Original Research Article

Comparison of post-transcriptional gene silencing (PTGS) strategies for developing transgenic plants resistant to Tomato leaf curl virus (ToLCV)

Abstract: Tomato (*Solanum lycopersicum* L.), an economically important crop is host to many whitefly transmitted geminiviruses including tomato leaf curl virus (ToLCV). Genetically engineering resistance of pathogen through Post-transcriptional gene silencing (PTGS/RNAi) is a powerful strategy that can provide alternative to existing methods of producing virus resistant plants. We cloned and characterized ToLCV-replicase (TRP) gene from a local Dharwad, Karnataka, India ToLCV isolate for development of transgenic tomato plants. Plant expression vectors carrying viral replicase (rep) gene in sense, antisense, ihp (intron spaced hairpin) and HUTR (inverted repeats of heterologous 3′-untranslated region) were constructed. Transgenic tomato plants carrying rep gene in different strategies when challenged with whiteflies carrying ToLCV showed varied degrees of resistance. Such plants were confirmed through PCR, GUS, Dot blot, Southern blot and semiquantitative PCR analysis. High degree of resistance was observed in the construct carrying both sense and antisense strand interrupted by intron (ihp). Our results demonstrate that, transgenic plants with simultaneous expression of sense and antisense strands are more efficient in gene silencing of ToLCV than those expressing either sense or antisense strand alone.

Keywords: Tomato leaf curl virus; Replicase; intron spaced hairpin RNA; silencing by heterologous 3’-UTR; Post-transcriptional gene silencing; Gemini virus

UNDER PEER REVIEW

Introduction

Tomato (*Solanum lycopersicum* L.) is an economically important vegetable crop in many countries and its cultivation is beset with problems of whitefly transmitted viral diseases such as tomato yellow leaf curl virus (TYLCV) and tomato leaf curl virus (ToLCV). Both these viruses belong to geminivirus group and can cause 100 percent yield losses under severe conditions, making tomato cultivation unprofitable (Nakhla and

Maxwell, 1998). Laboratories, worldwide have been trying to produce virus resistant tomatoes but achieving only limited success. However, the first TLCV field- tolerant cultivars developed through antisense technology showed mild or delayed symptoms (Day *et al*., 1991).

The use of gene encoding viral coat protein (CP) to develop viral resistant plants was first demonstrated in tobacco against tobacco mosaic virus (TMV) by Powell *et al*., (1986) is one of the pioneered successes achieved in plant biotechnology. Following this, several important crop plants have been engineering of viral resistance using this approach. Coat protein mediated resistance for geminiviruses achieved when transgenic tomato plants carrying TYLCV capsid protein showed resistance against TYLCV (Kunik *et al*., 1994). Other viral genes are also the target of such studies, Replicase is one of the important protein encoded by the viral genome which plays a major role in replication of virus by acting as the transcriptional regulator, as stimulator of viral transcription through recruitment of post translational modification machinery and also as suppressor of gene silencing (Ruhel and Chakraborty, 2019). In another study, Walsh et al. (2019) used transgene derived RNA hairpin, homologous to overlapping region of the South African cassava mosaic virus (SACMV) replication associated protein and virus suppressor of silencing proteins (AC1 and AC4) to develop resistance against SACMV in a susceptible cultivar.

Faiz and Abhinav (2021) have developed transgenic *Nicotiana benthamiana* plants resistant to cotton leaf curl virus (CLCuV) by using antisense AC4 construct and the transgenic lines showed no symptoms upon inoculation with viruliferous whiteflies. Antisense construct targetting βC1 gene of CLCuV was developed and transgenic *N. benthamiana* lines were raised, followed which no visible symptoms were observed upon inoculation with viruliferous whiteflies (Kumar *et al*., 2020). Though different PTGS processes have been described in many systems it has been proven that a common triggering factor for all these related phenomena is the double stranded RNA. This dsRNA in turn gets converted into siRNA (small interfering RNA) molecules that in turn get associated with RISC (RNA induced silencing complex) and bring about the degradation of the target mRNA. Several strategies and techniques have been developed to induce the formation of dsRNA molecules in vitro against target mRNA sequences

UNDER PEER REVIEW

(Agrawal *et al*., 2003). The hpRNA constructs are effectively used to get silenced plant for every gene that targeted, irrespective of whether it was a viral gene, transgene or endogenous gene and the silencing appears to be uniform within tissue in which the hpRNA is expressed (Wesley *et al*., 2001). The silencing was much more profound with ihp constructs than either sense or antisense constructs (Wagner *et al*., 2005)

With this background an effort has been made here to demonstrate the efficiency of different PTGS strategies in conferring resistance against ToLCV. We report here the development and validation of constructs for different strategies of PTGS against ToLCV. We sought to test whether the introduction of gene constructs that produced mRNA transcript capable of forming a duplex would be more or less effective at generating PTGS than constructs producing either sense or antisense strand of the target gene.

Materials and methods

Virus isolate

Tomato plants infected with ToLCV were collected from Dharwad, Karnataka, INDIA. The native viral culture was maintained in greenhouse tomato plant by serial transmission with whiteflies (*Bemesia tabaci*). Total DNA from virus infected leaf samples was extracted by CTAB method as described in Sambrook *et al*. (1989).

Construction of gene silencing vectors

Specific primers for ToLCV-*rep* gene were designed from the available sequence data (Dry *et al*., 1993; NCBI Acc No. S53251) and the complete *rep* coding region was PCR amplified by forward (5' ACGCACGGCAAATCAATTCTCTTC 3') and the reverse primers (5'ACCCCAGACACCGATTCATTTACC 3'). The amplicon was cloned into pTZ57R/T vector (Qiagen, Germany) as per manufacturer's instructions. *E. coli* DH5α cells were transformed with the ligation mix and recombinants were confirmed by restriction analysis of the isolated plasmid DNA.

