-*rep* (sh-*rep*) को नियोजित किया गया। RNAi संरचनाओं द्वारा, रूपान्तरण की सभी अवस्थाओं में रूपान्तरण दक्षता के लिए भिन्न प्रकार का व्यवहार प्रदर्शित किया गया। Ihp-*rep* ने 60% उच्चतम रूपान्तरण दक्षता दर्शाई इसके बाद T-*rep* और प्त-तमच द्वारा क्रमश: 55% और 45% दक्षता प्रदर्शित की गई। sh-*rep* संरचना में रूपान्तरण पर न्यूनतम 13% दक्षता प्रदर्शित हुई। विषाणु जिनोम के दमन के प्रति संरचनाओं में भिन्नता पाई गई। पौधों के 95% Ihp-*rep* संरचना के रूपान्तरण पर फिनोटाइप पुनप्राप्ति प्रदर्शित हुई जबकि अन्य RNAi संरचनाओं T-*rep*, sh-*rep* और IR-*rep* द्वारा पौधों की, लक्षणों के आधार पर क्रमश: 80, 90 और 91% पुनप्राप्ति अभिव्यक्त की गई। इन रणनीतियों से स्पष्ट होता है कि ToLCV *rep* चालित (ड्रिवन) RNAi, AC4 का लक्षित करते हुए और विषाणु क्रमण को संरक्षित करते हुए, ToLCV संक्रमण के विस्तृत स्पेक्ट्रम के दमन के लिए और पराजीवी स्थिर गुणों के विकास के लिए एक आशाजनक अविधारण उपलब्ध कराता है। अध्ययन के दौरान फिनोटाइप (शीर्षस्थ) एपोटोसिस, घटता हुआ एपिकल क्षेत्र, असमान पत्ती आकार/ वक्रता, एग्रेवीट्रॉपिक जड़े, घटती हुए पत्रदल, घटता हुआ आकार और विपथी पत्ती आकृति आदि से sh-*rep* रूपान्तरणों मे गैर लक्षित दमनित प्रभाव परिलक्षित हुए। ये गैर लक्षित प्रभाव, टमाटर के नए पहचाने गए पुट-miR3 के अभिव्यक्तित्व करण पर एक अर्न्तदृष्टि उपलब्ध कराते हैं।

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APPENDIX-I

siRNA design rules

1. Tom Tuschl's rules (MPI rules: Tuschl *et al.*, 2003)

a) Select targeted region from a given cDNA sequence beginning 50-100 nt downstream of start codon

b) First search for 23-nt sequence motif AA (N_{19}). If no suitable sequence is found, then,

c) Search for 23-nt sequence motif NA(N_{21}) and convert the 3' end of the sense siRNA to TT

d) Or search for NAR(N_{17})YNN

e) Target sequence should have a GC content of around 50%

A = Adenine; T = Thymine; R = Adenine or Guanine (Purines); Y = Thymine or Cytosine (Pyrimidines); N = Any.

2. Reynolds siRNA design Rules (Reynolds *et al.*, 2004)

Reynolds et al. identified eight characteristics associated with siRNA functionality. These characteristics are used by rational siRNA design algorithm to evaluate potential targeted sequences and assign scores to them. Sequences with higher scores will have higher chance of success in RNAi. The table below lists the 8 criteria and the methods of score assignment.

Criteria	Description	Score	
		Yes	No
1	Moderate to low (30%-52%) GC Content	1 point	
2	At least 3 A/Us at positions 15-19 (sense)	1 point /per A or U	
3	Lack of internal repeats (Tm*<20¡ãC)	1 point	
4	A at position 19 (sense)	1 point	
5	A at position 3 (sense)	1 point	
6	U at position 10 (sense)	1 point	
7	No G/C at position 19 (sense)		-1 point
8	No G at position 13 (sense)		-1 point

A sum score of 6 defines the cutoff for selecting siRNAs. All siRNAs scoring higher than 6 are acceptable candidates.

Tm = 79.8 + 18.5$\log_{10}([Na^+])$ + (58.4 * GC%/100) + (11.8 * $(GC\%/100)^2$) - (820/Length)

3. Ui-Tei siRNA design rules (Ui-Tei *et al.*, 2004)

Highly effective RNAi was found to occur in mammalian cells if siRNA satisfying the four following sequence conditions

a) A/U at the 5′ end of the AS;
b) G/C at the 5′ end of the SS;
c) AU-richness in the 5′ terminal, 7 bp long region of the AS; and
d) The absence of any long GC stretch of more than 9 bp in length

Rational siRNA design Rules

a) siRNA targeted sequence is usually 21 nt in length.
b) Avoid regions within 50-100 bp of the start codon and the termination codon
c) Avoid intron regions
d) Avoid stretches of 4 or more bases such as AAAA, CCCC
e) Avoid regions with GC content <30% or > 60%.
f) Avoid repeats and low complex sequence
g) Avoid single nucleotide polymorphism (SNP) sites
h) Perform BLAST homology search to avoid off-target effects on other genes or sequences
i) Always design negative controls by scrambling targeted siRNA sequence. The control RNA should have the same length and nucleotide composition as the siRNA but have at least 4-5 bases mismatched to the siRNA. Make sure the scrambling will not create new homology to other genes.

1: AF524893. Reports Tomato leaf curl...[gi:21930131]

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LOCUS       AF524893                1086 bp    DNA     linear   VRL 20-
APR-2005
DEFINITION  Tomato leaf curl virus replicase associated protein (AC1)
gene,
            complete cds.
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REFERENCE   1  (bases 1 to 1086)
  AUTHORS   Dasgupta,A., Sinha,S.K. and Praveen,S.
  TITLE     Structure of replication initiator protein unites diverse
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  JOURNAL   Plant Sci. 166 (4), 1063-1067 (2004)
REFERENCE   2  (bases 1 to 1086)
  AUTHORS   Praveen,S., Dasgupta,A. and Varma,A.
  TITLE     Phylogenetic analysis and homologies of the replicase of
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  JOURNAL   Virus Genes 28 (2), 195-199 (2004)
   PUBMED   14976419
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  AUTHORS   Praveen,S., Sinha,S.K. and Varma,A.
  TITLE     AC1 ORF of tomato leaf curl virus-IARI-Isolate-India
  JOURNAL   Unpublished
REFERENCE   4  (bases 1 to 1086)
  AUTHORS   Praveen,S., Sinha,S.K. and Varma,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-JUN-2002) ACPV, IARI, Pusa, New Delhi, Delhi
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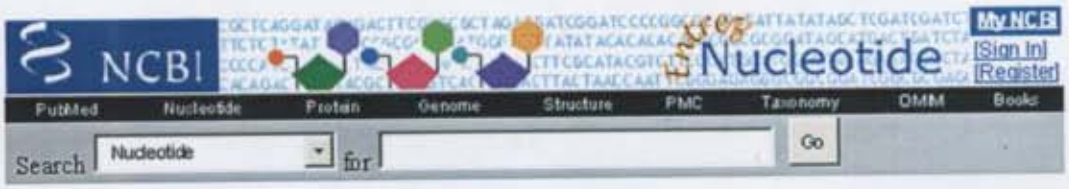


