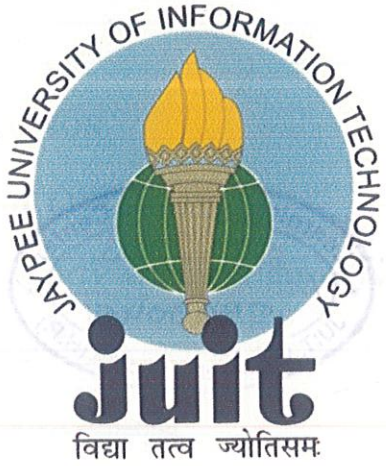
OPTIMIZATION OF VIRUS INDUCED GENE SILENCING OF PHYTOENE DESATURASE IN TOBACCO AND CAPSICUM

Enrollment no. - **091713, 091726**

Name of the students- **Mamta Mishra, Madhusudan Bairagi**

Name of the supervisor- **Dr. Anil Kant**



May 2013

Submitted in partial fulfillment of the Degree of

Bachelor of Technology,

Department of Biotechnology,

Jaypee University of Information Technology,

Waknaghat

TABLE OF CONTENTS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

juit

विद्या तत्व ज्योतिसम

CERTIFICATE

This is to certify that the work titled "**Optimization of Virus Induced Silencing (VIGS) of PDS gene in *Nicotiana tabacum* and *Capsicum annum***" submitted by "**Mamta Mishra and Madhusudan Bairagi**" in partial fulfillment for the award of degree of **Bachelor of Technology in Biotechnology** of **Jaypee University of Information Technology, Waknaghat** has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor .....

Name of Supervisor Dr. Anil Kant

Designation Sr. Lecturer

Date 28.5.2013

Acknowledgement

It is our great pleasure and privilege to work under the guidance of Dr. Anil Kant as our project guide who has been incessantly motivating and encouraging us since the inception of the project to work hard and put in every possible effort to make the project a success.

We are also thankful and indebted to PhD scholar Mr. Aseem Chawla who has been supervising us day and nightand has been helping us at every odd hour acquainting us with the basics and subtleties of molecular biology and genetic engineering.

Signature of the student	Mamta Mishra	Madhusudan Bairagi
Name of Student	Mamta Mishra	Madhusudan Bairagi
Date	27/05/13	27/05/13

SUMMARY

Virus-induced gene silencing (VIGS) is a technology that exploits an RNA-mediated antiviral defense mechanism. In plants infected with unmodified viruses the mechanism is specifically targeted against the viral genome. However, with virus vectors carrying inserts derived from host genes the process can be additionally targeted against the corresponding mRNAs. VIGS has been used widely in plants for analysis of gene function and has been adapted for high-throughput functional genomics. Until now most applications of VIGS have been in *Nicotiana benthamiana*. However, new vector systems and methods are being developed that could be used in other plants, including *Arabidopsis*. In this study optimization of PDS gene silencing in *Capsicum annum*was attempted. Similar study was also conducted on tobacco and tomato. Syringe infiltration and vacuum infiltration were used to infiltrate the plants with agrobacterium harboring TRV1 and TRV2. Successful standardization of PDS silencing in capsicum will allow analysis of other important genes in capsicum through VIGS technology.

Signature of Student	Signature of Student	Signature of Supervisor
Name- Mamta Mishra	Name- Mamta Mishra	Name-Dr. Anil Kant
Date- 27/05/13	Date- 27/05/13	Date-

LIST OF FIGURES

LIST OF SYMBOLS AND ACRONYMS

VIGS- Virus Induced Gene Silencing

PDS- Phytoene Desaturase

PTGS- Post Transcriptional Gene Silencing

dsRNA- Double stranded RNA

RNAi- RNA Interference

siRNA- Small Interfering RNA

RISC- RNA Induced Silencing Complex

MES-Methanoethane sulfonic Acid

Chapter 1

INTRODUCTION

Gene silencing is a general term describing epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic modification. That is, a gene which would be expressed ("turned on") under normal circumstances is switched off by machinery in the cell.

Many early experiments in plants had demonstrated that over-expression of either sense or antisense RNAs could induce post-transcriptional silencing and did so at similar, relatively low frequencies. Testing the hypothesis that double stranded RNAs resulting from the production of both sense and antisense RNAs might trigger post-transcriptional gene silencing, Waterhouse *et al.* showed that the frequency of viral or reporter gene silencing was dramatically increased if transgenes were engineered to produce duplex RNAs in the cell. This could be triggered by crossing transgenic lines, one expressing a sense transcript and the other an antisense transcript. Likewise, high frequency silencing was conferred by transgenes that expressed a transcript containing an inverted repeat such that the transcript could fold back on itself to form a double-stranded RNA (dsRNA). These experiments showing that dsRNA triggers RNA elimination plants occurred nearly simultaneously with those of Fire et al. that demonstrated that hairpin RNA molecules induce RNA interference in *C. elegans*. These and subsequent findings meshed well with the Dougherty lab's hypothesis that dsRNA molecules initiated the cascade of events leading to silencing. Subsequent biochemical and genetic experiments in *Neurospora*, *Drosophila*, mammals and plants have further supported Dougherty and colleagues' hypothesis. Indeed, the requirement for RNA-dependent RNA polymerase activity in RNAi has been firmly established, as has the involvement of nucleases that cleave the dsRNAs into small RNA molecules. Resulting small RNA fragments may then be targeted for degradation, or may bind to homologous RNAs, priming production of new dsRNAs and amplifying the silencing signal.

Greater than ninety percent of all plant viruses are RNA viruses that replicate through a dsRNA intermediate. So, it seems likely that RNA interference in plants has evolved, in part, as a means for protection against viral infection and retro transposon proliferation. Indeed, small interfering RNAs (a hallmark of RNAi) homologous to viral sequence have been found systemically in viral

infected plants and mutations in genes involved in RNAi lead to increased susceptibility to disease and to transposon proliferation. Likewise, some plant viruses encode genes which counteract the plant's RNAi surveillance system, thus evading degradation. Important for its proposed role in combating disease, RNAi can act systemically throughout a plant. One clear demonstration came from grafting the root and lower portion of a plant in which a reporter gene was silenced by RNAi to a shoot in which the same reporter was active. Amazingly, a silencing signal traversed the graft junction to cause silencing in the shoot. Likewise, infecting a reporter gene-expressing plant with a virus that included sequences matching the reporter gene caused reporter gene silencing far beyond the site of infection. Thus it is thought that cells that initiate RNAi, as in the response to virus infection, can generate small RNAs, or some other mobile signal, which traffic via the phloem throughout the plant and help target related RNAs, thus protecting against a spreading RNA virus.