Standard gene cloning methods (Sambrook *et al*., 1989) were used to make the constructs. The generic vectors derived from pRT100 (Reinhard *et al*., 1987; Ashfaq *et al*., 2007) were used for developing generic vector constructs. Unique restriction endonuclease sites at 5' (*Xho*I, *Apa*I, *Nco*I) and 3' (*Kpn*I, *Sma*I, *Bam*HI, *Xba*I) of the generic ihp vector provided multiple options during the cloning of targeted gene

UNDER PEER REVIEW

segments. Gene segments were PCR amplified by using primers that contain these unique restriction sites and cloned directly into generic vector. For the sense construct, the fragment was amplified from a clone carrying *rep* gene using PCR primers Rp1 (5'CG**GGATCC**ACGCACGGCAAATCAATTCTCTTC3') and Rp2 (5' CG**GGTACC**ACCCCAGACACCGATTCATTTACC 3') and cloned into *Bam*HI-*Kpn*I site. For the Antisense construct, the fragment was amplified using PCR primers Rp3 and Rp4 (Rp3: 5' CG**CCATGG**ACGCACGGCAAATCAATTCTCTTC3'; Rp4: 5' CG**CTCGAG**ACCCCAGACACCGATTCATTTACC 3') and cloned into *Nco*I-*Xho*I site. To generate ihp vector, the fragment was amplified using PCR primers Rp1-Rp2 and Rp3-Rp4 and were cloned in both the direction spaced by a functional intron at *Bam*HI-*Kpn*I and *Nco*I-*Xho*I site, respectively. While for SHUTR construct, the fragment was amplified using PCR primers Rp3 and Rp4 and cloned only in antisense direction at *Nco*I-*Xho*I site. The entire gene cassettes of sense, antisense, ihp and HUTR were exuded from the vector using *Hind*III digestion and cloned into the T-DNA of a binary vector pCAMBIA1305.1 next to an expression cassette for the *hpt*II selectable marker gene. The binary vector was introduced into *Agroabcterium* strain LBA4404 by triparental mating, with pRK2013 as helper plasmid.

Plant transformation

Tomato cultivar Pusa Ruby was transformed and regenerated into whole plants essentially as described by McCormick *et al*. (1991). Healthy seeds of Pusa Ruby were surface sterilized and germinated on half strength MS medium (Murashige and Skoog *et al*., 1962). Cotyledonary segments of 15 days old seedlings were pre-cultured on MS medium for two days, floated for 30min on overnight grown *Agrobacterium* culture supplemented with 200 μM acetosyringone with gentle shaking in dark. The blot dried explants were returned to the same plates used for pre-culturing and incubated in dark for two days at 25+/-2℃. After cocultivation, the cotyledonary leaf segments were transferred (placed upside-down) on to selection medium (MS containing Gamborg's B5 vitamins, 50 mg/L ascorbic acid, 2 mg/L Zeatin, 0.1 mg/L IAA, 7.5 mg/L hygromycin and 500 mg/L cefotaxime). The cultures were incubated for regeneration at 25℃ and 16h/8h (light/dark) photoperiod in a culture room. Sub-culturing was done at 3-4 weeks intervals and explants showing shoot regeneration were transferred to the same medium

UNDER PEER REVIEW

with different growth regulator combination (0.1 mg/L Zeatin and 0.1 mg/L IAA) for shoot development. The shoots were rooted on MS medium containing Gamborg's B5 vitamins, 0.05 mg/L IBA and 3.5 mg/L hygromicin. After hardening for 8-10 days in thumb pots with sterile peat, the plants were shifted to green house for establishment.

Viral inoculation

The T_0-plants along with control plants were challenged with whiteflies carrying ToLCV (2-4 leaf stage) and were observed for degree of resistance/susceptibility. The phenotypically superior T_0-plants were selfed to get T_1 seeds and similar inoculation study was done in T_1 plants and data was recorded for further analysis.

Polymerase chain reaction (PCR), Dot and Southern blot analyses

PCR analysis was done to select the putative transgenic plants of both T_0 and T_1 for further analysis. Since transgenics were challenged with virus particles initially at 2-4 leaf stage, PCR analysis was done using *hpt*II gene specific primers instead of gene specific primers to avoid false positive results from infected virus particles. DNA isolated from transgenic plants from each construct was tested for the presence of insert by PCR using *hpt*II specific primers (FP-5' CGACCTGATGCAGCTCTCGGAGGGC3'; RP-5' CGATTGCGTCGCATCGACCCTGCGC 3') in a 20 µl PCR reaction containing 1U of *Taq* DNA polymerase, 2mM dNTP mix, 5 pmoles of each primer, 1x *Taq* assay buffer. Vector DNA and non-transgenic plant DNA were used as positive and negative controls, respectively.

DNA from PCR positive T_0 and T_1-plants (pre-challenged with virus) was subjected to Dot blot analysis to check for the presence of insert. In order to avoid false positive signal from infected viral particles *hpt*II specific probes were used instead of gene specific probes. A digoxigenin labelled probe was prepared from PCR amplified fragment of *hpt*II using random priming method. About 1µg of purified DNA from transgenic and non-transgenic plants was spotted on positively charged Nylon membrane and fixed by UV cross-linking for 5 min. Prehybridization and hybridization with labelled *hpt*II probe was done at high stringency and immunological detection was completed as prescribed by the DIG-DNA labelling and detection kit (Roche Diagnostics, Germany).

UNDER PEER REVIEW

Dot blot positive plants of T_0 and T_1-plants were subjected to southern blot analysis as described by Southern (1979) with some modifications to check the copy number insertion. About 10 µg of DNA from each plant was digested with *Eco*RI, size fractioned on 0.8% agarose gel, and transferred to positively charged nylon membrane (Nytron) following the manufacturer's instructions (New England Biolabs, UK). The blots were hybridized to DIG labelled *hpt*II DNA probe and washed to stringency of 0.5x SSC, 0.1% SDS at 68℃. Probe preparation, pre-hybridization and hybridization at high stringency and immunological detection were completed as above.

GUS histochemical assay

GUS histochemical assay for T_1-plants was performed as described by Jefferson *et al*. (1987) with some modifications. The leaves from PCR positive plants were cut radially, washed for 30-60 min with several changes of ice-cold distilled water and transferred and held in incubation medium in dark at 37℃ until the appearance of distinct staining. The tissue was held in the distilled water with a couple of changes and incubated with absolute alcohol for several hours until all the chlorophyll content was removed. The ethanol was then removed and the tissue was mounted in 100% glycerol on a slide and observed under the microscope.

Semi quantitative PCR analysis

To quantify viral titre in the T_1-transgenic plants, hundred nanograms of DNA from transgenic lines from each construct was used as a template in a 20 µl PCR reaction containing 1U of *Taq* DNA polymerase, 2 mM dNTP mix, 5 pmoles of each primer and 1x *Taq* assay buffer. For the PCR reaction, the following conditions were used with coat protein gene specific primers (CpF: 5' AGGGATTGCATTGGGGTTGTTAG 3'; CpR: 5' GCGATACACAAATGCTTCCTGGAC 3'): 94℃ for 5 min, 10 to 40 cycles of 55℃ for 1min, 72℃ for 2 min extension, final extension of 72℃ for 10 min. DNA from ToLCV infected control plants was used as positive control. The PCR reaction was performed for different number of cycles (10, 20, 30 and 40) and the amplicons were separated on 1% agarose gel for quantification.