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FEB-2006
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            Viruses; ssDNA viruses; Geminiviridae; Begomovirus.
REFERENCE   1  (bases 1 to 177)
  AUTHORS   Kumari,P., Ramesh,S.V. and Praveen,S.
  TITLE     AC4 ORF of tomato leaf curl virus-IARI isolate- New Delhi
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 177)
  AUTHORS   Kumari,P., Ramesh,S.V. and Praveen,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-JAN-2006) Division of Plant Pathology, Indian
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896	CTATCTTCCTGTGCAATAAAG (-> sense) GTGATAGAAGGACACGTTATT (<- antisense)	0	0	38.1	Yes	No	0.00
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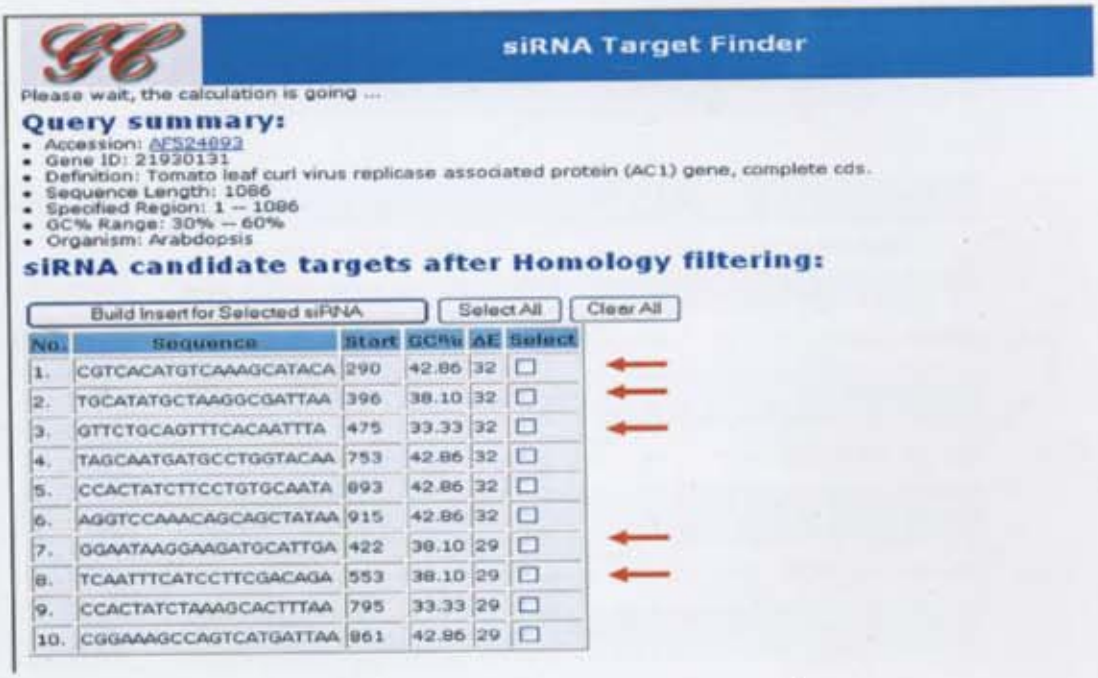
The potent siRNAs generated by replication initiator protein gene as input query in design algorithm DEQOR along with their characteristic features A potent siRNA that targets the conserved *replicase* and embedded ORF AC4, encoding for a putative suppressor portein is highlighted

(a)



siRNA Target Finder

Please wait, the calculation is going ...

Query summary:

- Accession: AF524893
- Gene ID: 21930131
- Definition: Tomato leaf curl virus replicase associated protein (AC1) gene, complete cds.
- Sequence Length: 1086
- Specified Region: 1 -- 1086
- GC% Range: 30% -- 60%
- Organism: Arabdopsis

siRNA candidate targets after Homology filtering:

