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Generation of cloning and silencing construct for transgenic lines of *Nicotiana benthamiana* F-box Kelch gene and biochemical investigation with cucumber mosaic virus

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Abstract
The construct has been prepared for generating the silent lines of the model plant *Nicotiana benthamiana*. F-box Kelch is an important protein that associates with the viral proteins and is an important factor that helps in the establishment of viral infection. A conserved portion of the gene was amplified in sense and antisense orientation and in a sequential manner it was cloned into the silencing vector pSAT4. The construct development was ascertained with the help of restriction digestion and sequence determination. The construct is ready to be transformed into plants. For the biochemical analysis, purified Cucumber mosaic virus (CMV) was inoculated at their 3-4 leaf stage and infection was confined by symptom development. Both infected and healthy plants were subjected to biochemical analysis of the phenolic compounds (total phenols, simple phenols, Ortho-dihydric phenols and bound phenols), alkaloids and total chlorophyll and carotenoids. It was observed that the total phenol content was increased in the infected plant in comparison to the healthy leaves. However, total chlorophyll and carotenoids were reduced in infected plants as compared to healthy leaves. This is expected as virus infection leads to loss of chlorophyll as reflected by mosaic symptoms that the virus produces.

Keywords: Silencing, *Nicotiana benthamiana*, CMV (cucumber mosaic virus), virus-induced gene silencing (VIGS), biochemical analysis

Introduction
Viruses are among the most damaging of plant pathogens and elicit a variety of responses from their hosts. Plant biologists have studied these responses for many years in attempts to engineer crops that are resistant to viruses and thereby decrease yield losses caused by these pathogens. Virus-induced gene silencing (VIGS) is a recently developed gene transcript suppression technique for characterizing the function of plant genes. The approach involves cloning a short sequence of a targeted plant gene into a viral delivery vector. The vector is used to infect a young plant, and in a few weeks natural defense mechanisms of the plant directed at suppressing virus replication also result in specific degradation of mRNAs from the endogenous plant gene that is targeted for silencing.

Nicotiana benthamiana, a close relative of tobacco and species of *Nicotiana* indigenous to Australia, is the most widely used experimental host in plant virology, due mainly to the large number of diverse plant viruses that can successfully infect it [2].

Plants are able to recognize and degrade double-stranded RNA molecules employing the post-transcriptional gene silencing (PTGS) mechanism. PTGS is both a vegetal defense strategy against viral infections and a conserved eukaryotic mechanism for regulate endogenous gene expression. The VIGS methodology (Virus Induced Gene Silencing) uses this mechanism to selectively silence genes employing viral vectors, which contain the target gene, becoming a tool for functional gene validation. The *Geminiviridae* family, with the *Begomovirus, Curtovirus, Mastrevirus* and *Topocuvirus* genera, encompass viruses of circular, single-strand DNA packed in icosahedric geminated particles and some of them had been evolved to infect particular plant species. Viruses are classified according to the genome organization, the type of host plants and the insect vector that transmits the virus. *Gemini viruses* are able to induce gene silencing (GS) and therefore they have been used to develop VIGS–based methodologies for functional gene silencing. RNA interference (RNAi) is a promising gene regulatory approach in functional genomics that has significant impact on crop improvement which permits down-regulation in gene expression with greater precise manner without affecting the

expression of other genes. RNAi mechanism is expedited by small molecules of interfering RNA to suppress a gene of interest effectively. RNAi has also been exploited in plants for resistance against pathogens, insect/pest, nematodes and viruses that cause significant economic losses. The Toll-interleukin-1 receptor/nucleotide binding site/leucine-rich repeat (TIR-NBS-LRR) encoding N gene was introduced into *N. benthamiana* which resulted in the acquirement of hypersensitivity response to tobacco mosaic virus (TMV) [7]. The main focus of the research expressed in this investigation is the inactivation of F-box Kelch gene present in the plant itself through the means of gene silencing (GS) and studying biochemical aspects of model plant used.

Although the main target is to achieve virus resistance in tomato, direct experiments on tomato plant cannot be performed by following the measures of maintaining plant genetic diversity. The model plant *N. benthamiana* is susceptible to a wide variety of plant-pathogenic agents that can be genetically transformed and regenerated with good efficiency and are amenable to facile methods for virus-induced gene silencing or transient protein expression [6].