For relative quantification of viral titre, densitometric analysis of amplicons obtained was done using AlphaEase FC software (Alpha innotech corporations, USA). DNA from infected control plants that exhibited clear symptoms of disease was used as a

UNDER PEER REVIEW

control. Densitometry values obtained from transgenic plants at different PCR cycles were subjected to background correction (as per manufacturer's instruction) and percent silencing was calculated in comparison to the control and values were plotted on a graph.

Results

Cloning of *rep* gene and construction of PTGS vectors ToLCV-*rep* amplicon (~1.1kb) obtained using specific primers from ToLCV infected tomato DNA was eluted from the gel and cloned into pTZ57R/T and transformed into competent *E. coli* DH5α cells. The cloning was confirmed using restriction analysis and sequencing (Fig.1a-d). This gene was cloned in different generic PTGS vectors to develop gene cassettes that were designed to silencing the replicase gene of ToLCV through different silencing modes such as co-suppression, homology dependent silencing, antisense suppression, intron-hairpin RNA and silencing by heterologous 3'-Untranslated region (SHUTR). The cassettes of all these clones were confirmed using restriction analysis and sequencing and were sub-coned within T-DNA region of binary vector at *Hind*III site. The developed gene cassettes are schematically represented in Figure 2a-d. The binary vectors carrying the respective expression cassettes were mobilized into *Agrobacterium* strain LBA 4404 by triparental mating.

Development of transgenic plants

Seven-day-old pre-cultured cotyledonary explants of Pusa Ruby were infected with *Agrobacterium* carrying different gene constructs and co-cultivated on MS medium. The treated explants produced multiple shoots within 4 weeks. Surviving green shoots, having well developed root system were transferred to sterile peat and shifted to green house. The results of survival of explants following co-cultivation at various stages are summarized in Table 1.

About 42% of the explants survived on selection medium after co-cultivation. Direct shoot initiation rather than the callus was frequently observed at the proximal end of the explants. About 43.3% of the selected explants showed multiple shoot induction. Healthy shoots were subsequently transformed to fresh selection medium for shoot elongation. During shoot elongation about 29% of the shoots showed shoot elongation. The elongated shoots were cultured in rooting medium. Root development from

UNDER PEER REVIEW

elongated shoots was achieved with reduced level (5mg/l) of hygromycin. The frequency of 39.7% of elongated shoots showed rooting. Finally, 44.3% of the rooted plantlets survived and established in greenhouse.

Virus inoculation

The T1-transgenic plants showed varied tolerance against ToLCV infection as compared to untransformed control plants (Fig.3a-c). Virus inoculation results indicated that, 48.0% from Sense 33.8% from Antisense, 22.0% of the plants from ihp and 28.3% of the plants from SHUTR showed early symptoms after 2-4 weeks of inoculation (WI) as against 70% of the control plants. Similarly, 35.7% from Sense, 41.8% from Antisense, 36.5% from ihp and 36.3% from SHUTR showed delayed symptoms at 4-6 WI. With all the control plants showing symptoms, 17.3% from Sense, 24.4% from Antisense, 41.5% from ihp and 35.4% from SHUTR did not show any visual symptoms even after 6 WI (Fig. 4). The degree of symptoms expressed by plantlets at various stage post inoculation is summarized in Table 2.

Transgene analyses

PCR analysis of putative transgenic plants using *hpt*II specific primers gave an expected PCR amplicon of ~800 bp, but no such amplicon was observed in ntransformed plants (Fig. 5a-d). Plants obtained from Sense, Antisense, ihp and SHUTR showed positive results for PCR analysis.

PCR positive transformed plants from Sense, Antisense, ihp and SHUTR, were positive for dot blot analysis (Fig. 5e). All the dot blot positive plants showed positive for southern analysis but no such signal was seen in negative plants (Fig. 5f-i). For southern analyses, *Eco*RI enzyme was chosen for digestion as the binary vector had a unique *Eco*RI site within the T-DNA region and the copy number of the transgene could be estimated by scoring the number of bands on the DNA gel blots. All bands were scored irrespective of size and intensity of hybridization. Majority of T0-transgenic plants had multiple copy insertions and showed varied integration patterns among the constructs tested. Similarly, the results were further confirmed in the T1 generation and showed presence of single or double copy insertions (data not shown).

The transgenic plants of Sense, Antisense, ihp and SHUTR were GUS positive, but no GUS activity was observed in control untransformed plants (Fig. 6a-e). Varied

UNDER PEER REVIEW

intensity of histochemical staining was observed among the transgenics obtained from different constructs indicating varied level of expression.

Viral titre analyses

Semi quantitative PCR was carried out to assess the quantum of virus present in each of the transgenic plants post inoculation. In the transgenic plants derived from different constructs, drastic reduction in the virus inoculum was observed as compared to control plants. Densitometric analysis revealed degree of gene silencing of ToLCV. Silencing of ToLCV ranging from 47-75% in Sense, 37-100% in Antisense, 66-100% in ihp and 65-91% in SHUTR was observed (Fig. 6f-i). Among different constructs tested, one plant from Sense and two plants from ihp were completely free of virus inoculum as indicated by the densitometric values (Table. 3, Fig. 7). The transgenic plants obtained by ihp construct showed significant silencing of ToLCV compared to other constructs tested. On an average ihp construct showed 82.75 % silencing followed by 73 % in Antisense, 67.4 % in SHUTR and 64 % in Sense.

Discussion

The present study was undertaken to assess the efficiency of four approaches of PTGS viz., co-suppression, antisense, intron-haiprin and silencing by heterologous 3'-UTR in effectively controlling the ToLCV disease in tomato using the transgenic approach. Transgenic constructs engineered to produce dsRNA as opposed to single stranded sense or antisense RNA are known to cause higher level of silencing (Tougou *et al*.,2006). Vector constructs containing an inverted repeat of the 3'-UTR (SHUTR) have been shown to operate effectively in *Arabidopsis* and tomato (Brummel *et al*., 2003), suggesting utility of this approach in silencing studies. In this study constructs were developed to suit different gene silencing strategies using ToLCV replicase gene, which is an important component in viral multiplication.

To explore the utility of available virus silencing strategies, *rep* gene was cloned into suitable vectors to produce four different silencing constructs that could effectively silence the TRP gene using different PTGS approaches. The regeneration protocol using cotyledonary explants of Pusa Ruby gave high frequency of transformants under

UNDER PEER REVIEW

hygromycin selection pressure. These results were in conformity with the results obtained by Raj *et al.* (2005) in transgenic tomato expressing the ToLCV-coat protein gene through *Agrobacterium* mediated transformation. Gogoi *et al*. (2019) found that transgenic tobacco plant expressing sense and antisense orientation of CP gene of Sri Lankan cassava mosaic virus (SLCMV) showed resistance to SLCMV.