RNAi has rapidly gained favour as a "reverse genetics" tool to knock down the expression of targeted genes in plants, as in other species, due to certain advantages that RNAi technology holds over gene disruptions caused by transposon or T-DNA insertion. The ability to target multiple gene family members with a single RNAi-inducing transgene is one such advantage. Another is that gene knockdowns due to RNAi are dominant, whereas insertional or other loss of function mutations are recessive. The dominant aspect of RNAi allows the knock down of genes in polyploid genomes that contain four or more orthologs and are thus refractive to traditional mutagenesis. Likewise, orthologs can be knocked down in F1 hybrids in which the RNAi-inducing transgene is introduced through only one of the parents. Finally, the dominance of RNAi allows one to save time by eliminating the additional generations needed to identify individuals that are homozygous for recessive loss-of-function alleles.

The term VIGS describes a technique employing recombinant viruses to specifically reduce endogenous gene activity and is based on Post-Transcriptional Gene Silencing (PTGS) .The viral vectors commonly used for VIGS are manipulated to incorporate a fragment of the host plant's target gene. Plants employ PTGS as their innate antiviral defence line to counteract viral proliferation, employing an RNA degradation mechanism very similar to the pathways of RNA interference. Vectors for VIGS are standard binary Ti-plasmid derived vectors used for *Agrobacterium tumefaciens* mediated plant transformation in which part of a viral genome is inserted. Within the viral genome a multiple cloning site is added to insert the endogenous target gene of the host plant. Fragments of 200 to 1300 bp length targeting the middle regions of mRNAs and devoid of homopolymeric regions, such as poly-A tails suffice for VIGS in *Nicotiana*

benthamiana. Plant inoculation with viral vectors is most commonly achieved via *Agrobacterium tumefaciens* infection. The T-DNA containing the viral genome is then integrated into the host genome of at least one cell, transcribed, and translated. This leads to the production of double-stranded RNAs (dsRNAs) from the viral ssRNA template by self-assembly of viral ssRNA into secondary structures or complementary sequences derived from longer positive and negative viral ssRNA strands. Dicer-like proteins cleave these viral dsRNAs into short interfering RNAs (siRNAs) duplexes which are approximately 21–24 nucleotides (nt) in length. These siRNA in turn are incorporated as single strand RNA molecules into RISC (RNA-induced Silencing Complex) which screens for and destroys RNAs complementary to the siRNA. In the special case of VIGS viral RNAs and the mRNA of the target gene are cleaved. The virus-derived silencing signal is further amplified and spreads systemically throughout the plant. siRNAs about 21 nt in length are assumed to mediate the short range transport and the RNA-dependent RNA Polymerase6(RDR6) is required for long-range transport, possibly by amplifying the silencing signal . This systemic spread of the silencing signal occurs regardless of the successful movement of the virus particles in the plant. When VIGS is applied to a susceptible plant, the host plant's target gene mRNA is degraded in large portions of the plant.

Many plant viruses have been used to develop VIGS vectors. Early vectors included tobacco mosaic virus (TMV), potato virus X (PVX) and tomato golden mosaic virus (TGMV). All these viruses may cause strong infection symptoms including chlorosis, leaf distortion and necrosis. In addition, they are incapable of infecting the apical meristem and are therefore unlikely to provide information about the genes involved in the identity and development of plant tissues and organs. Tobacco rattle virus (TRV)-based vectors have been suggested to have potential for use with a wide range of plant species due to the wide host range of TRV. These TRV vectors overcome many of the difficulties associated with PVX, TMV and TGMV. For example the TRV vector include mild symptoms, infects large areas of adjacent cells and silences expression of gene in the meristem. The TRV based VIGS system has been shown to function effectively in *Arabidopsis, Nicotiana benthamiana*, tomato, lettuce, *Aquilegia* and California poppy.

TRV is a two particle positive sense RNA rod type virus. RNA1 encodes two replicase proteins, a movement protein, and a cysteine rich protein. RNA2 encode the coat protein a two known structural proteins. Because TRV RNA1 can replicate and move systemically in the plant in the absence of RNA 2, researchers reasoned that RNA 2 could be modified for the insertion of fragments of genes targeted for silencing. They constructed binary transformation vectors using T-DNA from agrobacterium engineered to carry 35S promoters and the RNA 1 and RNA2 of

TRV. The 35S promoters simulate initial viral transcription, thus ensuring rapid infection once plant cells have been infected by the agrobacterium. The RNA 2 genes encoding non-structural proteins were replicated with a multiple cloning site (MCS) into which fragments of target genes can rapidly be inserted.

Because VIGS typically results in chimeric plants with some portions uninfected and therefore unsilenced, it is essential to incorporate the silencing of a reporter gene as a tool to indicate sites of silencing. Early studies used the silencing of the PDS to show the effect of VIGS; infected plants show characteristic photo bleaching symptoms resulting from the inhibition of biosynthesis of protective carotene. While silencing of PDS serves as a clean reporter, the phenotype has obvious disadvantages, as the photo bleaching is concomitant with destruction of photosynthetic apparatus.

The objectives of the study are :

1. Cloning of Capsicum PDS gene
2. Optimization of VIGS of PDS gene in Tobacco and Capsicum

Chapter 2

REVIEW OF LITERATURE

Virus induced gene silencing (VIGS) is a fast an efficient functional genomics tool and a method for rapid silencing of plant genes in order to discover their function. VIGS is based on Post-Transcriptional Gene Silencing (PTGS) .Plants exploit PTGS as their innate antiviral defence line to encounter viral multiplication, employing defence mechanism very similar to the pathways of RNA interference which refers to interference in gene expression, mediated by small RNA in a sequence specific manner. Post-transcriptional gene silencing is the result of mRNA of a particular gene being destroyed or blocked. The destruction of the mRNA prevents translation to form an active gene product (in most cases, a protein). A common mechanism of post-transcriptional gene silencing is RNAi. VIGS process begins with the *Agrobacterium*-mediated introduction of modified virus-based cDNA constructs that also contain fragments of endogenous gene sequences. Once expressed *in vivo*, dsRNAs are generated from an encoded viral polymerase as the virus replicates and spreads through the plan. These dsRNAs are then targeted by DICER-like enzymes and degraded into siRNA. In turn, the siRNA molecules provide a template for degradation of complimentary RNAs, including complimentary endogenous mRNAs, by the RNA-induced silencing complex (RISC). Silencing persists until proliferation of viral RNAs is overcome by the silencing response.