The conjugated and bound phenols found in genus *Nicotiana* produce antioxidants in intestine in response to bacterial infection [11]. The leaves of genus *Nicotiana* exhibit a structural and functional acclimation of the photosynthetic apparatus to the light intensity helping in their growth and also revealed. that photosynthesis is the basic process during which light energy is absorbed and converted into organic matter in all chloroplast-containing plants. Total Chlorophylls are used as the basis for the calculation of photosynthetic and respiratory rates in genus *Nicotiana* [10].

Materials and Methods

Plant materials

The present investigation was conducted in Biochemistry laboratory, Department of Chemistry and Biochemistry, College of Basic Sciences, CSKHPKV, Palampur in collaboration with CSIR-IHBT (Institute of Himalayan Bio-resource Technology) for molecular characterization of F-box Kelch gene and biochemical evaluation of *Nicotiana benthamiana*.

Growing the plants of *Nicotiana benthamiana*

Seeds of *N. benthamiana* were germinated inside poly-house under controlled temperature of 24 °C in pots having pot size of (5×6×3) cm. The leaf samples were collected from these plants. Wild-type seeds were washed with absolute ethanol for 3 min. Then seeds were rinsed with autoclave water 3 times. Mercuric chloride (0.05%) treatment was given to the seeds for 5 min. Again, seeds were rinsed with autoclave water thrice. Sterilized seeds were transferred to MS media containing flasks and then shifted to growth chamber at a temperature of 22±2 °C and 16 hrs light period and 8 hrs dark periods. Seeds were allowed to germinate till they attained 3-4 leaf stage.

Methods of gene cloning

Total RNA was isolated by using Nucleospin® RNA extraction kit (Macherey Nagel, Hildon Germany). First strand cDNA synthesis was carried out from total RNA with gene-specific reverse primer (F-box Kelch -RP) using Prime Script reverse transcriptase (Takara Bio, Japan). Ligation of F-box Kelch eluted product was firstly carried in pJET Cloning Vector. After ligation was confirmed the reaction mixture was incubated at 4 °C and 16 °C. To transfer the ligated product into bacterial cells, the *E. coli* strain DH5α culture competent cells played their role in transformation. The method of competent cell preparation was adapted from Inoue *et al*. (1990) [4]. Transformation was carried in frozen competent cells of *E. coli* DH5α. For screening of recombinant colonies, the colony PCR was carried out in order to check whether the colony contained plasmid with insert. To execute the restriction digestion of pure plasmid**,** the purity and quantity of plasmid was confirmed with the help of Nanodrop (Thermo Scientific, USA) and the plasmid was digested separately followed by alcohol precipitation with SalI and BamHI restriction enzyme for antisense strand and digestion with HindIII followed by alcohol precipitation with XhoI enzyme for sense strand. The reaction was incubated at 37 °C for 3-4 hrs. This digested product was loaded on 1% agarose gel. Expected band size for insert and vector is ~ 300 bp and ~3.0 kb, respectively. In case of sense stand HindIII restriction digestion was followed by alcohol precipitation, followed by second digestion with XhoI.

Gene Sequencing

Recombinant plasmid of antisense F-box Kelch gene was isolated using NucleoSpin® Plasmid QuickPure kit (Macherey-Nagel, Germany). The sequences of the cloned DNA were obtained by sequencing the recombinant plasmids using M13 forward and M13 reverse primer.

Sequencing PCR was performed using parameters as 95 °C for 10 sec; 40 cycles of 50 °C for 40 sec and 60 °C for 4 min. The sample was washed with montage PCR cleaning kit for removal of surplus dye and other ions (Millipore, USA). Sequencing was done at ABI PRISM™, 3110 Genetic Analyzer.

Biochemical analysis

For biochemical analysis, the fresh leaves were oven dried at a temperature of 40-45 °C for 24 hrs

Extraction

Finely ground dried sample (0.2 g) was taken in a beaker to which 10 ml of 70 percent acetone was added. Extraction was carried out for two hrs in a water bath shaker maintained at 37 °C. The beaker was covered with aluminum foil to avoid evaporation. After extraction the contents were centrifuged at 10,000 rpm for 20 min and the supernatant was used for the estimation of total phenols and tannins.