T_1-transgenic plants obtained from each strategy showed variable degrees of resistance when challenged with whiteflies carrying virus particles. Among the infected plants, some were absolutely free of infection even after 6 weeks of infection. Transgenic plants developed to produce ihp RNA against replicase showed high degree of resistance to ToLCV followed by SHUTR, Antisense and Sense. Guo *et al.* (2015) developed the transgenic sugarcane expressing CP gene of Sorghum mosaic virus (SrMV) based on the RNA silencing approach. It was reported that transgenic line expressing the RNA cassettes showed resistance against SrMV upon artificial inoculation. Similarly, tobacco plants transformed with hpRNA containing CP gene of TSV through the Agrobacterium, mediated transformation exhibited resistance to TSV upon mechanical inoculation (Rajamanickam *et al*., 2015). Recently, genetically modified tomato plants resistant to the potato spindle tuber viroid (PSTVd) were developed by encoding hpRNA from PSTVd sequences (Nora S *et al.,* 2009).

The transgene integration and expression in plant genome was confirmed through PCR and histochemical assay respectively, showed varied intensity of GUS activity among different transformants, as shown by Elseu *et al.* (1994) and Eapen and George (1994). Among the PCR positive plants tested the frequency of GUS positive shoots varied with different constructs (data not shown). Expression of GUS activity in the regenerated shoots was not directly correlated with hygromycin resistance. The copy number and location of insertion and subsequent rearrangement can significantly affect expression level of the gene (Battraw and Hall, 1990).

Majority of the T0-trangenic plants subjected to Southern blot gave multiple bands in each case when genomic DNA was digested with *EcoR*I that had single site within T-DNA region. The results indicate the multiples of the transgene in the genome. Similarly, Southern blot analysis of selected T1-transgenic plants gave positive results with either single or two copy insertions (data not shown). Transformants with the same

UNDER PEER REVIEW

TRP transgene integration pattern did not necessarily display the same phenotype upon inoculation as reported earlier (Aida *et al.,* 2000; Ingelbrecht *et al.,* 1999). In fact, all the four constructs had both resistant and susceptible phenotypes upon inoculation and a few of them were immune to ToLCV. It has been previously reported that isogenic transgenic lines can display different levels of resistance upon virus inoculation in dicots (Sijen *et al.,* 1996). An artificial micro-RNA (amiRNA) method was recently employed to repress 4 members of the Polyphenol oxidase gene family, either separately or in group, as a result, potatoes with few DNA inserts are produced that are low in browning (Chi *et al*., 2014).

Plants with highly related transgene integration pattern, most likely, originated from the same transformation event. Semi quantitative PCR analysis carried out with viral coat protein gene specific primers showed varied levels of gene silencing among transformants developed with different strategies of silencing. Densitometric results indicated a drastic reduction in the virus inoculum among the transgenic plants obtained from each construct. Among different strategies tested for transgenic resistance, the transgenic plants developed by ihp construct showed significant resistance against ToLCV followed by SHUTR, Antisense and Sense. Transgenic plants obtained by Sense construct yielded least percentage of resistance compared to the other constructs. Thus, constructs that led to production of double stranded RNA molecules against replicase were more effective than those produced single stranded RNA molecules, either sense or antisense. Such observations were obtained by earlier studies when RNAi constructs carrying P7, a key structural protein of rice dwarf virus was used to induce post-transcriptional gene silencing of virus by Tenllado *et al.* (2004) and Zhong *et al.*(2005). Silencing through the antisense construct of replicase gene also showed quite a high degree (25%) of silencing of ToLCV. Such results have also demonstrated by Praveen *et al.* (2006), using replicase (*rep*) gene sequence of ToLCV. Senthilraja et al. (2018) investigated the development of transgenic peanuts by expressing the hpRNA cassettes containing CP gene of TSV through Agrobacterium-mediated transformation. Chen *et al.* (2019) found that chimeric hpRNA comprising CP genes of Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV) expressed in tobacco showed

UNDER PEER REVIEW

normal growth and produced no symptoms when inoculated with CymMV and ORSV as mixed inoculum.

Though, the highest level of silencing of *rep* gene was seen with the construct that produced ihp RNA against target gene. Transgenic plants with an inverted repeat of 3'-UTR also showed significant level of resistance as compared to transgenes that produced either sense or antisense transcripts of *rep* gene. Since in the SHUTR strategy the construct hairpin structure formed sense and antisense from the 3’-UTR system provides priming to produce dsRNA by plant RdRP activity and is likely to lead to better silencing than the conventional antisense approaches. Similar kind of results have been reported by Singh *et al.,* (2020) the hairpin RNA construct was utilised to clone the target ODC gene fragment, which was then used to create transgenic tomatoes. Small interfering RNAs were produced by the RNAi transgene lines, which showed medium to high resistance to fusarium wilt in transgenic tomatoes. Previous studies of transgene induced PTGS in plants have suggested that gene silencing is initiated by the 3' region of the target gene (Dalakouras *et al*., 2020).

Thus, our results have demonstrate that the construct with simultaneous expression of sense and antisense strands are more efficient in gene silencing than those expressing either sense or antisense strand alone. The results obtained from different transgenic plants showed variation in the degree of silencing of ToLCV, within and among the constructs tested. These results suggest that gene specific PTGS against ToLCV was induced when ToLCV-*rep* gene was targeted. Similar kind of results were previously demonstrated by Ammara *et al*. (2015) the hpRNAi strategy as a means to control the TYLCV complex in Oman by targeting four regions of the TYLCVOM genome.