Induction of VIGS is a useful alternative to the often difficult and laborious process of generating stably transformed plants, and offers the ability to overcome functional redundancy by suppressing all or most members of a gene family. VIGS vectors have been developed from viral systems that affect many plant hosts including many dicots and several monocots (barley and wheat). Here we demonstrate VIGS in the species *Aquilegia vulgaris* using vectors based on the bipartite genome of the tobacco rattle virus (TRV). This system uses two vectors, derived from binary transformation plasmids, which have cDNAs encoding the TRV RNA1 (TRV1) and TRV RNA2 (TRV2) inserted into the T-DNA region. Both vectors contain a duplicated 35S promoter and a self-cleaving ribozyme sequence to enable rapid generation of intact viral transcripts. Genes essential for plant to plant transmission of TRV through its nematode vector have been deleted from TRV2. The TRV system has the advantage of being able to penetrate meristematic cells and

overall causes mild viral symptoms in a wide range of susceptible hosts. Although TRV-VIGS has primarily been used in the Solanaceae (tobacco), it has also been successfully applied in *Arabidopsis* and the lower eudicot *Papaver* (poppy).

TRV is a bipartite virus and, as such, two different *A. tumefaciens* strains are used for VIGS. One carries pTRV1, which encodes the replication and movement viral functions while the other, pTRV2, harbors the coat protein and the sequence used for VIGS. Inoculation of *Nicotiana tabaccum* and tomato seedlings with a mixture of both strains results in gene silencing. Silencing of the endogenous *phytoene desaturase* (*PDS*) gene, which causes photo bleaching, is used as a control for VIGS efficiency. It should be noted, however, that silencing in tomato is usually less efficient than in *N. benthamiana*. RNA transcript abundance of the gene of interest should always be measured to ensure that the target gene has efficiently been down-regulated. Nevertheless, heterologous gene sequences from *N. benthamiana* can be used to silence their respective orthologs in tomato and vice versa

Virus-induced gene silencing (VIGS) offers a rapid and high throughput technique platform for the analysis of gene function in plants. Although routinely used in some Solanaceous species, VIGS system has not been well established in Arabidopsis thaliana . We have recently reported some factors that potentially influence tobacco rattle virus (TRV)-mediated VIGS of phytoene desaturase (PDS) and actin gene expression in Arabidopsis. In this study, we have further established that the Agrobacterium strain used for agro-inoculation significantly affects the VIGS efficiency. Strain GV3101 was highly effective; C58C1 and LBA4404 were invalid, while EHA105 was plant growth stage-dependent for TRV-induced gene silencing. Furthermore, the VIGS procedure optimised for the PDS gene was applied for the functional analysis of the disease resistance gene RPS2-mediated resistance pathway(Xin Zhong et al,December 2005).

Virus-induced gene silencing (VIGS) is an effective new tool to study the function of orthologs of floral homeotic genes such as DEFICIENS (DEF) in non-model systems. We used a tobacco rattle virus (TRV)-based VIGS approach to study the function of the Nicotiana benthamiana DEF ortholog (NbDEF). Silencing of NbDEF in N. benthamiana using TRV-VIGS was similar to that of Antirrhinum def and Arabidopsis ap3 mutants and caused transformation of petals into sepals and stamens into carpels. Molecular analysis of the NbDEF-silenced plants revealed a dramatic reduction of the levels of NbDEF mRNA and protein in flowers. NbDEF silencing was specific and has no effect on the mRNA levels of NbTM6, the closest paralog of NbDEF. A dramatic

reduction of the levels of N. benthamiana GLOBOSA (NbGLO) mRNA and protein was also observed in flowers of NbDEF-silenced plants, suggesting that cross-regulation of this GLO-like gene by NbDEF. Taken together, our results suggest that NbDEF is a functional homolog of Antirrhinum DEF. Our results are significant in that they show that TRV efficiently induces gene silencing in young and differentiating flowers and that VIGS is a promising new tool for analyses of developmental gene function in non-model organisms (Yule Liu et al, January 2004).

The lower eudicot genus *Aquilegia*, commonly known as columbine, is currently the subject of extensive genetic and genomic research aimed at developing this taxon as a new model for the study of ecology and evolution. demonstrate the effective use of a reverse genetic technique, virus-induced gene silencing (VIGS), to study gene function in this emerging model plant. Using *Agrobacterium* mediated transfer of tobacco rattle virus (TRV) based vectors, we induce silencing of *PHYTOENE DESATURASE* (*AqPDS*) in *Aquilegia vulgaris* seedlings, and *ANTHOCYANIDIN SYNTHASE* (*AqANS*) and the B-class floral organ identity gene *PISTILLATA* in *A. vulgaris* flowers. For all of these genes, silencing phenotypes are associated with consistent reduction in endogenous transcript levels. In addition, we show that silencing of *AqANS* has no effect on overall floral morphology and is therefore a suitable marker for the identification of silenced flowers in dual-locus silencing experiments (Billie Gould et al,April 2007).

Using a tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) system, expression of phytoene desaturase (PDS) and ribulose-1,5-bisphosphate carboxylase small-subunit (rbcS) genes was suppressed in Nicotiana benthamiana and pepper plants (Capsicum annuum L. cv. Bukang). The silenced phenotypes of pale yellow (rbcS), and photo bleached leaves (PDS), were invariably obvious 2 weeks after inoculation with the TRV-based vector. In a parallel experiment, the same set of genes was silenced in N. benthamiana and yielded identical phenotypes to pepper 1 week after inoculation. Northern blot analyses showed that the endogenous levels of CarbcS and CaPDS transcripts were dramatically reduced in the silenced leaf tissues. These observations confirm that the silenced phenotype is closely correlated with the pattern of tissue expression. To our knowledge, this is the first high frequency VIGS method in pepper plants. It should provide a tool for large-scale gene silencing studies in pepper functional genomics (Chung et al, April 2004).

Chapter 3

Materials and Methods

3.1 Plant material

For this research *Nicotiana tabacum* and *Capsicum annum* were used. Plants were grown in temperature controlled (25°C). Green house and plants at different leaf stages were used in VIGS experiments.