Total Phenols

Total and simple phenols were estimated in powdered dried sample *Nicotiana benthamiana* leaves by the method of Makkar *et al*. (1993) [9].

Tannins

Total tannins were calculated after subtracting simple phenols from total phenols.

Total Tannins = Total Phenols – Simple Phenols

Ortho-dihydric Phenols

Ortho-dihydric phenols were estimated by method of Mahadevan and Sridhar 1986 [8].

Bound Phenols
Bound phenols were estimated by method of Chattopadhyay and Samaddar, 1980 [1].

Alkaloids: Alkaloids were estimated by Harborne, 1973 [3].

Total Chlorophyll and Carotenoids
Chlorophylls and carotenoids in fresh leaves of *Nicotiana benthamiana* were estimated spectrophotometrically by the method of Jayaraman and Davies (1981) [5].

Results
Characterization of F-box Kelch gene from *Nicotiana benthamiana*
RNA Isolation
The quality of the extracted RNA was confirmed by electrophoresis in 1.5 percent agarose gel. The quantity of extracted RNA from 2 samples (as shown above) was 194 ng/µl and 315 ng/µl obtained from per 100 mg leaves sample and it showed A260/A280 value on average basis 2.1.

First Strand cDNA Synthesis
RNA obtained from *N. benthamiana* plants were converted to first single-strand cDNA by using reverse transcriptase.

Polymerase Chain Reaction (PCR) for Sense Strand Amplification
Amplification of the amplicon was better achieved at 52 °C and therefore it was selected as the optimum annealing temperature (Fig.1)

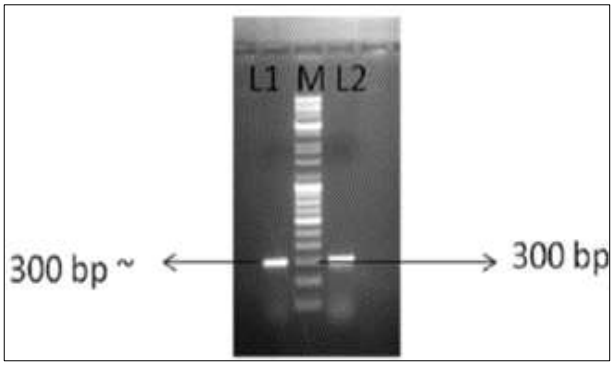

Fig 1: Amplification of sense strand of F-box Kelch gene (Lane1 and Lane 2) (M: 100 bp ladder)

Polymerase Chain Reaction (PCR) for Antisense Strand Amplification
PCR for antisense strand is depicted (Fig. 2)

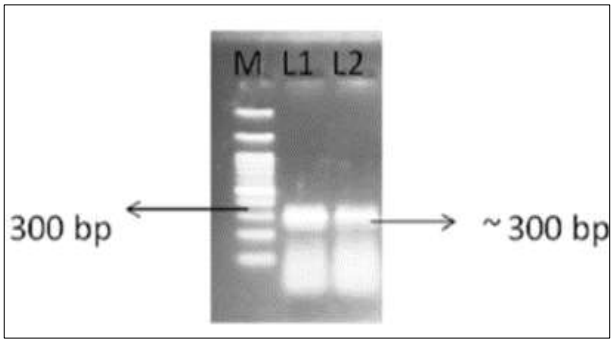

Fig 2: Amplification of antisense strand of F-box Kelch gene (Lane1-2) (M: 100 bp ladder)

Cloning of F-box Kelch Antisense strand in pJET Cloning Vector
The amplified antisense product was eluted from the agarose gel by using NucleoSpin® Gel with PCR Clean-up kit and ligated into pJET Cloning Vector. The ligated product was then transformed into DH5α strain of *E. coli*. Recombinant colonies were further confirmed through colony PCR using gene-specific primers and were checked on 1 percent agarose gel.