Build Insert for Selected siRNA | Select All | Clear All

No.	Sequence	Start	GC%	AE	Select
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2.	TGCATATGCTAAGGCGATTAA	396	38.10	32	☐
3.	GTTCTGCAGTTTCACAATTTA	475	33.33	32	☐
4.	TAGCAATGATGCCTGGTACAA	753	42.86	32	☐
5.	CCACTATCTTCCTGTGCAATA	893	42.86	32	☐
6.	AGGTCCAAACAGCAGCTATAA	915	42.86	32	☐
7.	GGAATAAGGAAGATGCATTGA	422	38.10	29	☐
8.	TCAATTTCATCCTTCGACAGA	553	38.10	29	☐
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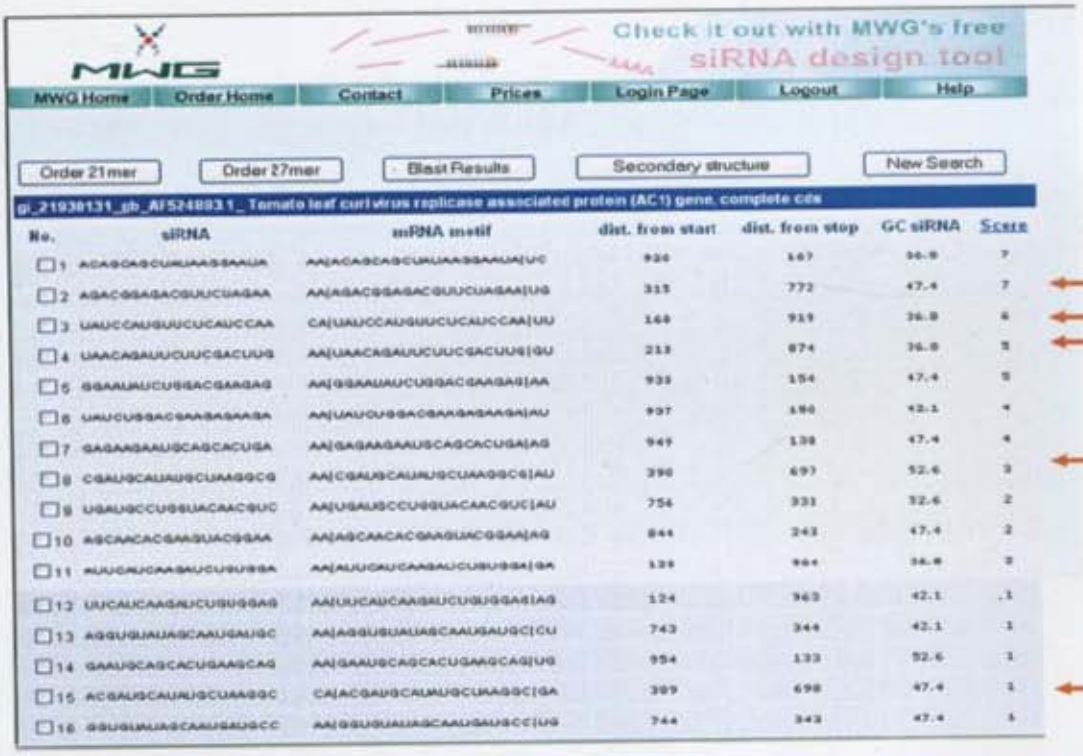
(b)



MWG

Check it out with MWG's free siRNA design tool

MWG Home | Order Home | Contact | Prices | Login Page | Logout | Help

Order 21mer | Order 27mer | Blast Results | Secondary structure | New Search

gi_21930131_gb_AF524893.1_ Tomato leaf curl virus replicase associated protein (AC1) gene, complete cds

No.	siRNA	mRNA motif	dist. from start	dist. from stop	GC siRNA	Score
☐1	ACAGCAGCUAUAASAAUA	AA[ACAGCAGCUAUAASAAUA]UC	920	167	36.9	7
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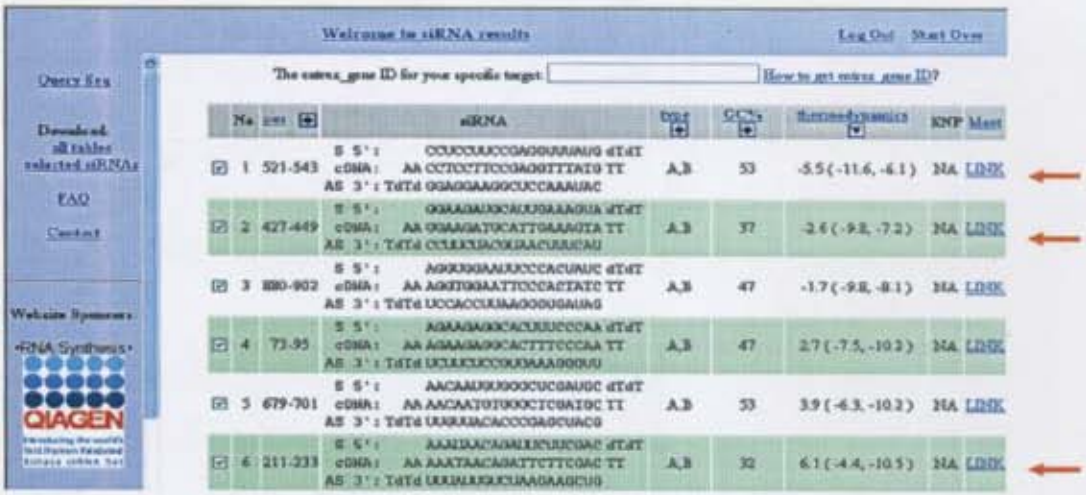
☐3	UAUCCAUGUUCUCAUCCAA	CA[UAUCCAUGUUCUCAUCCAA]UU	168	919	36.8	6
☐4	UAACAGAUUCUUCGACUUG	AA[UAACAGAUUCUUCGACUUG]GU	213	874	36.8	5
☐5	GGAAUAUCUGGACGAAGAG	AA[GGAAUAUCUGGACGAAGAG]AA	933	154	47.4	5
☐6	UAUCUGGACGAAGAGAAGA	AA[UAUCUGGACGAAGAGAAGA]AU	937	150	42.1	4
☐7	GAGAAGAAUGCAGCACUGA	AA[GAGAAGAAUGCAGCACUGA]AG	949	138	47.4	4
☐8	CGAUGCAUAUGCUAAGGCG	AA[CGAUGCAUAUGCUAAGGCG]AU	390	697	52.6	3
☐9	UGAUGCCUGGUACAACGUC	AA[UGAUGCCUGGUACAACGUC]AU	754	333	52.6	2
☐10	AGCAACACGAAGUACGGAA	AA[AGCAACACGAAGUACGGAA]AG	844	243	47.4	2
☐11	AUUCAUCAAGAUCUGUGGA	AA[AUUCAUCAAGAUCUGUGGA]GA	139	944	36.8	2
☐12	UUCAUCAAGAUCUGUGGAG	AA[UUCAUCAAGAUCUGUGGAG]AG	124	963	42.1	1
☐13	AGGUGUAUAGCAAUGAUGC	AA[AGGUGUAUAGCAAUGAUGC]CU	743	344	42.1	1
☐14	GAAUGCAGCACUGAAGCAG	AA[GAAUGCAGCACUGAAGCAG]UG	954	133	52.6	1
☐15	ACGAUGCAUAUGCUAAGGC	CA[ACGAUGCAUAUGCUAAGGC]GA	389	698	47.4	1
☐16	GGUGUAUAGCAAUGAUGCC	AA[GGUGUAUAGCAAUGAUGCC]UG	744	343	47.4	1

Potent siRNAs generated by replicase protein gene as input query in the web based design algorithms (a) siRNA target finder and (b)MWG siRNA design tool The potent siRNAs in the T-*rep* region are shown with red arrows

(a)