3.2 Total plant RNA isolation

For total plant RNA isolation, Genei Plant RNA isolation kit was used. All the reagents as well as plastic ware should be DEPC treated before proceeding for RNA isolation. Following protocol was used for RNA isolation:

1. Chill the pulverizer with liquid nitrogen and pulverize the plant tissue (1g) to fine powder in the absence of lysis buffer.
2. Add prewarmed buffer A + phenol mixture (80°C) in 1:4 ratio to the powdered tissue (i.e. for every 1g of tissue add 4 ml of the mixture) in a 30 ml polypropylene tubes and mix for 5 minutes.
3. Add equal volume of chloroform (4-7 ml/g of powder or tissue).
4. Incubate at RT for 30 minutes with intermittent mixing.
5. Centrifuge at 5000 rpm for 15 minutes.
6. Transfer the upper phase to the new tube and add 1/3rd volume of precipitation solution (buffer B), mix well & incubate atleast for 4 hrs. at 4°C (for better yield incubate overnight at 4°C).
7. Precipitate the RNA by centrifugation at 7000 rpm for 20 minutes.
8. Discard the supernatant & dissolve the precipitate in 1-2 ml of wash buffer (buffer c), allow time to suspend the pellet completely & transfer to 1.5 ml vial.
9. Centrifuge at 7000 rpm for 20 minutes.
10. Repeat step 8 & 9 for effective precipitation of RNA (minimum 5 washes is recommended).
11. Wash the pellet with 70 % ethanol twice &finally with 100 % ethanol (mix well & centrifuge at 7000 rpm for 10 minutes).

12. Air dry the pellet for 15-20 minutes. Dissolve the pellet in 150-200 µl of nuclease free water (for 1g of tissue).
13. Evaluate the quality & quantity of RNA in the extracts by measuring the absorbance at 260 & 280 nm ratio & subsequently by agarose gel electrophoresis.
14. The RNA obtained can be used immediately for downstream applications or can be stored at -70°C. (store as aliquots)
15. In case of DNA contamination in the preparation treat the sample with DNase I-RNase free (1U/ug RNA) at 37°C for 1 hour then perform phenol-chloroform extraction and ethanol precipitation. Suspend the pellet in minimum amount of nuclease free water.

3.3 Single strand cDNA synthesis

Single stranded cDNA was performed using Mmlv reverse transcriptase. Before proceeding with the cDNA synthesis quantification of the RNA was performed using Thermo scientific nanodrop 2000c. RNA was taken with the concentration of 1 µg/µl.

1. In a nuclease free micro vials add the following components

   RNA - 4.0 µl

   Oligo d (T) – 1.0 µl

   DEPC H_2O - 9.2 µl
2. Mix the contents briefly, spin and incubate at 65^0C for 5 minutes.
3. Add the following components to the vial in order

   5X RT buffer – 4.0µl

   0.1 M DTT – 1.0 µl

   DNTP – 0.5 µl

   MMlv RT – 0.3 µl
4. Mix gently and centrifuge the vial.
5. Incubate it at 37^0C for 60 minutes.
6. Terminate the reaction by heating at 70^0C for 10 minutes.

3.4 Amplification of PDS gene of California wonder

The Phytoene desaturase gene is responsible for the photo bleaching phenotype. This gene is conserved throughout the plant species. Universal primers (Forward primer5'-CGG TCT AGA

GGC ACT CAA CTT TAT AAACC-3' and Reverse primer 5'-CGG GGA TCC CTT CAG TTT TCT GTC AAA CC-3') were designed for from the conserved regions of this gene and were used to amplify PDS gene from California wonder. PCR amplification was performed with initial denaturation at 94^{0}C for 5 min followed by 35 cycles of 94^{0}C for 1 min, 57^{0}C for 1 min, 72^{0}C for 2 min and final extension at 72^{0}C for 7 min before cooling at 4^{0}C.

	1X	5X
10X PCR buffer	1.5 µl	7.5 µl
dNTPs	0.3 µl	1.5 µl
Primer F	1 µl	5 µl
Primer R	1 µl	5 µl
DNA template	0.5 µl	1 µl
Taq polymerase	0.15 µl	0.75 µl
Autoclaved, distilled water	10 µl	50 µl
Total	14.5 µl	70.75 µl

PCR master mix

3.5 Cloning of PDS gene in pGEM-T vector (TA cloning)

The amplified PDS gene fragment was cloned in the pGEM-T vector. pGEM-T easy vector cloning kit (Promega) was used for this purpose.

1. Briefly centrifuge the pGEM-T Easy Vector and PDS gene fragment DNAtubes to collect the contents at the bottom of the tubes.
2. Set up ligation reactions as described below.

Reaction Component	Standard Reaction
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl
pGEM-T Easy Vector (50ng)	1µl
PCR product	2µl
T4 DNA Ligase (3 Weiss units/µl)	1µl
Nuclease-free water	1µl

3. Mix the reactions by pipetting. Incubate the reactions for 16 hours at 16^0C.

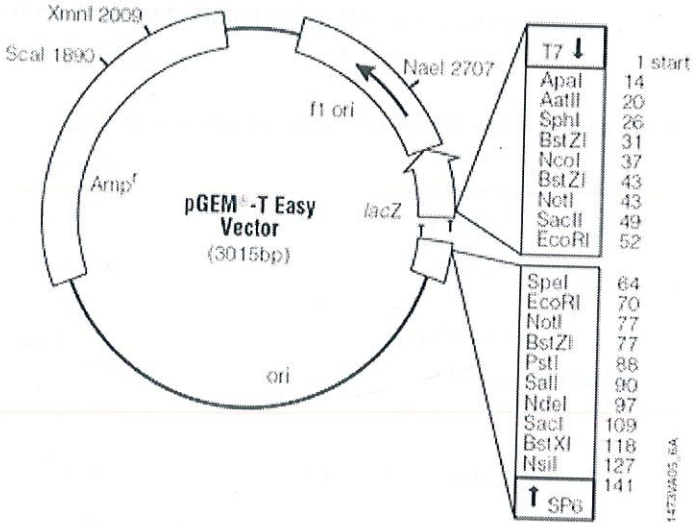


Figure 3.5.1: Map of pGEM-T Easy vector

3.6 Transformation using the pGEM-T easy vector ligation reactions

The ligation reaction had been transformed into chemically competent DH5α Ecoli cells. The LacZ scorable marker was used to select the putative transformants carrying the PDS gene fragment

3.6 a) Preparation of *E. coli* competent cells by calcium chloride method

1. Grow E. coli DH5α cells at 37^0C until the O.D.$_{600nm}$reaches 0.6 (for about 4 hours)
2. Remove the flask from the shaker and keep it at 4^0Cto stop further growth of the cells.
3. Divide the culture in 50ml falcon tubes and spin at 7000 rpm at 4^0C for 15 minutes.