Cloning of F-box Kelch Antisense strand in pJET Cloning Vector
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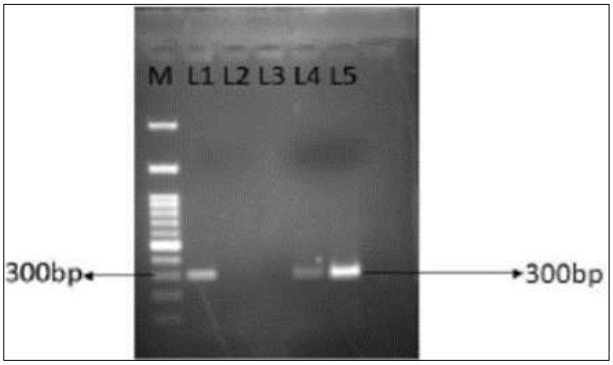

Fig 3: Recombinant colonies were screened through colony PCR using F-box Kelch antisense strand-specific primers in pJET vector (Lane 1-5), M: 100 bp ladder. Lane 1, Lane 4 and Lane 5 recombinant colonies showed positive amplification of expected size 300bp.

The pure plasmid was isolated using Nucleospin ® Plasmid QuickPure kit (Macherey-Nagel, Germany). The recombinant plasmid was digested using *Sal* I and *Bam HI* to confirm cloned gene in vector (Fig.4).

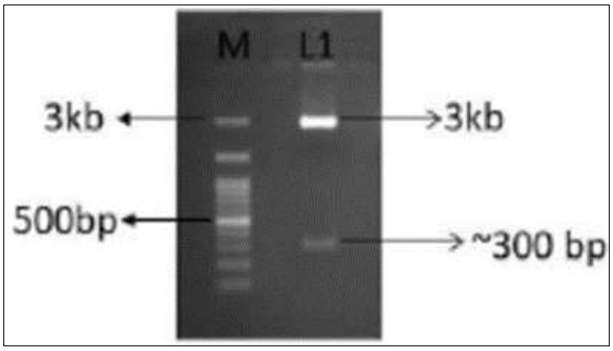

Fig 4: The recombinant plasmid was digested using *Sal* I and *Bam HI* to confirm cloned gene in vector are presented

Sequencing of Antisense Strand in pJET and its Analysis
The cloned DNA was sequenced by using vector-specific primers. The sequence of the insert was analyzed by using NCBI-BLAST. The obtained sequence showed 100 percent sequence similarity with *N. benthamiana* F-box Kelch gene, confirming cloning of the correct gene (Fig. 5).