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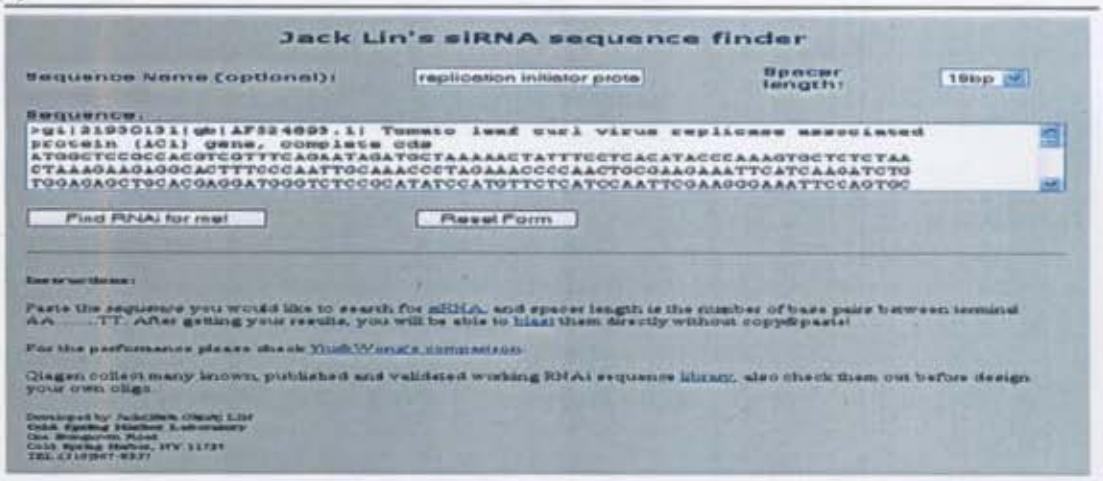

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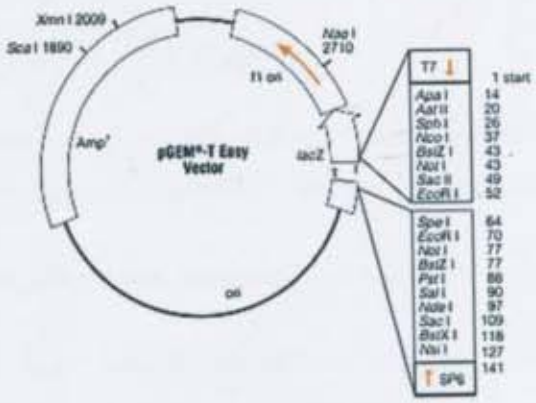
AAAGGAGAGGCACTTTCCCAATT	168	47%
AATTAGCCCCAAAAGATTACGTT	648	42%
AAAGGAATTAGCCCCAAAAGATT	643	42%
AAGGAAGATGCATTGAAAGTATT	520	37%
AAAAATAACAGATTCTTCGACTT	304	32%
AATACCAATTTAGATCGTATTTT	588	26%
AAATACCAATTTAGATCGTATTT	588	26%

Potent siRNAs generated by replicase gene as input query in the web based design algorithms (a) siRNA at WHITEHEAD and (b) Jack Lin's siRNA sequence finder
The potent siRNAs targeting T-*rep* region are shown with red arrows

(a)



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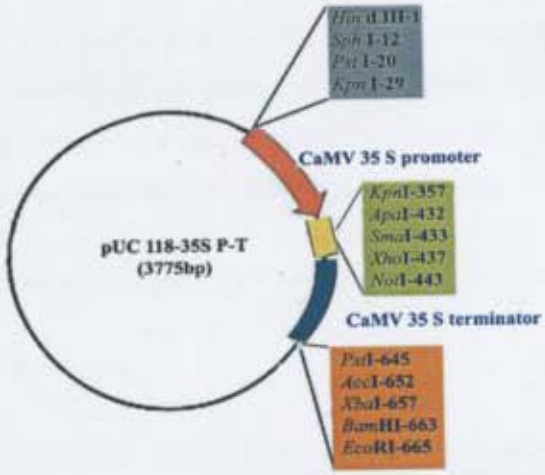

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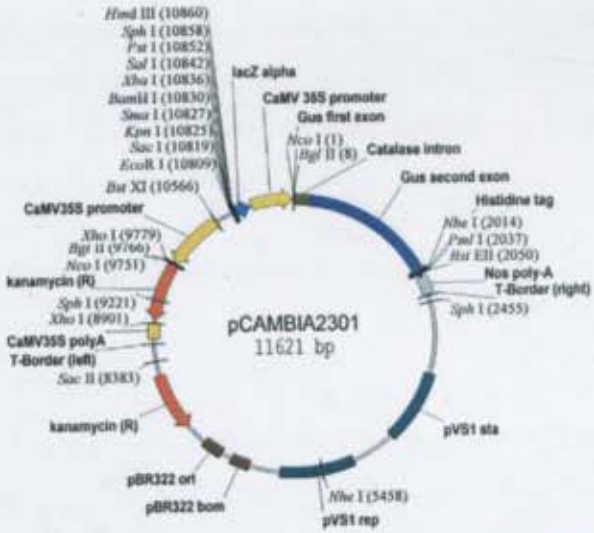

Maps of the plasmids employed in generation of the RNAi constructs
(a) pGEM-T-Easy (b) pUC-118-35S-P-T (c) pCAMBIA 2301

APPENDIX-II

1. Test Plants

Tomato (*Solanum lycopersicum*) plants naturally infected by *Tomato leaf curl virus* (ToLCV) were maintained in glass house under insect free conditions.

2. Isolation of Total DNA from Infected Leaf Sample

Total DNA from infected tomato leaf samples, showing symptoms, were isolated by DNeasy Plant Mini Kit provided by Qiagen.

- 100 mg of washed and dried leaf samples were ground in liquid nitrogen to a fine powder using a sterile mortar and pestle. Sample powder and liquid nitrogen were transferred to an appropriate size sterile eppendorf tube and the liquid nitrogen was allowed to evaporate but care was taken so that sample does not thaw.

- Subsequent steps were followed as per manufacturer's instructions.

3. Agarose Gel Electrophoresis

Total DNA isolated from infected leaf samples were mixed with 4 µl of 6X loading dye and sterilized water to make up the volume to 20 µl and were loaded onto wells in 1% agarose gel, prepared in 1X Tris-acetate-EDTA (TAE) buffer containing EtBr (0.5 µg/ml). Electrophoresis was carried out at 60 V for 1-2 hr. An aliquot (500 ng) of 1 kb DNA ladder (MBI Fermentas) was similarly mixed with dye and electrophoresed to serve as molecular size marker.