4. Remove the supernatant completely and to the pellet add 25 ml of ice cold $MgCl_2$:$CaCl_2$ (80mM: 20mM) mixture.
5. Vortex the vials vigorously to re-suspend the pellet. Keep the suspended pellet on ice for 30 minutes.
6. Centrifuge the vials at 7000 rpm for 15 minutes at 4^0C.
7. Discard the supernatant. To each 50ml vial, add 2ml of 0.1 M $CaCl_2$and 40µl of DMSO..
8. Make 200 µl aliquots of cells and store at -80^0C.

3.6 b) Transformation of chemically competent *E.coli* cells by Heat Shock method.

1. Prepare two LB/ampicillin (100µg/µl) /IPTG/X-Gal plates for each ligation reaction, plus two plates for determining transformation efficiency. Equilibrate the plates to room temperature.
2. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom. Add 2µl of each ligation reaction to the thawed *E.Coli* competent cells.
3. Mix the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile.
4. Place micro centrifuge tubes on ice for 20 minutes.
5. Heat-shock the cells for 90 seconds in a water bath at exactly 42°C.
6. Immediately return the tubes to ice for 2 minutes.
7. Add 950µl room-temperature LB medium to the tubes containing cells transformed with ligation reactions.
8. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
9. Plate 100µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates.
10. Incubate the plates overnight (16–24 hours) at 37°C.

3.7 Plasmid Isolation by Alkaline Lysis Method

1. Take 1 ml culture in 1.5 ml eppendorf. Centrifuge at 10000 rpm for 5 minutes at 4°C.
2. Discard the supernatant. Ensure that all the supernatant is removed and pellet is dry.
3. Add 100 µl of ice cold solution 1. Vortex.
4. Add 200 µl of solution 2 and invert 20 times.
5. Add 150 µl of ice cold solution 3 and invert 5 times.

6. Keep on ice for 5 minutes. Centrifuge at 10000 rpm for 5 minutes at 4°C.
7. Transfer supernatant to fresh eppendorf. Add 450 µl of chloroform: isoamyl alcohol.
8. Vortex and centrifuge at 10000 rpm for 5 minutes at 4°C.
9. Transfer aqueous layer to fresh eppendorf. To the aqueous layer add 900 µl of absolute ethanol. Leave eppendorfs on ice for 5 minutes.
10. Centrifuge at 10000 rpm for 5 minutes at 4°C.
11. Discard supernatant .Add 1 ml of 70 % ethanol. Centrifuge at 10000 rpm for 5 minutes at 4°C.
12. Discard supernatant. Let all the ethanol evaporate from pellet.
13. Add 50 µl of autoclaved distilled water. Store at -20°C.

3.8 Syringe Infiltration of Tobacco, Tomato and Capsicum plants

Preparation of MES buffer:-MES buffer =10mM $MgCl_2$+10mM MES (pH of the mixture 5.5)

1. Weigh 213 mg of MES (Morpholinoethanesulfonic acid) and dissolve in 10 ml of 100mM $MgCl_2$. Add 80 ml distilled water and set pH of the solution to 5.5.
2. Centrifuge TRV1, TRV2-Empty and TRV2-Ntpds cultures at 6000 rpm for 15 minutes at 4°C.
3. Discard the supernatant and blot dry the pellet. Add 25 ml of MES buffer to each of the pellet.
4. Mix TRV1+TRV2empty solutions and TRV1+TRV2ntpds solutions respectively.
5. Add 200µl acetosyringone to each. Incubate and keep at RT for 2-3 hrs.
6. The above incubated mixtures are injected on lower surface of leaf in 4-5 leaf stage tobacco, tomato and capsicum plants. As a control water is injected in some plants in place of TRV cultures. Cover the plants by plastic bags to maintain the humidity.
7. The above injected plants are placed in the glass house and observed after 3 weeks for photo bleaching.

3.9 Vacuum Infiltration in tomato saplings

1. 50 ml cultures of TRV1, TRV2empty and TRV2ntpds in vials centrifuged at 6000 rpm for 20 minutes at 4^0C.
2. Add 50 ml of MES to pellets of TRV1 and TRV2ntpds.Vortex and mix well.

3. Add 200 µl of acetosyringone prepared in DMF to mixture of TRV1 and TRV2ntpds.Mix well. Keep at 28^0C in shaker for 2 hours to incubate.
4. Take O.D. at 600 nm with MES as reference.
5. 30 saplings of tomato washed. Placed in hood of the vacuum infiltration apparatus for vacuum infiltration in mixture of TRV1+TRV2ntpds in a beaker.200 µl of Triton X-100 was added to vial mixture.
6. Another 30 saplings taken. Process repeated.
7. Placed in BOD incubator in an autoclavable bag. Subsequently growth is monitored

Chapter 4

RESULTS AND DISCUSSION

4.1 RNA isolation from *Capsicum annum*

RNA was isolated from the fresh leaf tissue of capsicum. The isolated RNA was checked by running on 1% formaldehyde agarose gel. The gel was analyzed for the presence of DNA and it was found that RNA samples were free from DNA and fit for downstream applications.

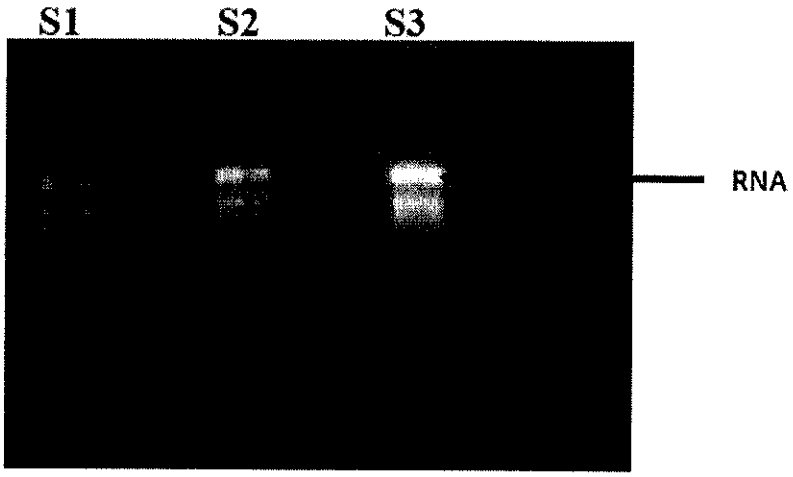


Figure 4.1.1: RNA isolated from leaf tissue of *Capsicum annum.* S1, S2 and S3 are Capsicum samples from which RNA is isolated

4.2 Single strand cDNA synthesis

cDNA was synthesized from the RNA with MmLv reverse transcriptase. The quality of the cDNA was checked by amplifying 26S rRNA gene. The successful amplification of this gene implies that cDNA is successfully synthesized and is ready for amplification of desired gene fragments.