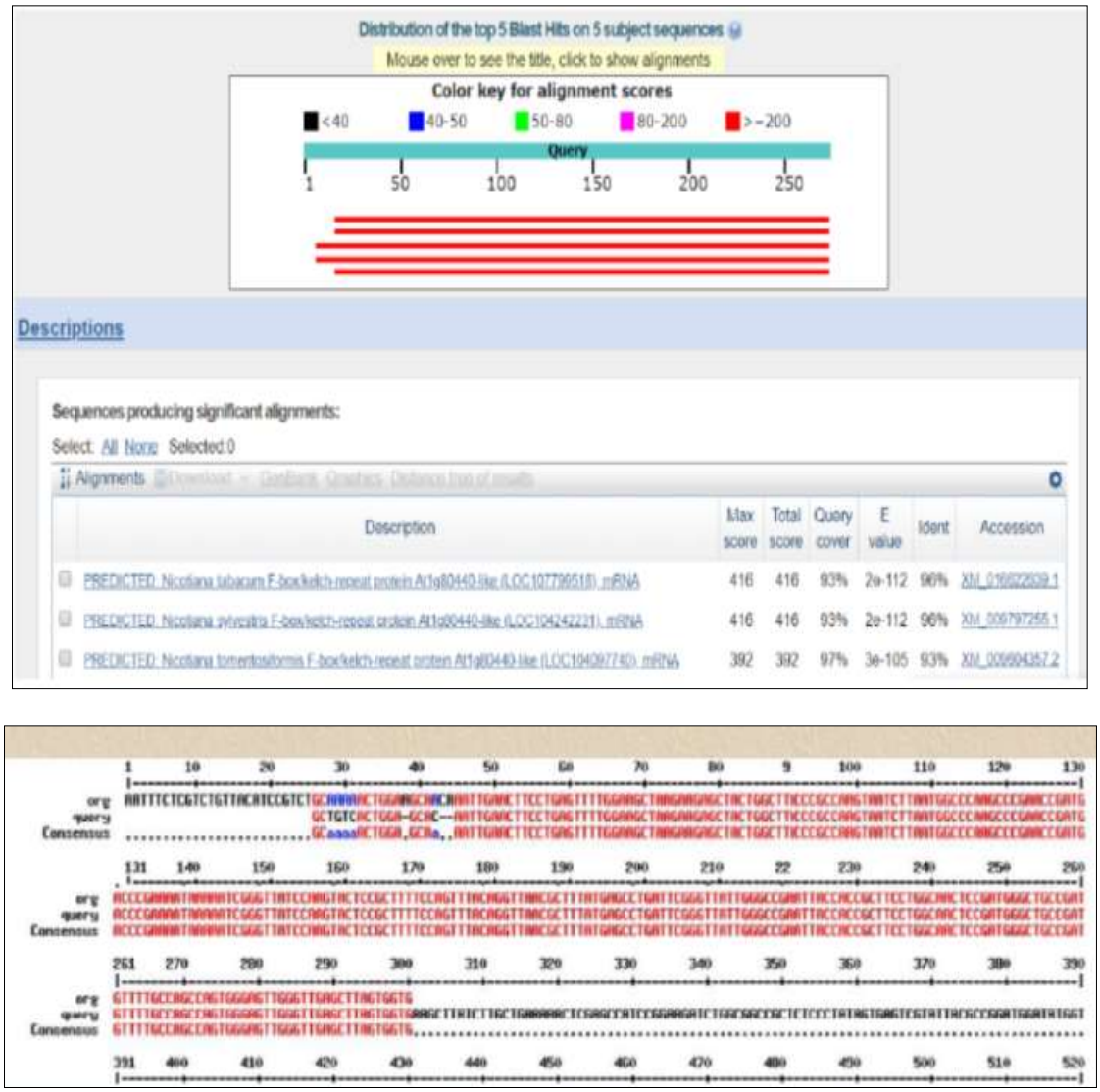

Fig 5: NCBI blast analysis of antisense strand in pJET generated sequence information.

Results showed that gene sequence showed hits (red line) with *N. benthamiana* F-box Kelch gene and its other orthologs. Similar homology was obtained when generated sequence was multi-aligned with *A. thaliana* F-box Kelch. The complete sequence was determined to be 300 bp.

Cloning of F-box Kelch Sense Strand in pJET Cloning Vector

The amplified sense product was eluted from the agarose gel by using NucleoSpin® Gel with PCR Clean-up kit and ligated in pJET Cloning Vector. The colonies were analyzed by restriction digestion of the purified plasmid preparation (Fig. 6).

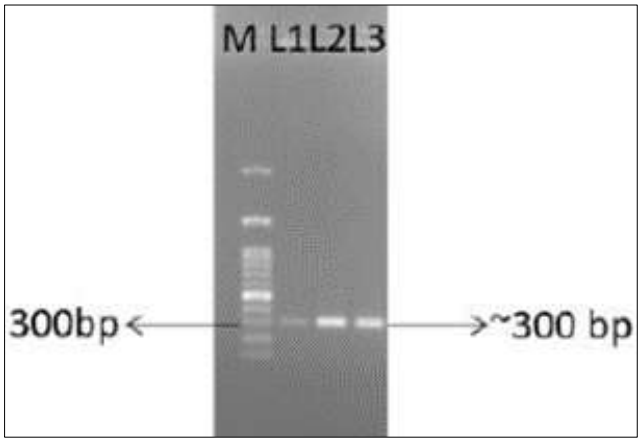

Fig 6: Colony PCR to screen recombinant clones using F-box Kelch sense strand specific primers in pJET vector (Lane 1-3), M: 100 bp ladder. (Lane 1-3) showed positive band of expected size 300bp.

Presence of the insert was confirmed by digestion with XhoI and *Hind* III (Fig. 7).