4. Polymerase Chain Reaction (PCR) Amplification

4.1. T-*rep* region

Specific primers were synthesized from Microsynth Pvt. Ltd. Sequences of primers were based on sequence of ToLCV (Sinha *et al.*, 2004). Using this specific primer, T-*rep* region was amplified from total DNA isolated from all the infected leaf samples mentioned above.

Primer sequences were

Forward primer	:	5' CATCAAGATCTGTGGAGAGAGC 3'
Length	:	22 mer
T_m	:	60
Reverse primer	:	5'CGTCGATTGGGTCT CGTCTA 3'
Length	:	20 mer
T_m	:	60

The PCR reaction mixtures were prepared as follows:

Total DNA (25 ng/µl)	2 µl
Forward primer (200 ng/µl)	1 µl
Reverse primer (200 ng/µl)	1 µl
10X PCR buffer	2 µl
10 mM dNTPs	1 µl
Taq DNA polymerase (5 U/µl)	1 µl
Sterile distilled water	12 µl
	Total 20 µl

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

- 1 cycle for denaturation of DNA at 94°C for 4 min.
- 30 cycles which had 3 segments

  Denaturation at 94°C for 30 sec.

  Annealing at 60°C for 40 sec.

  Primer extension at 72°C for 30 sec.
- Final primer extension was at 72°C for 5 min

4.2. 35 S P-T primers

Specific primers were synthesized from Microsynth Pvt for 35 S Promoter and terminator region flanking the MCS in pUC 118 vector hence it can be used for the screening of RNAi inducing inverted repeat constructs (IR-*rep*, Ihp-*rep* and sh-*rep*) clones or for their integration in tomato plants

Primer sequences were

Forward primer (35 S -P)	:	5' ATTGCGATAAAGGGAAGGCC 3'
Reverse primer (35 S-T)	:	5' CCTGCAGGTACCACTGGATT 3'

The PCR reaction mixtures were prepared as follows:

Total DNA (25 ng/µl) (plasmid or Plant genomic DNA)	2 µl
Forward primer (200 ng/µl)	1 µl
Reverse primer (200 ng/µl)	1 µl
10X PCR buffer	2 µl
10 mM dNTPs	1 µl
Taq DNA polymerase (5 U/µl)	1 µl
Sterile distilled water	12 µl
	Total 20 µl

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

- 1 cycle for denaturation of DNA at 94^{O}C for 4 min.
- 30 cycles which had 3 segments

  Denaturation at 94^{O}C for 30 sec.

  Annealing at 58^{O}C for 40 sec.

  Primer extension at 72^{O}C for 30 sec.
- Final primer extension was at 72^{O}C for 5 min

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

5. PCR Purification of Amplified Product

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

6. Cloning of amplified T-*rep* region in pGEM-T Easy Cloning Vector

pGEM-T Easy vector (3015 bp) from Promega is convenient system for the cloning of PCR products. Successful clones of the T-*rep* region insert in the pGEM-T Easy vectors is identified by colour screening on indicator plates containing ampicillin, X-gal and IPTG, as the vector contains the ampicillin resistance marker gene and the insert interrupts the coding sequence of β-galactosidase thus producing white colonies upon overnight incubation at 37°C temperature. PCR amplified T-*rep* region of ToLCV was taken for cloning.

6.1. Optimization of Insert: Vector Molar Ratios

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

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

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

pGEM-T Easy vector (50 ng/µl)	1 µl
PCR amplified product (50 ng/µl)	3 µl
10X Ligation buffer	1 µl
10 mM ATP	1 µl
T_4 DNA ligase (3U/µl)	1 µl
Sterile distilled water	3 µl
	Total 10 µl

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

6.3 Preparation of Competent Cells

The competent cells were prepared by $CaCl_2$ method described by Mandel and Higa (1970).

- 50 ml Luria Broth (LB) was inoculated with overnight grown culture of DH5α strain of *Escherichia coli* and incubated at 37° C for 1 h and 15 min. with constant shaking at 200 rpm in a shaker incubator till the bacterial growth as measured by optical density reached 0.3 O.D. at 600 nm.

- The culture was then asceptically transferred to 40 ml sterile screw capped tubes and kept on ice for 10 min.

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

- The cells were resuspended gently in 10 ml ice cold 0.1 M $MgCl_2$ solution and centrifuged at 5000 rpm for 10 min. at 4° C.

- The pellet was resuspended in 10 ml ice cold 0.1 M $CaCl_2$ solution and kept on ice for 1 h.

- The cells were recovered by centrifuging at 5000 rpm for 10 min. at 4° C and the pellets were resuspended in 1 ml of chilled 0.1 M $CaCl_2$ and used for transformation after keeping on ice for 1 h.

6.4 Transformation of Competent Cells

- 200 µl competent cells were added to 20 µl of each of the ligation mixtures in two separate sterile microfuge tubes and were gently mixed and kept on ice for 1 h.

- Next the competent cells were given heat shock at 42^{O} C for 90 sec. 1 ml of LB medium was then added and the transformants were allowed to grow at 37^{O} C for 1 h in shaker incubator at 200 rpm.

- Two sets of 200 µl of serially diluted cell suspensions were asceptically plated on Luria Agar (LA) plates separately containing ampicillin, X-gal and IPTG (50 µl of 50 µg/ml ampicillin, 100 µl of 2 per cent X-gal and 10 µl of 0.1 M IPTG in 50 ml LA).

- The plates were incubated overnight at 37^{O} C.

6.5 Selection of Transformants

The transformants were selected on the basis of blue/white colonies. The white colonies were selected and subsequently streaked on LA Plates (master plates) containing IPTG, X-gal and ampicillin.

7. Rapid Screening for the Recombinant Clones by Colony PCR Method

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

Following was the colony PCR reaction master mix:

Forward primer (200 ng/µl)	20 µl
Reverse primer (200 ng/µl)	20 µl
10X PCR buffer	40 µl
10 mM dNTPs	10 µl
Taq DNA polymerase (5U/µl)	10 µl
Sterile distilled water	300 µl
	Total 400 µl

Aliquot of 40 µl of master mix was taken in ten different PCR tubes and the white colonies were taken one in each tube. The tubes were then placed in the same thermocycler. The temperature profile and cycle were same as used in amplification of gene.