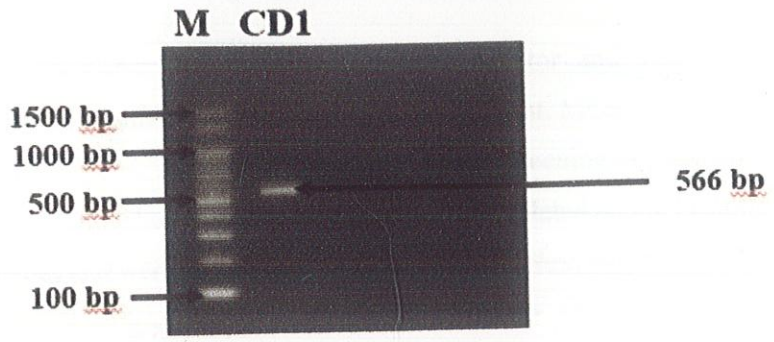


Figure 4.2.1: 26S rRNA gene amplified from the cDNA of *Capsicum annum*. M = 100 bp ladder, CD1 = *Capsicum annum*c DNA sample 1

4.3 Amplification of PDS gene of California wonder

PDS gene was amplified from the cDNA of *Capsicum annum*. 444 bp of PDS gene fragment was amplified by the universal primers.

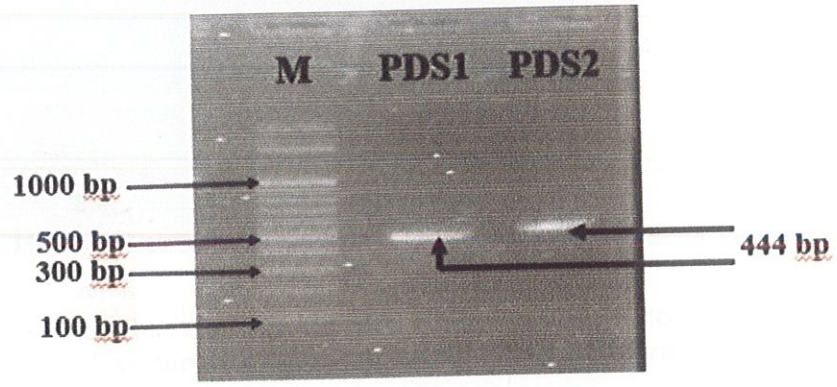


Figure 4.3.1: PDS gene fragment of Cap*sicum annum*(444bp). M = 100 bp ladder, PDS1 = *Capsicum annum*PDS fragment sample 1, PDS2 = *Capsicum annum* PDS fragment sample 2

4.4 Cloning of PDS gene in pGEM-T vector and transformation in E.Coli

The PDS gene fragment was cloned in pGEM-T vector and was transformed in E.coli DH5α.Ampicillin(100µg/ml) was used as the selective agent. MCS of pGEM-T vector is located in-between the LacZ gene, which allows the blue white screening of putative colonies harboring gene of interest. After the heat shock treatment, cells were plated on the LB/ampicillin (100µg/µl) /IPTG/X-Gal plates and were incubated at 37°C overnight. Next day white colonies were streaked on LB/ampicillin (100µg/µl) /IPTG/X-Gal plates (master plate). After overnight incubation streaks showing consistent white colonies were selected for further testing.

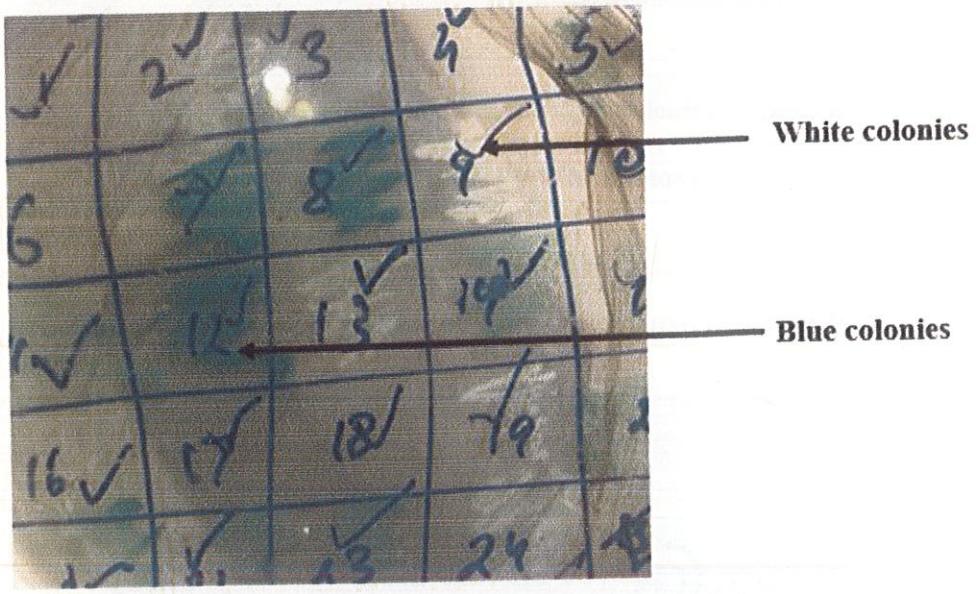


Figure 4.4.1: Master plate showing blue and white colonies of *E.coli* harbouring pGEM-T vector with *Capsicum annum*PDS gene fragment

4.5 Colony PCR of Putative *E.coli* colonies harbouring PDS gene fragment

For confirmation of the white colonies which have PDS gene fragment, colony PCR was performed. Universal primers were used for confirmation of these colonies. After the confirmation of colonies, identified colonies were inoculated in LB media for glycerol stock as well as plasmid isolation.