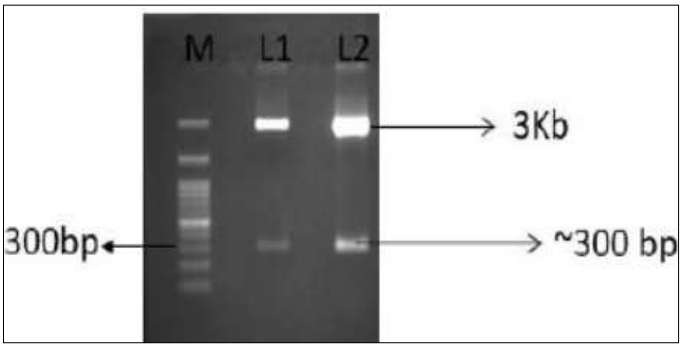

Fig 7: Restriction digestion for analyzing the recombinant clone of F-box Kelch with XhoI and HindIII restriction enzyme (Lane 1-2): fragment corresponding to the expected size of F-box Kelch gene was observed on gel after restriction digestion M: - 100 bp ladder.

Sequencing of Sense Strand in pJET and its Analysis

The cloned DNA was sequenced by using vector specific primers. The sequence of the insert was analyzed by using NCBI-BLAST (Fig. 8). The obtained sequence showed 100 percent sequence similarity with *N. benthamiana* F-box Kelch gene, confirming cloning of the correct gene.

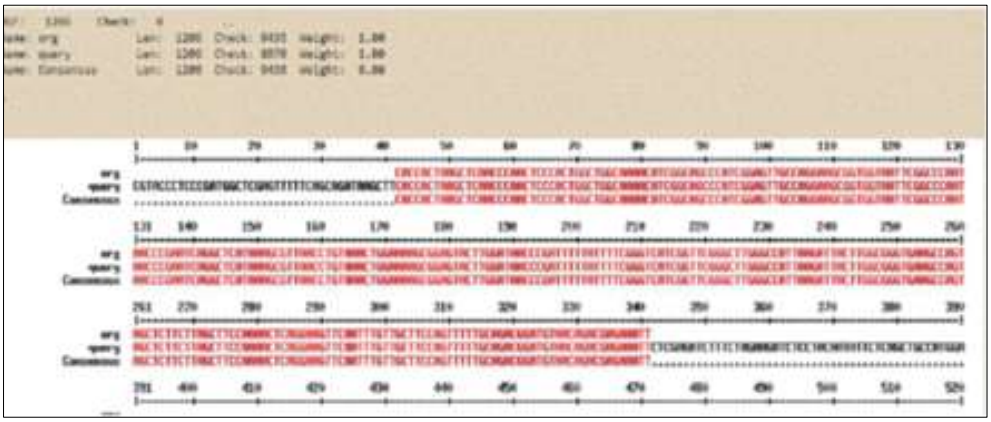

Fig 8: NCBI blast analysis of sense strand in pJET generated sequence information

Cloning of F-box Kelch Antisense Strand in pSAT4CHSRNAi Silencing Vector

The pSAT4CHSRNAi plasmid and recombinant plasmid was isolated by using NucleoSpin® Plasmid QuickPure kit (Macherey-Nagel). The concentration of plasmid was determined by spectrophotometric analysis to be 183.5 ng/µl of pSAT4CHSRNAi plasmid and 531.7 ng/µl of recombinant plasmid (fragment in pJET).

Restriction digestion of pSAT4CHSRNAi

pSAT was sequentially digested with SalI and BamHI restriction enzymes. Initially, it was digested with SalI restriction enzyme and alcohol precipitated.

This was followed by with BamHI digestion and was loaded on 1% agarose gel (Fig.9).