8 Isolation of Recombinant Plasmid DNA by Miniprep Method

The Recombinant DNA from the p-GEMT-T-*rep* clones was isolated following the modified alkaline lysis method (Birnboim and Doly, 1979).

- Selected white colonies of p-GEMT-T-*rep* clones, found positive in colony PCR reaction were individually inoculated in 2 ml of LB medium containing ampicillin (50 µg/ml) in sterile capped culture tubes.

- Tubes were then incubated overnight at 37^{O} C at 200 rpm in a shaker incubator.

- The overnight grown bacterial cells were then transferred to 1.5 ml sterile eppendorf tube and cells were harvested by centrifuging in a table top centrifuge for 1 min. Care was taken to remove the medium adhering to the cell pellet.

- The pellet was resuspended in 100 µl of solution I and mixed vigorously by vortexing.

- The 200 µl of freshly prepared lysis solution (solution II) was then added and mixed gently.

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

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

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

- The supernatant was collected and equal volume of phenol: chloroform: isoamyl alcohol mixture (25:24:1) was added. It was

vortexed well, centrifuged in a tabletop centrifuge for 15 min. at room temperature. The clear aqueous phase was transferred to fresh eppendorf tube.

- The DNA in aqueous phase was precipitated by adding 0.8 volume of isopropanol and kept on ice for 10 min.
- The mixture was then centrifuged at 15000 rpm for 20 min at 4°C.
- To the pellet 200 µl of 70% ethanol was added. The tube was rotated well so that the pellet from the wall gets suspended in 70% alcohol. This ensures removal of adhering salts by 70% alcohol. DNA was then pelletized by centrifuging at 15000 rpm for 5 min.
- The pellet was finally suspended in 30 µl sterile double distilled water.

9. Release of Insert with Restriction Enzyme

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

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

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

10. Cloning of T-*rep* in pUC 118-35 S-P-T vector

The T-*rep* region was cloned in pUC118-35S P-T vector.

10.1 Isolation of Recombinant Plasmid from pGEM-T–T-*rep* clone

Plasmid isolation was done by the modified alkaline lysis method (Birnboim and Doly, 1979) as stated earlier. Isolated plasmid was checked on 1% agarose gel.

10.2 Release of T-*rep* region from the pGEM-T- T-*rep* clone

Recombinant plasmids from p-GEMT T-*rep* were subjected to digestion with *Not*I restriction enzyme as above (9).

10.3 Gel Purification of Restricted Product

Released insert (~ 500 bp) was excised from the agarose gel and purified using Qiagen Gel Purification kit following manufacturer's instructions.

10.4 Linearization of Vector pUC118-35S-P-T

The vector pUC118 was linearized with enzyme *Not* I produce cohesive ends on the vector. The reaction mixture was prepared as follows

Vector DNA (0.5 mg/ml)	2 µl
Enzyme *Not* I (5 U/ml)	1 µl
10X reaction buffer	2 µl
Sterile distilled water	15 µl
	Total 20 µl

The reaction mixture was incubated at 37° C overnight.

10.5 Ligation of Insert with pUC118 -35S-P-T Vector DNA

Ligation reaction between gel purified DNA and vector DNA was carried out using T4 DNA ligase by following cohesive end ligation method. The reaction mix was prepared as follows

Gel purified fragment (~100 ng/ml)	10µl
Vector (~100 ng/ml)	2 µl
Ligase (high concentration) (200 U/ml)	1 µl
10X reaction buffer	2 µl
10 mM ATP	1 µl
Sterile distilled water	4 µl
	Total 20 µl

In addition to standard reaction mix, positive and negative control mixes were also prepared by adding control insert and no DNA in ligation mix respectively. The ligation mixtures were incubated at 16^{O}C overnight. Transformation of the *E. coli* (DH5α) competent cells was done as described in 6.3-6.4. The cell suspensions after transformation was asceptically plated on Luria Agar (LA) plates separately containing ampicillin (50 µl of 50 µg/ml ampicillin in 50 ml LA). The plates were incubated overnight at 37^{O}C. The colonies obtained were screened for the presence of the insert by restriction analysis.

10.6 Selection of Transformants

The transformants were selected on the basis of restriction with *Not* I. The recombinant DNA from the pUC118-T-*rep* clones were isolated and restricted with *Not*I as described above (8-9).After restriction digestion, the products were electrophoresed in 1% agarose gel. Fragment size was assessed in comparison with 1 kb DNA molecular weight marker.

11. Cloning of constructs into Binary Vector

Besides T-*rep* construct, RNAi inducing inverted repeats construct which were generated in pUC118-35S-P-T vector were also sub cloned in pCAMBIA 2301, a plant transformation compatible binary vector as follows.

11.1 Isolation of Recombinant Plasmid from pUC118-recombinant Clone:

Plasmid isolation was done by the modified alkaline lysis method (Birnboim and Doly, 1979) as stated earlier. Isolated plasmid was checked on 1% agarose gel.

11.2 Release of Insert with Restriction Enzyme

T-*rep* construct (CaMV 35S P + T-*rep* region + CaMV 35S ter) was released from the vector by double digestion with the enzymes *Bam*H1 and *Hin*dIII. These enzymes were used because they have sites in both pUC118-35S-P-T and pCAMB1A 2301 vector and these sites were absent in insert. In case of RNAi inducing inverted repeats constructs the restriction or release of the insert was carried with *Eco*R I and *Hin*d III. The reaction mix prepared was as follows.

Recombinant plasmid (0.5 mg/ml)	10 µl
Bam HI or *Eco* RI (10U/ml)	0.5 µl
Hin d III	0.5 µl
10X reaction buffer	2.0 µl
Sterile distilled water	7.0 µl
	Total 20 µl

The mixture was incubated at 37° C overnight, and the restricted product was then subjected to 1% agarose electrophoresis.

11.3 Gel Purification of Restricted Product

Released insert was (~1.1Kb) with T-*rep* sub cloning, was excised from the agarose gel and the DNA was purified from the gel using Qiagen Gel Purification kit by following manufacturer's instructions. In RNAi constructs the insert releases were ~720 bp, ~ 850 bp, and ~650 bp for IR-*rep*, Ihp-*rep* and sh-*rep* recombinant pUC 118-35S-P-T plasmids.