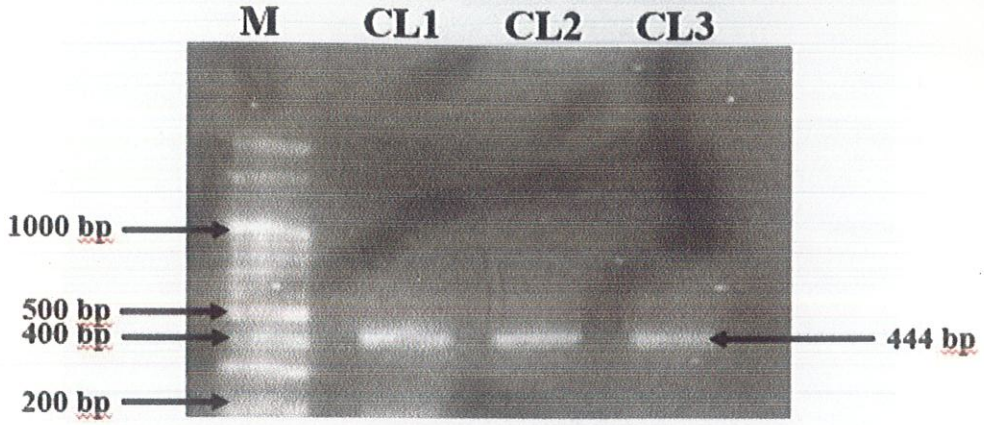


Figure 4.5.1: Colony PCR for confirmation of white colonies harbouring PDS gene fragment of Cap*sicum annum* (444bp). M = 100 bp ladder, CL1 = White colony 1, CL2 = White colony 2, CL3 = White colony 3

4.6 Syringe In-filtration in Tobacco, Tomato, Capsicum plants

Figure 4.6.1-Infiltration in tobacco plants through injection

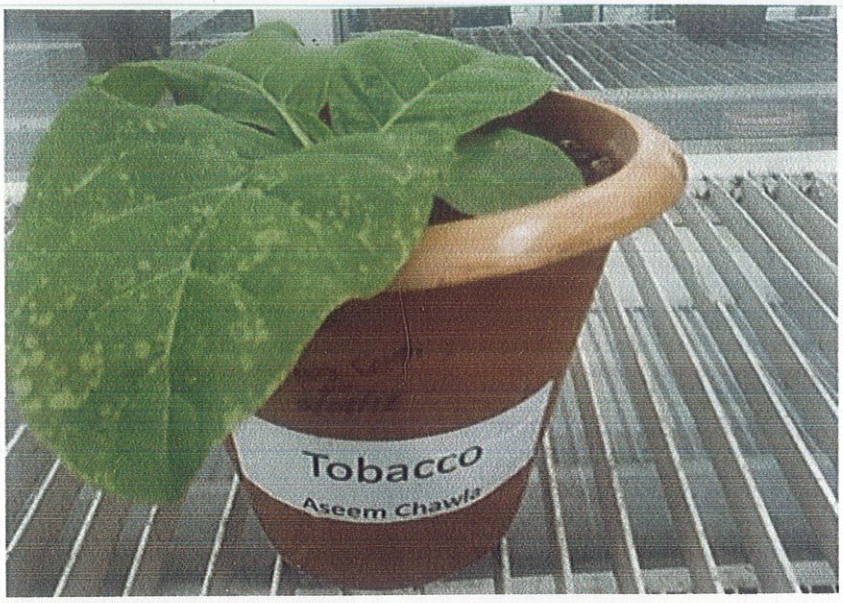


Figure 4.6.2- Injected tobacco plant observed after two weeks. It shows no photo bleaching which would have occurred as a pale uniform patch on the leaf. Non-uniform white spots on the leaf are due to infection.

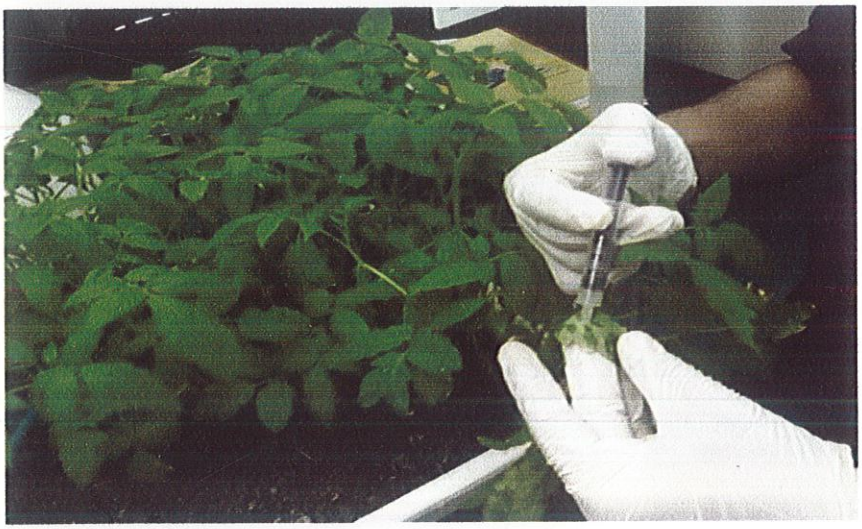


Figure 4.6.3- Infiltration in tomato plants through injection on the lower surface of the leaf.

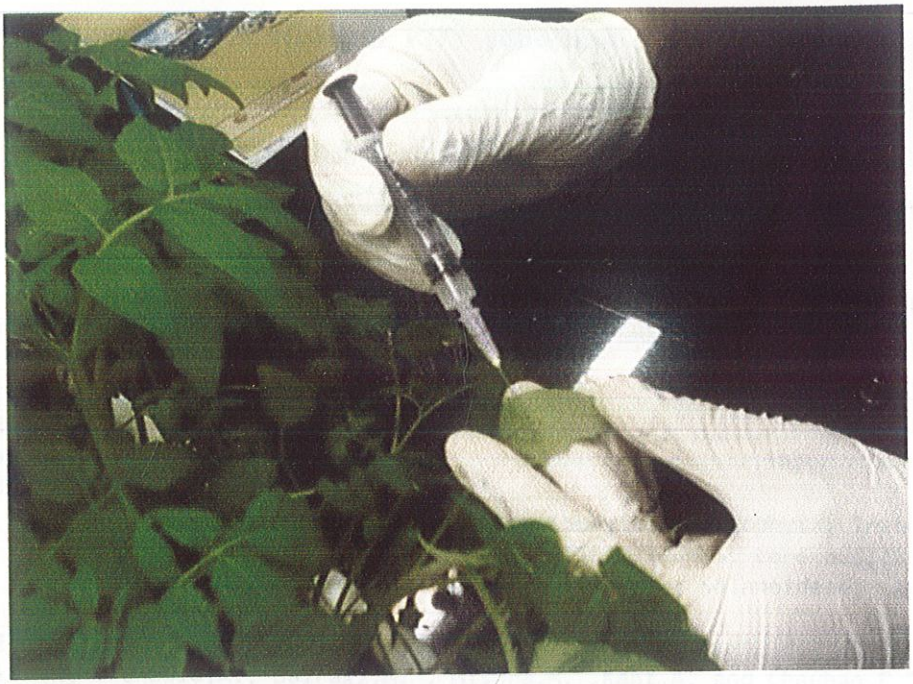


Figure 4.6.4-Before injection, mild injury is made on the lower surface of the leaf for the solution to infilter into the leaf.