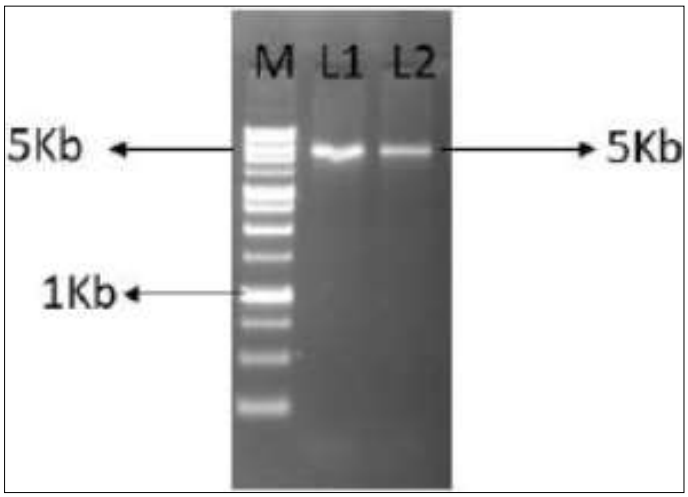

Fig 9: Restriction digestion of pSAT4CHSRNAi with SalI and BamHI restriction enzymes (Lane 1-2): Double digested pSAT4CHSRNAi

Restriction Digestion of Recombinant Plasmid

The recombinant plasmid of F-box Kelch antisense strand already cloned in pJET vector was also digested with SalI and BamHI restriction sites. Initially, it was digested with SalI restriction enzyme and alcohol precipitated. This was followed by with BamHI digestion and was loaded on 1 (%) agarose gel (Fig. 10).

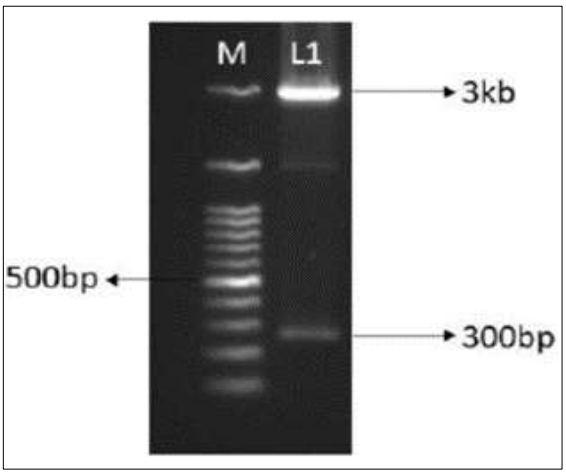

Fig 10: Restriction digestion for analyzing the recombinant clone of F-box Kelch antisense strand cloned in pJET with SalI and BamHI restriction enzymes. (Lane 1) recombinant colonies with desired insert; M: - 100 bp ladder.

Ligation and Transformation

Eluted F-box Kelch antisense product and digested pSAT were ligated and transformed in DH5α strain of *E. coli*. Colony PCR was carried out in order to determine integration of the gene Fig. 11). From the total colonies that appeared, one of the colonies was selected for further analysis.

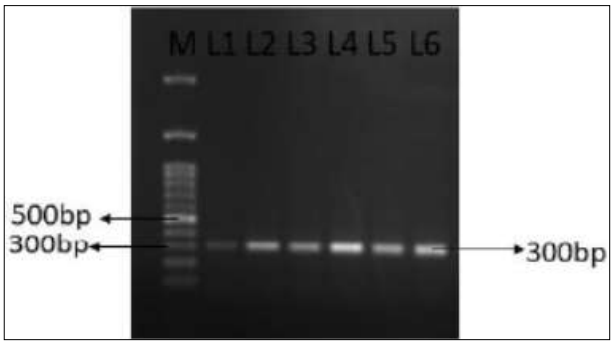

Fig 11: Colony PCR of F-box Kelch antisense cloned in p SATvector. Figure showed that expected gene was found successfully cloned in analyzed colonies (Lane 1-6). M: - 100 bp ladder.

Plasmid Isolation and Restriction Digestion

From the colony PCR, one of the positive colony was selected randomly. It was picked and put into LB containing antibiotic (Ampicillin) for its overnight growth. Plasmid was isolated and quantified spectrophotometrically by Nanodrop. Its concentration was 163.9 ng/µl. To reconfirm the recombinant nature of the plasmid, it was digested with *Sal* I followed by *Bam* HI restriction enzyme and loaded on 1 percent agarose gel (Fig. 12).