11.4 Linearization of Vector pCAMBIA 2301

The *Agrobacterium tumefaciens* Ti plasmid derived binary vector pCAMBIA 2301 was linearised using *Bam* HI or *Eco*RI (as the case may be) and *Hin*dIII enzyme, producing cohesive ends on the vector. The reaction mixture was prepared as follows.

Vector DNA (0.5 mg/ml)	2 µl
Bam HI or *Eco* RI (10U/ml)	0.5 µl
Hin d III	0.5 µl
10X reaction buffer	2 µl
Sterile distilled water	15 µl
	Total 20 µl

The reaction mixture was incubated at 37° C overnight.

11.5 Ligation of Insert with pCAMBIA Vector DNA

Ligation reaction between gel purified insert and vector DNA was carried out using T_4 DNA ligase by following cohesive end ligation method as above. The ligation mixture was prepared as follows.

Gel purified fragment (~100 ng/ml)	12µl
Vector (~100 ng/ml)	3 µl
T_4 DNA Ligase (high concentration) (200 U/ml)	2 µl
10X reaction buffer	2 µl
10 mM ATP	1 µl
	Total 20 µl

In addition to standard reaction mix, positive and negative control mixes were also prepared by adding control insert and no DNA in ligation mix respectively. The ligation mixtures were incubated at 16^{o} C overnight. Transformation of the *E.coli* (DH5α) competent cells was done as described in 6.3 - 6.4. The cell suspension after transformation was aseptically plated on Luria Agar (LA) plates separately containing kanamycin, (25 µl of 50 µg/ml kanamycin in 50 ml LA). The plates were incubated overnight at 37^{o} C.

11.6 Selection of Transformants

The colonies obtained were screened for the presence of the insert by colony PCR and restriction analysis with *Bam*H1 or (*Eco*RI for RNAi constructs) and *Hin*dIII. The recombinant plasmid DNA from pCAMBIA 2301 clones were isolated and restricted with *Bam*H1 (*Eco*RI for RNAi constructs) and *Hin*dIII as described above (11.2). After restriction digestion, the products were electrophoresed in 1% agarose gel. Fragment size was assessed in comparison with 1 kb DNA marker.

12. Mobilization of the constructs into *Agrobacterium tumefaciens* LBA 4404

12.1 Preparation of competent cells

An overnight grown culture of *Agrobacterium* was diluted in 50 ml Luria broth (LB) and incubated at 28° C for 3-4 hours. Logarithmically growing cells were centrifuged at 5500 rpm for 20 minutes at 4° C. The pellet was washed once in 10 ml precooled TE (10mM Tris-HCl, pH 7.5 ; 1 mM EDTA) and resuspended in 20 ml LB medium. Aliquots of 500 µl were used directly for transformation.

12.2 Transformation

Stored cells were thawed on ice prior to transformation. Competent *Agrobacterium* cells are mixed with 0.5 - 1.0 µg recombinant pCAMBIA-2301 DNA. The cells were subjected successively to 5 minutes ice, 5 minutes liquid nitrogen and 5 minutes 37° C treatment. After dilution in 1 ml LB medium the cells were shaken 2-4 hr at 28° C. Aliquots of 200 µl were plated on LA plate containing Kanamycin (50mg/l) and streptomycin (100 mg/ml) and incubated for two days at 28° C.

12.3 Screening for recombinant clones by colony PCR

The colonies obtained were screened for the presence of the insert by colony PCR with T-*rep* specific primers or using 35 S P-T primers to screen for the presence of entire cassette.

APPENDIX-III

Common Reagents, Buffers and Media Used

I. Antibiotics

Ampicillin	Stock solution (50 mg/ml) of the antibiotic was made in double distilled water, filter sterilized (through 0.22 micron filter) and distributed into 200 µl aliquots and stored at -20° C. It was used at a concentration of 50 µg/ml.
Kanamycin	Stock solution (50 mg/ml) prepared similarly and stored at -20° C. It was used at a concentration of 25 µg/ml.
Streptomycin	Stock solution (100 mg/ml) prepared similarly and stored at -20° C. It was used at a concentration of 100 µg/ml.

II. Electrophoresis Reagents

50X TAE	Tris base	242.03
	Glacial acetic acid	57.1 g
	0.5 M EDTA (pH 8.0)	100 ml
	Distilled water to 1 litre	
Loading dye	1% Bromophenol blue	200 µl
	Glycerol	200 µl
	10% SDS	60 µl
	0.5 M EDTA	50 µl
	10X TAE	60 µl
	Distilled water	30 µl

III. Media

Luria Agar Medium	Bacto-tryptone	10.0 g
	Bacto-yeast extract	5.0 g
	NaCl	10.0 g
	Agar	15.0 g
	Deionized water	950 ml

pH was adjusted to 7.0 with 5 N NaOH and volume made up to 1L with deionized water. It was dispensed in 100 ml aliquots in 250 ml flasks and was sterilized by autoclaving for 20 min at 15 p.s.i.

Luria Broth medium	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Deionized water	950 ml

pH was adjusted to 7.0 with 5N NaOH and volume made up to 1 L with deionized water. The medium was aliquoted into 50 ml in 250 ml flasks and sterilized by autoclaving for 20 min at 15 p.s.i.

IV. Plasmid Isolation Buffers

Solution I (Resuspension buffers)	25 mM Tris HCl pH (8.0) 50 mM Glucose 10 mM EDTA
Solution II (Lysis buffer)	0.2 N NaOH 1% SDS
Solution III (Neutralization buffer)	3 M Sodium acetate pH 4.8

V. Buffer

Ligation buffer 10X	0.5 M Tris HCl (pH 7.8) 0.5 M $MgCl_2$ 0.1 M Dithiothreitol 500 $\mu g\ ml^{-1}$ Bovine serum albumin