UNDER PEER REVIEW

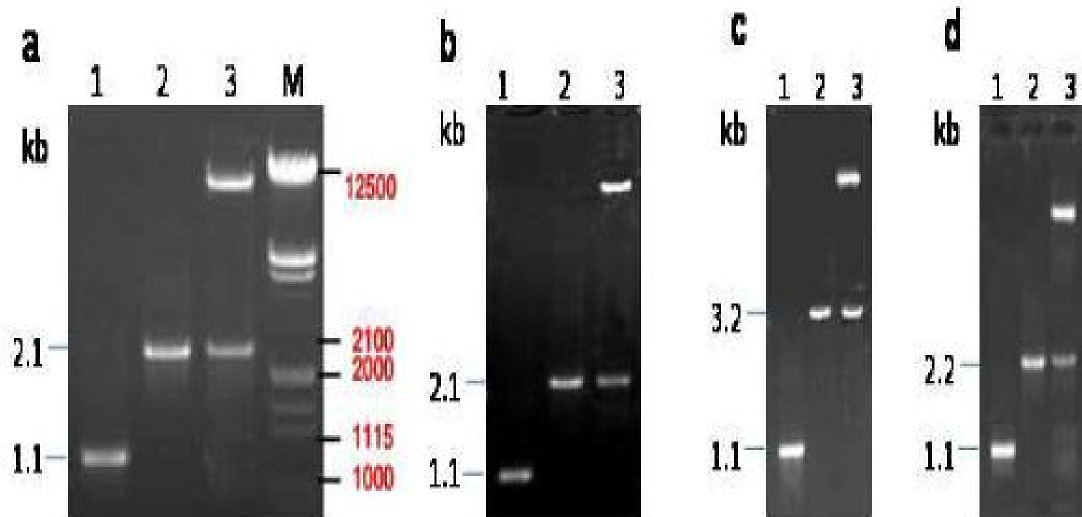

Fig. 1 Restriction digestion of PTGS vectors with HindIII. (**M**) ë *Eco*RI+ *Hind*III digest Marker. Restriction digestion of PTGS vector containing *rep* gene in sense (**a**), antisense (**b**), ihp (**c**) and SHUTR (**d**) approach; PCR product of *rep* gene(a1,b1,c1,d1), *rep* gene containing cassette (positive control) (a2,b2,c2,d2), *rep* cassette containing clone (a3,b3,c3,d3). Fig1a and b shows release of 2.1kb sense and antisense cassette, Fig1c shows release of 3.2kb ihp cassette; Fig1d shows release of 2.2 kb of SHUTR cassette and 1.1 kb of rep amplicon in Fig1a-d.

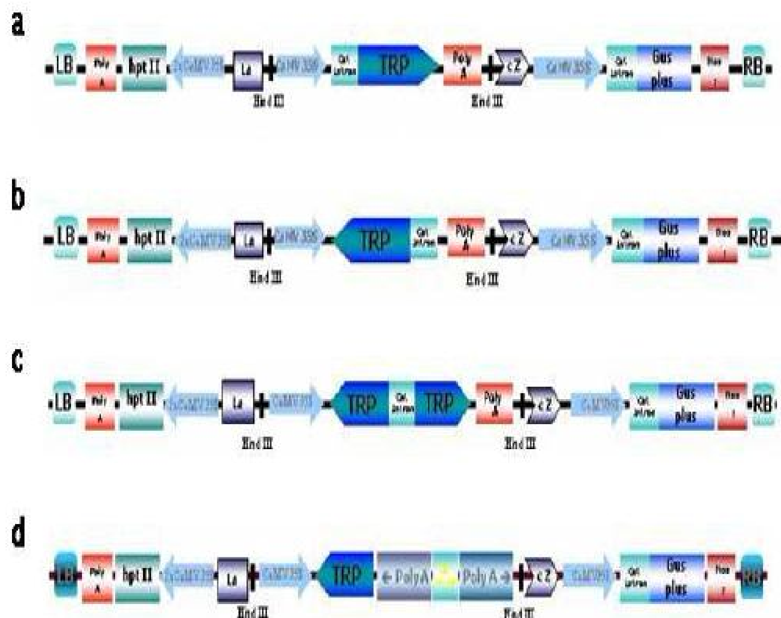

Fig. 2 Construct maps of PTGS showing different approaches. Construct carrying *rep* gene in

sense (**a**), antisense (**b**), ihp (**c**) and SHUTR (**d**) approaches. LB: left border, RB: right border, PolyA: 3' UTR, *hpt*II: hygromycin resistant gene, 2xCaMV35S: 35S double promoter, Cat: catalase intron, *Lac*Z: reporter gene used for selection of recombinants by insertional inactivation, GUS plus: GUS reporter gene, TRP: replicase gene inserted in different orientation.

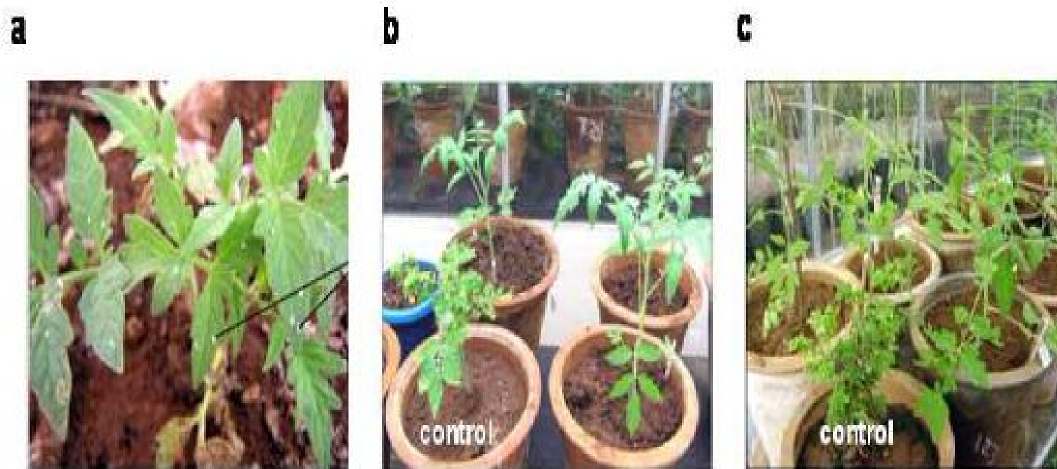

Fig. 3 Transgenic tomato plants showing resistance to virus infection. Transgenic plants challenging with whiteflies carrying virus particles (a), Transgenic plants showing tolerance to ToLCV after 4 weeks of inoculation (b) and after 6 weeks of inoculation(c).

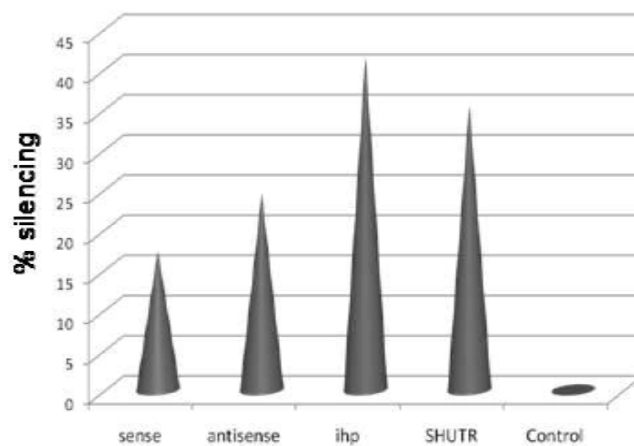

Fig. 4 Percent silencing of ToLCV after 6 weeks of virus inoculation.