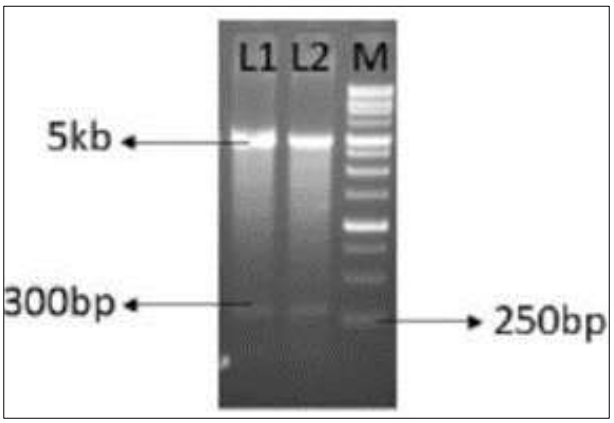

Fig 12: Restriction digestion for confirmation of the recombinant clone (Lanes 1 and 2); M: -1kb ladder

Biochemical Characterization

Phenolic compounds

Total Phenols and Simple Phenols

Table 1 shows the values of total phenols which were having lower value of 4.43±0.88 mg/g in healthy plants of *N. benthamiana* than 7.41±0.57 mg/g in infected *N. benthamiana* plants.

Table 1 shows the values of simple phenols which were having lower value of 4.65±0.05 mg/g in healthy plants of *N. benthamiana* than 5.59±0.07 mg/g in infected *N. benthamiana* plants.

Alkaloids

Table 1 shows the values of alkaloids which were having lower value of 44.0±0.57 mg/g in healthy plants of *N. benthamiana* than 52.0±1.52 mg/g in infected *N. benthamiana* plants.

Total Chlorophyll and Carotenoids

Table 1 shows the values of chlorophyll 'a' which were having higher value of 5.04±1.3 in healthy plants of *N. benthamiana* than 3.72±0.9 mg/g in infected *N. benthamiana* plants. The chlorophyll 'b' having higher value of 1.19±0.9 mg/g in healthy plants of *N. benthamiana* than 1.31±0.8 mg/g in infected plants of *N. benthamiana*.

Table 1 shows the values of carotenoids which were having higher values of 2.90±2.01 µg/g in healthy plants of *N. benthamiana* than 2.09±0.80 µg/g in infected leaves of *N. benthamiana* plant.

Table 1: Phenolic compounds, alkaloids in leaves of *N. benthamiana* (Values on dry weight basis) and Total chlorophyll and carotenoids in leaves of *N. benthamiana* (Values on fresh weight basis)

Nicotiana benthamiana	Total Phenols (mg/g)	Simple phenols (mg/g)	Ortho-dihydric phenols (mg/g)	Bound phenols (mg/g)	Alkaloids (mg/g)	Chl a. (mg/g)	Chl b. (mg/g)	Total Chlorophyll (mg/g)	Carotenoids (µg/g)
Infected	7.41±0.88	5.59±0.07	4.83. ±0.03	4.41±0.06	52.0±1.52	3.72±0.91	1.31±0.80	4.53±0.62	2.09±0.80
Healthy	4.43±0.57	4.65±0.05	1.85±0.02	2.47±0.05	44.0±0.57	5.04±1.30	1.19±0.90	5.84±0.68	2.90±.2.01

Conclusion

Gene amplification and Silencing Construct Preparation

The conserved portion of the gene was amplified and cloned into pSAT vector in sense (S) and antisense (AS) orientation. The fragments were initially mobilized into pJET cloning vector. The pSAT vector with sense portion of the gene was initially prepared from pJET. This was confirmed by restriction analysis and sequencing, for including the AS part into it. The final construct development was ascertained with the help of restriction digestion and sequence determination. The construct is ready to be transformed into plants.

Variation in Different Biochemical Constituents in Leaves of *Nicotiana benthamiana* due to virus infection

Both infected and healthy plants were subjected to biochemical analysis of the phenolic compounds (total phenols, simple phenols, Ortho-dihydric phenols and bound phenols), alkaloids and total chlorophyll and carotenoids. It was observed that the total phenol content was increased in the infected plant in comparison to the healthy leaves. However, total chlorophyll and carotenoids were reduced in infected plants as compared to healthy leaves. This is expected as virus infection leads to loss of chlorophyll as reflected by mosaic symptoms that the virus produces.

Acknowledgement

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