Figure 4.6.5- *Capsicum annum* plants observed one week after injection. No photo-bleaching is observed.

4.7 Sequencing of Capsicum PDS gene and submission to NCBI genbank database

The amplified fragment of the Capsicum PDS gene was sent for sequencing. The sequence of the gene fragment was submitted to NCBI gene bank (**Accession No. KC754703**)

```
LOCUS       seq1                    497 bp    mRNA    linear   PLN 08-MAR-2013
DEFINITION  Phytoene desaturase Capsicum annuum mRNA, partial cds.
ACCESSION   seq1
VERSION
KEYWORDS    .
SOURCE      Capsicum annuum
  ORGANISM  Capsicum annuum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            asterids; lamiids; Solanales; Solanaceae; Solanoideae; Capsiceae;
            Capsicum.
REFERENCE   1  (bases 1 to 497)
  AUTHORS   Chawla,A., Bairagi,M. , Mishra,M. ,Kant,A. and Chauhan,R.S.
  TITLE     Capsicum annuum PDS gene
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 497)
  AUTHORS   Chawla,A., Bairagi,M. , Mishra,M. , Kant,A. and Chauhan,R.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (08-MAR-2013) Department of Bioinformatics and
            Biotechnology, Jaypee University of information and Technology,
            P.O. Waknaghat, Waknaghat, Himachal Pradesh 173234, India
COMMENT     Bankit Comment: ALT EMAIL:aseemchawla2004@gmail.com.
            Bankit Comment: TOTAL # OF SEQS:1.

            ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..497
                     /organism="Capsicum annuum"
                     /mol_type="mRNA"
                     /cultivar="California Wonder"
                     /db_xref="taxon:4072"
                     /tissue_type="leaf"
     gene            215..>496
                     /gene="PDS"
     CDS             215..>496
                     /gene="PDS"
```

```
     CDS             215..>496
                     /gene="PDS"
                     /codon_start=1
                     /product="Phytoene Desaturase"
                     /translation="MRNNEMLTWPEKIKFAIGLLPAMVGGQAYVEAQDGLSFKEGMEK
                     QGVPERVTDEVFIAMSKALNFINPDKLSMHCILIALNRFLQEKHGSKMDE"
BASE COUNT      144 a     89 c    129 g    135 t
ORIGIN
        1 gcggtagacg ggaaaagggt gtgttactgg tgtacatatt ttcttcggtg cttaaccgaa
       61 tgtgcagaat tatttggaga acgggggatc aatgatcggt tgcagtggaa ggaacactcc
      121 agattttaag ctatgccaag taaacctgga gaatttagta gatttgactt cccagatgtc
      181 ctaccagcac ccttaactcg ttatttaggc tattttgcgg aacaacgaga tgctgacatg
      241 gccagagaaa ataaagtttg ctattggact tttgccagcc atggtcggcg gtcaggctta
      301 tgttgaggcc caagatggtt tatcattcaa agaagggatg gaaaagcagg gagtacctga
      361 gcgcgtgacc gacgaggtgt ttattgccat gtcaaaggcg ctaaacttta taaaccctga
      421 taaactgtca atgcattgca ttttgatagc tttgaaccgg tttcttcagg aaaaacatgg
      481 ttcaaaaatg gacgaaa
//
```

References

1. Billie Gould and Elena M Kramer;Virus-induced gene silencing as a tool for functional analyses in the emerging model plant *Aquilegia* (columbine, Ranunculaceae), April 2007.

2. Xin-Zhang Cai,Qiu Fang Xu,Chang-Chun Wang,Zhong Zheng; Development of a virus-induced gene-silencing system for functional analysis of the RPS2-dependent resistance signalling pathways in Arabidopsis,2006.

3. Yule Liu, Naomi Nakayama, Michael Schiff, Amy Litt, Vivian F. Irish and S.P. Dinesh Kumar; Virus induced gene silencing of a DEFICIENS ortholog in Nicotiana Benthamiana, April 2004.

4. Chung E, Seong E, Kim YC, Chung EJ, Oh SK, Lee S, Park JM, Joung YH, Choi D: A method of high frequency virus-induced gene silencing in chili pepper (Capsicum annuum L. cv. Bukang),April 2004.

5. Fang-I HO, Yong-Yi CHEN, Yu-Mei LIN, Chiu-Ping CHENG, Jaw- Fen WANG; A tobacco rattle virus-induced gene silencing system for a soil-borne vascular pathogen *Ralstonia solanacearum,* April 14,2009.

6. Jaberolansar N Hayati J, Rajabi-Memari H, Hosseini-Tafreshi SA, Nabati- Ahmadi D; Tomato and Tobacco PhytoeneDenaturase Gene Silencing(VIGS) Technique, 2010.

7. Ariel A. Bazzini, Vanesa C. Mongelli, H. Esteban Hopp, Mariana del Vas, Sebastian Asurmendi; A practical approach to the understanding and teaching of RNA silencing in plants, November 2006.

8. Eunsook Chung, EunsooSeong, Yeong-Cheol Kim, EunJoo Chung, Sang-Keun Oh, Sanghyeob Lee, JeongMee Park, Young HeeJoung, and Doil Choi; A method of high frequency Virus-induced Gene Silencing in Chilli Pepper(Capsicum annuum L. cv. Bukang), February,2004.

PUBLICATIONS

RESARCH ARTICLES – **NA**

CONFERENCE PROCEEDINGS - **NA**

OTHERS

1. **NCBI genbank Sequence submission**

Phytoene desaturase Capsicum annuum mRNA, partial cds

Chawla,A., **Bairagi,M. , Mishra,M.** , Kant,A. and Chauhan,R.S.

Accession No. **KC754703**