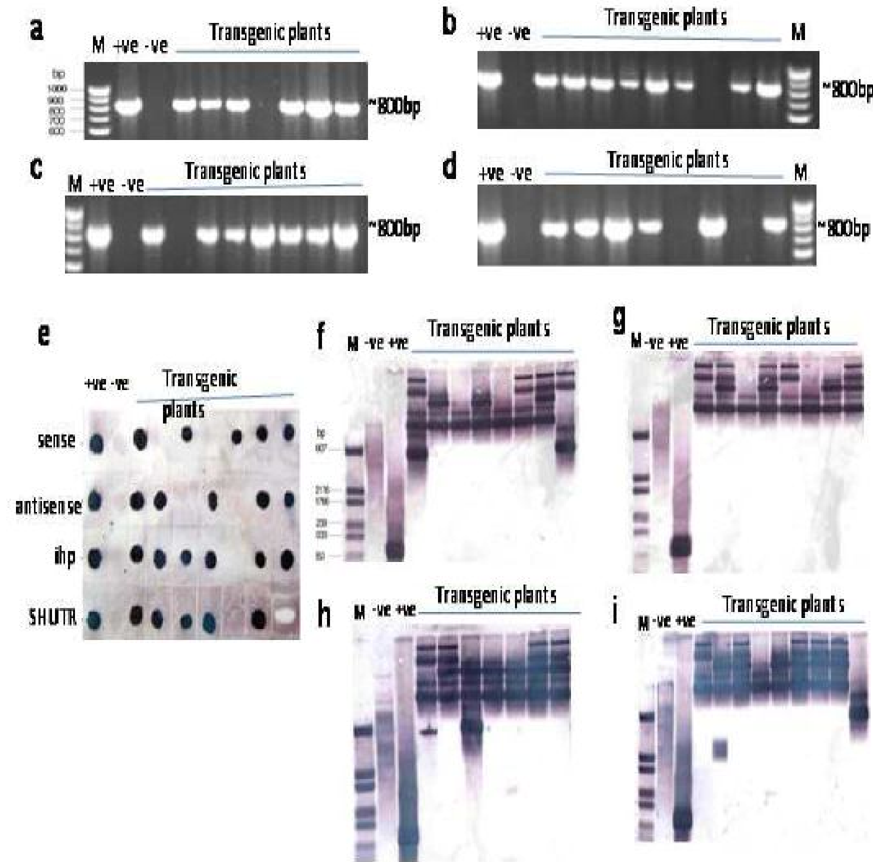

Fig. 5 Transgene integration analysis. PCR amplification of *hpt*II gene showing integration of sense (**a**), antisense (**b**), ihp (**c**) and SHUTR (**b**) cassettes in transgenic plants, infected plant DNA and nontransgenic plant DNA was used as positive (+ve) and negative (-ve) controls, respectively. (**e**) Dot blot analysis showing integration of PTGS cassettes using hptII specific probe. Southern blot analysis showing integration of sense (**f**), antisense (**g**), ihp (**h**) and SHUTR (**i**) cassettes in transgenic plants using hptII specific probe, nontransgenic plant DNA and *rep* gene PCR product was used as negative (-ve) and positive (+ve) controls, respectively.

UNDER PEER REVIEW

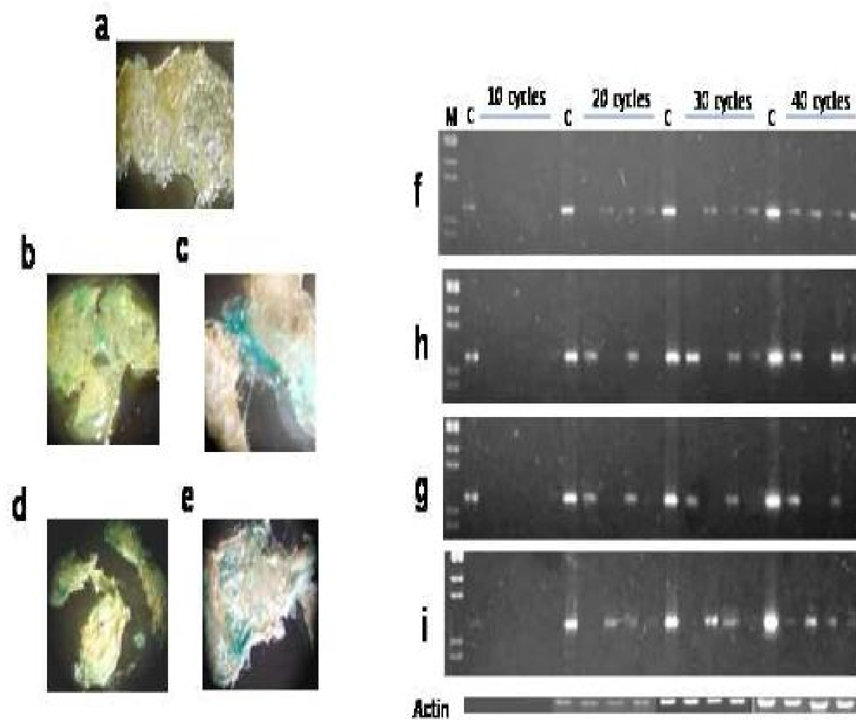

Fig. 6 Transgene expression analysis. GUS histochemical analysis of transgenic plants showing varied expression status of sense (**b**), antisense (**c**), ihp (**d**) and SHUTR (**e**) cassettes, whereas, no such expression was observed in control plants (**a**). Semiquantitative PCR analysis (targeting viral coat protein gene) of four phenotypically superior transgenic plants obtained by sense (**f**), antisense (**g**), ihp (**h**) and SHUTR (**i**) cassettes showing viral titer levels at different cycling conditions against infected plant DNA sample as control.