VI. DNA Molecular Weight Marker

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

VII. Preparation of commonly used stock solution

Solution	Method of preparation
0.1 M Adenosine triphosphate (ATP)	60.0 mg of ATP was dissolved in 0.8 ml of distilled water. The pH was adjusted to 7.0 with 0.1 N NaOH and volume made up to 1 ml with distilled water. The solution was dispensed into small aliquots and stored at -70° C.
1 M $CaCl_2$	54.0 g of $CaCl_2.2H_2O$ was dissolved in 200 ml of pure water. The solution was sterilized by passing through a 0.22 micron filter and stored in 1 ml aliquots at 4° C.
0.5 M EDTA (pH 8.0)	186.1 g of ethylenediamine tetra acetic acid disodium salt $2H_2O$ was added to 800 ml of distilled water, stirred vigorously on a magnetic stirrer, pH was adjusted to 8.0 with NaOH (20.0 g of NaOH pellets). Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
Ethidium bromide (10 mg ml^{-1})	1.0 g of ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The solution was transferred to a dark bottle and stored at room temperature
Phenol : chloroform : isoamyl alcohol	Buffer saturated phenol, chloroform and isoamyl alcohol were mixed in the ratio of 25:24:1. The equilibrated mixture was stored under a layer of 0.01 M Tris-HCl (pH 7.6) at 4° C in dark glass bottle.
IPTG (Isopropyl--b-D-thiogalacto-pyranoside)	A solution of IPTG was made by dissolving 2.0 g of IPTG in 8 ml of distilled water. Volume was made upto 10 ml with distilled water and sterilized by filtration through a 0.22 μ disposable filter.

	The solution was dispensed into 1 ml of aliquots and stored at -20° C.
1M $MgCl_2$	203.3 g of $MgCl_2.6H_2O$ was dissolved in 800 ml of distilled water. The volume was made upto 1L, dispensed into aliquots and sterilized by autoclaving.
3M Sodium acetate (pH 4.8)	408.1 g of $NaOAc.3H_2O$ was dissolved in 800 ml of distilled water. The pH was adjusted to 4.8 with glacial acetic acid. Volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
5M NaCl	233.8 g of NaCl was dissolved in 800 ml of distilled water, volume made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
10 N NaOH	Dissolve 400 g of NaOH in 800 ml of distilled water and make up the volume to 1 L with distilled water.
1M Tris-HCl	121.1 g of Tris base was dissolved in 800 ml of distilled water. pH was adjusted to the desired value by adding concentrated HCl (for pH 7.4, HCl 70 ml; for pH 8.0, HCl 42 ml). The solution was allowed to cool down to room temperature before making final adjustment to the pH. The volume was made upto 1 L with distilled water, dispensed into aliquots and sterilized by autoclaving.
X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside)	The stock solution was made by dissolving X-gal in dimethyl formamide to make a 20 mg/ml solution and stored at -20° C.

VIII. Medium and solutions for tissue culture

1. Composition of MS Medium

	Concentration of ingredients in the medium (mg/L)
Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$CaCl_2.2H_2O$	440
Micronutrients	
$MnSO_4.4H_2O$	22.3
$NZSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.25
$Na_2MoO_4.2H_2$	0.83
Cu Compounds	
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Fe-EDTA	
Na_2 EDTA.$2H_2O$	37.30
$FeSO_4.7H_2O$	27.80
Organic compounds	
Glycine	2.0
Nicotinic acid	0.5
Pyridoxin-HCl	0.5
Thiamin-HCL	0.1
Myo-inositol	100
Other	
Agar-2	8 g/L
Sucrose	30 g/L

2. Composition of regeneration medium and cocultivation medium

MS salts	4.4 g/L
BAP	1.0 mg/L
IBA	0.4 mg/L
Agar-2	8 g/L
$CaCl_2$	440 mg/L

3. Composition of selection medium

MS-salt	4.2 g/L
BAP	1 mg/L
IBA	0.5 mg/L
Agar-2	8 g/L
$CaCl_2$	440 mg/L
Sucrose	30 g/L
Kanamycin	100 mg/L
Augmantine	250 mg/L

4. Composition of shooting medium

MS salt	4.2 g/L
BAP	2.5 mg/L
IBAS	0.2 mg/L
Kinetin	1 mg/L
$CaCl_2$	440 mg/L
Sucrose	30 mg/L
Kanazmycin	100 mg/L
Augmantine	250 mg/L
Agar-2	8 g/L

5. Composition of rooting medium

MS salt	2.1 gm/L
$CaCl_2$	600 mg/L
IAA	0.5 mg/L
Sucrose	30 mg/L
Agar	6 g/L

IX. Solutions for Southern blotting

1. Denhardt's solution (50x)

Ficoll (type 400) 1 %	5.0 gm
PVP (sigma) 1%	5.0 gm
BSA 1%	5.0 gm
Distilled water	up to 500 ml

2. Neutralization solution

NaCl	1.5 M
Tris-HCl (7.4)	1.0 M

3. Pre-hybridization Solution

Denhardt's	5X
SSC	6X
SDS	0.2 %
Yeast RNA / Salmon sperm	200 mg/ml

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

Materials	Source	Catalogue
Vector		
pGEM®-T-Easy	Promega	A 1360
pUC118		
pCAMBIA 2301		
Cells		
Escherichia coli DH5a	Stratagene, USA	200233
Nucleic acid marker		
1 kb DNA ladder	MBI Fermentas	SM 0311
100 bp DNA ladder	Promega	G2101

Enzymes		
*Not*I	MBI Fermentas	ER 0591
*Hind*III	MBI Fermentas	ER0501
Bam HI	MBI Fermentas	ER0051
*Eco*RI	MBI Fermentas	ER0271
Nuclease		
RNase A	Qiagen	19101
Other enzyme		
T_4 DNA ligase	MBI Fermentas	EL 0011
Klenow (DNA pol I)	Genei	MME2S
Other reagents		
5-bromo-4-chloro-3-indoly-b-D-galactopyranoside (X-gal)	MBI Fermentas	R0402
Isopropyl-b-D-thiogalacto-pyranoside (IPTG)	MBI Fermentas	R0392
Adenosine-5-triphosphate (ATP)	HIMEDIA	RM 439
PCR Reagents and Taq DNA polymerase	MBI Fermentas	EP0071
D-19 Developer powder	Kodak	901 -1320
X-ray acid fixing salt	Kodak	900-0720
Antibiotics		
Ampicillin sodium salt	HIMEDIA	RM645
Kanamycin monosulphate	Sigma	K4000
Kits		
DNeasy Plant Mini Kit (50)	Qiagen	69104
QIAquick®PCR purification kit (50)	Qiagen	28104
MinElute Gel Extraction Kit	Qiagen	28604
QIAprep Spin Miniprep Kit (250)	Qiagen	27106