UNDER PEER REVIEW

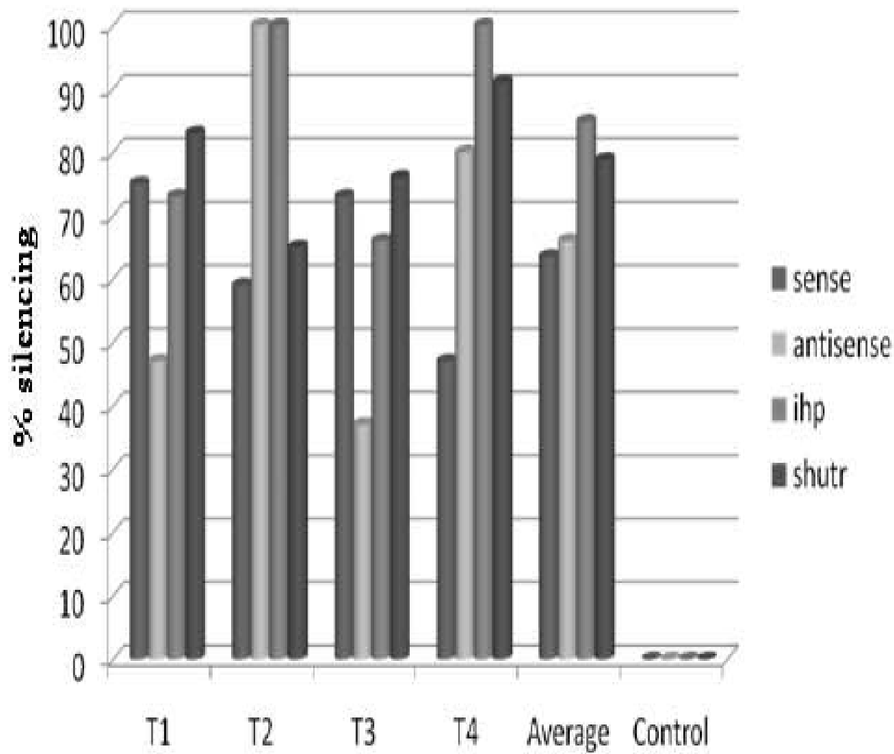

Fig. 7 Densitometry analysis of amplicons obtained from semi quantitative PCR showing percent silencing of ToLCV. Densitometry scanning of PCR amplicons from four phenotypically superior (T1, T2, T3 and T4) transgenic plants obtained from sense, antisense, ihp and SHUTR cassettes were converted to percent silencing by comparing them against respective controls. Average percent silencing of four phenotypically superior transgenic lines tested in each strategy tested is also shown.

UNDER PEER REVIEW

Table 1: Percent response of explants, shoots and plantlets for co-cultivation

	Response of explants to co-cultivation (based on values of previous stages)				
Constructs	Sense	Antisense	ihp	SHUTR	Average
No. of explants	387	407	423	289	377
Co-cultivation	36.8	55.2	47.8	27.4	41.8
Shoot initiation	42.4	43.3	48.4	39.1	43.3
Shoot elongation	24.6	28.0	33.0	30.3	29.0
Rooting	40.3	27.1	55.3	36.2	39.7
Hardening in peat	37.4	40.8	38.4	31.2	37.0
Greenhouse establishment	41.2	53.3	45.0	37.5	44.3

UNDER PEER REVIEW

Table 2: Transgenic plants showing symptom expression after virus inoculation

PTGS approaches	Percent degree of silencing		
	2-4 WI[a] (early symptom)	4-6 WI[a] (delayed symptom)	>6 WI[a] (no symptom)
Sense	48.0	35.7	17.3
Antisense	33.8	41.8	24.4
ihp	22.0	36.5	41.5
SHUTR	28.3	36.3	35.4
Control	70.0	100	100

[a] **WI:** Weeks post inoculation

Table 3: Densitometry analysis of transgenic plants showing viral titer

No. of PCR cycles	Selected transgenic plants	PTGS constructs			
		Sense[a]	Antisense[a]	ihp[a]	SHUTR[a]
10 cycles	Control	9800 (0)	13536 (0)	13650 (0)	13104 (0)
	T1	0 (100)	0 (100)	0 (100)	0 (100)
	T2	0 (100)	0 (100)	0 (100)	0 (100)
	T3	0 (100)	0 (100)	0 (100)	0 (100)
	T4	0 (100)	0 (100)	0 (100)	0 (100)
20 cycles	Control	14256 (0)	20857 (0)	20759 (0)	19460 (0)
	T1	0 (100)	8648 (60)	7556 (65)	0 (100)
	T2	9504 (33)	0 (100)	0 (100)	7683 (38)
	T3	6844 (52)	9779 (55)	8671 (60)	6112 (61)
	T4	5643 (60)	0 (100)	0 (100)	2210 (75)
30 cycles	Control	24056 (0)	27608 (0)	27608 (0)	26568 (0)
	T1	3279 (86)	14980 (46)	8052 (71)	0 (100)
	T2	11980 (50)	0 (100)	0 (100)	9776 (29)
	T3	8554 (63)	16778 (39)	9310 (66)	6700 (57)
	T4	7614 (68)	4399 (84)	0 (100)	2540 (79)

[a] Percent silencing of ToLCV are shown in the parenthesis

UNDER PEER REVIEW

References

Agrawal N, Dasaradhi PV, Mohmmed A, Malhotra P, Bhatnagar RK, Mukherjee SK (2003) RNA interference: biology, mechanism, and applications. Microbiol Mol Biol Rev 67**:**657–685.

Aida R, Kishimoto S, Tanaka Y and Shibata M (2000) Modification of flower color in Torenia (*Torenia fournieri* Lind.) by genetic transformation. Plant Science 153: 33-42.

Ammara, Shahid Mansoor, Muhammad Saeed, Imran Amin, Rob W Briddon and Abdullah Mohammed Al-Sadi (2015) RNA interference-based resistance in transgenic tomato plants against Tomato yellow leaf curl virus-Oman (TYLCV-OM) and its associated betasatellite. *Virology Journal*, 12:38.

Battraw MJ and Hall TC (1990) Histochemical analysis of CaMV 35S promoter- α-glucuronidase gene expression in transgenic rice plants. Plant Molecular Biology 15: 527-538.

Brummell DA, Balint-Kurti PJ, Hapster MH, Palys JM, Oeller PW and Gutterson N (2003). Inverted repeat of a heterologous 3'-untranslated region for high-efficiency, high throughput gene silencing. Plant Journal 33: 793-800.

Chen, T. Y., Pai, H., Hou, L. Y., Lee, S. C., Lin, T. T., Chang, C. H., Hsu, F. C., Hsu, Y. H. and N. S. Lin. (2019). Dual resistance of transgenic plants against Cymbidium mosaic virus and Odontoglossum ringspot virus. Scienti□c Rep.,9: 10230

Chi M, Bhagwat B, Lane WD, Tang G, Su Y and Sun R (2014) Reduced polyphenol oxidase gene expression and enzymatic browning in potato (*Solanum tuberosum* L.) with artificial microRNAs. BMC Plant Biol,14:62.

Dalakouras A, Wassenegger M, Dadami E, Ganopoulos I, Pappas ML and Papadopoulou K (2020) Genetically modified organism-free RNA interference: exogenous application of RNA molecules in plants. Plant physiology.;182(1):38-50.

Day AG, Bejarano ER, Buck KW, Burrell M and Lichtenstein CP (1991) Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. Proceeding of National Academy of Science 88:6721-6725.

UNDER PEER REVIEW

Dry IB, Ridgen JE, Krake LR, Mullineaux PM, Rezaian MA (1993) Nucleotide sequence and genome organization of tomato leaf curl geminivirus. J. Gen. Virol. 74**:** 147– 151.

Eapen S and George L (1994) *Agrobacterium tumefacines* mediated gene transfer in peanut (*Arachis hypogeae*). Plant Cell Reports 13: 582-586.

Elseu S, Figueria Filho, Lucio FA, Figueiredo and Damaras C and Monteneschich (1994) Transformation of potato using *Agrobacterium tumefacines* and evaluation of herbicide resistance. In vitro Cellular and Developmental Biology 28: 121-129.

Faiz, H., & Abhinav, K. (2021). Development of immunity against cotton leaf curl virus using antisense AC4 gene. *Research Journal of Biotechnology Vol*, *16*, 9.

Gogoi, A., Kaldis, A., Dasgupta, I., Borah, B. K. and A. Voloudakis. 2019. Sense and antisense-mediated resistance against Sri Lankan cassava mosaic virus (SLCMV) in Nicotiana benthamiana. Biol. Plantarum, 60:455-464

Guo, J., Gao, S., Lin, Q., Wang, H., Que, Y. and L. Xu. (2015), Transgenic sugarcane resistant to Sorghum mosaic virus based on coat protein gene silencing by RNA interference. BioMed Res. Int., 86: 1-9

Ingelbrecht I, Van Houdt H, Van Montagu M and Depicker A (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proceedings of National Academy of Science 91: 10502-10506.

Jefferson RA, Kavanagh TA and Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO journal 6:3901-3907.

UNDER PEER REVIEW

Kumar, A., Ahmad, J., & Khan, J. A. (2020) Engineering resistance against cotton leaf curl disease with the application of antisense technology. *Research Reports*, *4*.

Kunik T, Salomon R, Zamir D, Navot N, Zeidan M, Michelson I, Gafni Y and Czosnek H (1994) Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein are resistant to the virus. Bio/Technology 12, 500-504.

McCormick S (1991) Transformation of tomato with *Agrobacterium tumefaciens*. Plant Tissue Culture Manual 227: 1229-1231.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiological Plant 15: 73-97.

Nakhla MK and Maxwell DP (1998) Epidemiology and management of tomato yellow leaf curl disease. Plant Virus Disease Control 565-583.

Nora S, Michèle Z, Asuka I, Biao D, Ming□Bo W, Gabi K And Michael W (2009) RNAi□mediated resistance to Potato spindle tuber viroid in transgenic tomato expressing a viroid hairpin RNA construct. *Mol Plant Pathol*.; 10(4): 459–469

Powell-Abel P, Nelson RS, De, B, Hoffmann N, Rogers SG, Fraley RT and Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232: 738-743.

Praveen S, Anil M and Ginny A (2006) Viral suppression in transgenic plants expressing chimeric transgene from Tomato Leaf Curl Virus and Cucumber Mosaic Virus. Plant Cell, Tissue and Organ Culture 84: 49-55.

Raj SK, Singh RSK, Pandey and Singh BP (2005) *Agrobacterium*-mediated tomato

UNDER PEER REVIEW

transformation and regeneration of transgenic lines expressing Tomato leaf curl virus coat protein gene for resistance against TLCV infection. Current Science 88: 1674-1679.

Rajamanickam, S., Chandrasekar, G. and G. Karthikeyan, (2015) Coat protein mediated resistance against Tobacco streak virus in Nicotiana tabacum L. through RNA silencing. The Bioscan, 10(4): 1787-1793

Reinhard T, Volker M, Bruno G, Jozef S and Hans-Henning S (1987) A set of plant expression vectors for transcriptional and translational fusions. Nucleic Acids Research 15: 5890.

Ruhel, R., & Chakraborty, S. (2019) Multifunctional roles of geminivirus encoded replication initiator protein. *VirusDisease*, *30*(1), 66-73.

Sambrook J, Fritsch EF and Mainiati T (1989) Molecular cloning: A Laboratory Manual. M. 2nd Edition. Cold Spring Harbor Laboratory PRESS, NEW YORK 480-481.

Senthilraja, C., Gurivi Reddy, M., Rajeswaran, J., Kokiladevi, E. andR. Velazhahan. (2018) RNA interference-mediated resistance to Tobacco streak virus in transgenic peanut. Australasian Plant Pathol. https://doi.org/10.1007/s13313-018-0549-9

Sijen T, Wellink J, Hirirt JB and Van Kammen A (1996) RNA-mediated virus resistance: role of repeated transgenes and delineation of targeted regions. Plant Cell 8: 2277-2294.

Singh N, Mukherjee SK and Rajam MV (2020) Silencing of the ornithine decarboxylase gene of *Fusarium oxysporum* f. sp. lycopersici by host-induced RNAi confers resistance to Fusarium wilt in tomato. Plant Molecular Biology Reporter.;38(3):419-429.

UNDER PEER REVIEW

Southern E (1979) Gel electrophoresis of restriction fragments. Methods in Enzymology 68: 152-176.

Tenllado F and Diaz RJR (2004) Double-stranded RNA-mediated interference with plant virus infection. Journal of virology 75:12288-12297.

Tougou, M., Furutani, N., Yamagishi, N., Shizukawa, Y., Takahata, Y. and S. Hidaka. (2006). Development of resistant transgenic soybeans with inverted repeat-coat protein genes of Soybean dwarf virus. Plant Cell Rep., 25:1213-1218

Wagner A, Phillips L, Narayan RD, Moody JM and Geddes B (2005) Gene silencing studies in the gymnosperm species *Pinus radiata*. Plant Cell Report 24: 95-102.

Walsh, H. A., Vanderschuren, H., Taylor, S., & Rey, M. E. C. (2019). RNA silencing of South African cassava mosaic virus in transgenic cassava expressing AC1/AC4 hp-RNA induces tolerance. *Biotechnology Reports*, *24*, e00383.

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG and Waterhouse PM (2001) Construct design for effective and high-throughput gene silencing in plants. Plant Journal 27: 581-590.

Zhong B, Yan-Wei Shen and Toshihiro Omura (2005) RNA-binding Domain of the Key Structural Protein P7 for the Rice dwarf virus Particle Assembly. Acta Biochimica et Biophysica Sinica 37:55-58.

UNDER PEER REVIEW