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ORIGINAL ARTICLE

Screening of a multi-virus resistant RNAi construct in cowpea through transient vacuum infiltration method

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Abstract Plant viruses are the most devastating pathogens causing substantial economic losses in many crops. Current viral disease management relies on prophylactics, roguing and insect vector control, since in most crops resistant gene pools for resistance breeding are unavailable. RNA interference, a sequence dependent gene silencing mechanism holds great potential in imparting virus resistance. In this study, the efficacy of a RNAi gene construct developed against four viruses commonly infesting tomato and chilli viz., capsicum chlorosis virus, groundnut bud necrosis virus, cucumber mosaic virus and chilli veinal mottle virus was evaluated. A 3546 bp dsRNA-forming construct comprising sense-intron-antisense fragments in binary vector pBI121 (hpRNAi-MVR) was mobilized into *Agrobacterium tumefaciens*. Cowpea (*Vigna unguiculata*) was used as an indicator plant for GBNV agroinfiltration to evaluate the efficacy of hpRNAi-MVR construct in conferring GBNV resistance. The type of agroinfiltration, bacterial concentration and incubation-temperatures were optimized. Vacuum infiltration of three pulses of 20–30 s at 66.66 kPa were effective than syringe infiltration. Of the five Agrobacterial concentrations, OD_{600} 0.5 was more efficient. Incubation temperature of 31 ± 1 °C was favorable for development of disease symptoms than 20 ± 1 °C and 26 ± 1 °C. ELISA revealed a 35% decline in viral load in hpRNAi-MVR infiltrated plants compared to vector control plants. Quantitative real time PCR results have shown a viral gene silencing to the extent of 930–990 folds in hpRNAi-MVR infiltrated plants compared to vector control. This approach is simple, rapid and efficient to screen the efficacy of RNAi constructs developed for the RNAi mediated plant virus management.

Keywords Agroinfiltration · Tospovirus · Cowpea · GBNV · Bacterial concentrations

Introduction

Chilli (*Capsicum annum* L.) and Tomato (*Solanum lycopersicum* L.) are commercially important vegetable crops grown globally. Viral diseases caused by some major viruses like tospovirus, cucumovirus and potyvirus results in huge loss in productivity. Tospovirus-CaCV and GBNV (capsicum chlorosis virus and groundnut bud necrosis virus) are negative sense single-stranded RNA viruses, belong to *Bunyavirales* family, consists of tripartite genome comprising three RNA segments named S (3 kb), M (4.8 kb) and L (9 kb) [14, 15, 34, 36, 42]. In nature GBNV are transmitted by 13 different types of thrips vectors in a propagative and persistent manner. Symptoms of GBNV infected plants exhibit, apical necrosis, concentric rings, necrotic and chlorotic spots on leaves, severe leaf distortion

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[44]. GBNV and CaCV affect mainly vegetable and ornamental crops and it is estimated that the GBNV alone causes losses of more than U.S. $89 million annually [53]. Cucumovirus (cucumber mosaic virus-CMV) is a tripartite single-stranded sense positive RNA virus of family Bromoviridae. The CMV genome is divided into three segments consisting of RNA1 (3357 nt), RNA2 (3050 nt), and RNA3 (2216 nt) [43]. The host range of CMV is broad, and it can infect about 1200 species. Causes, severe damage to the members of Cucurbitaceae and Solanaceae families [48]. Symptoms of CMV in field include yellowing, mottling of leaves, stunting, shoe stringing of leaf blades or extreme filiformity [8, 19]. Potyvirus (chilli venial mottle virus-ChiVMV), belongs to the family *Potyviridae*, is a linear, single, positive-sense, single-stranded RNA (ssRNA) of about 10 kb, the genomic RNA of potyvirus is encapsidated by coat protein (CP) and its open reading frame (ORF) encodes large polyprotein members [1, 3]. ChiVMV was first reported on Solanaceous crop in Western Malaysia and it causes nearly 50% crop loss in peppers [46, 47]. Potyvirus and Cucumovirus are transmitted by aphids in a non-persistent manner [8]. Virus management has been challenging in recent years due to intensive crop cultivation that concomitantly resulted in disease outbreaks and also a spurt in the insect vectors transmitting these broad group of viruses. Various strategies have been employed to combat viral diseases, among which, RNAi technology is promising in conferring broad spectrum viral resistance in many susceptible crop plants [17, 58].

RNA interference (RNAi), is a natural gene regulation mechanism also involved in defense against viruses that can be efficiently employed to silence specific target genes. Thus, the RNAi mediated silencing of invading virus genes is a key step in imparting virus resistance. The potential of RNAi has been well established on virus resistance in plants [17, 45, 51, 58]. The expression of virus-specific dsRNA as hairpin structures guide the host RNA induced silencing complex (RISC) to cut and degrade the cognate viral RNA rendering these plants resistant to virus infection [5, 30].

Many transformation methods have been used to transfer the dsRNA forming transgene into the cells. Stable transformation system is a formidable approach for gene function studies in plants, but is limited by its requirement for increased men, material, effort and time. While, the transient gene expression is fast, less arduous, and more simplistic [37, 41].

Agrobacterium based transient transformation can be achieved in various tissues, it can be infiltrated by syringe or vacuum application into the apoplast, hence called "agroinfiltration". Transient Agrobacterium mediated transformation has been widely used to analyze the gene silencing [9], gene function [51, 55], and to study the host:pathogen interactions and virulence genes [31, 63, 64]. Agroinfiltration was first performed on tobacco (*Nicotiana benthamiana*) [64], and now it is widely applied on many plants including Arabidopsis (*Arabidopsis thaliana*) [63, 70], tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa*) [70], switch grass (*Panicum virgatum*) [11], potato (*S. tuberosum*) [4], grapevine (*Vitis vinifera*) [55], and tobacco (*N. tabacum*) [71].

Agroinfiltration, is commonly used for transient expression of hairpin RNAi constructs and the infiltrated plants exhibited resistance to potyvirus and tobamovirus [61], similarly in tobacco, transient RNAi mediated broad spectrum resistance was achieved against three viruses viz., Tospovirus by targeting N gene of tomato chlorotic spot virus (TCSV), groundnut ringspot virus (GRSV) and tomato spotted wilt virus (TSWV) [16], pepper mild mottle virus (PMMoV), tobacco etch virus (TEV), and alfalfa mosaic virus (AMV) [62] and potato virus Y (PVY) [66]. Challenge inoculation by the above viruses followed by agroinfiltration with cells containing hairpin RNAi construct indicated various degrees of virus resistance as assessed by systemic and local symptoms.

In this study, we have developed hpRNAi-MVR vector by targeting seven genes from four viruses belonging to three groups viz, Tospoviruses-groundnut bud necrosis virus (GBNV) and capsicum chlorosis virus (CaCV), Cucumovirus-cucumber mosaic virus (CMV), and Potyvirus-chilli venial mottle virus (ChiVMV). In order to evaluate the efficacy of hpRNAi-MVR construct, transient expression by agroinfiltration was employed using cowpea as host plants and the resistance to one of the target viruses, GBNV in these plants was assessed by visual symptoms, ELISA and by qRT-PCR determining of the extent of silencing of the viral gene. This strategy is fast, efficient and less laborious than stable integration and can be employed as a preliminary step in evaluating the efficacy of RNAi gene constructs against plant viruses in imparting RNAi mediated virus resistance.

Materials and methods

Preparation of Agrobacterium culture and agroinfiltration

Agrobacterium cells containing plasmids, hpRNAi-MVR developed previously (Supplementary Fig. 1) [40] and pBI121 (vector control) were used for agroinfiltration. A 5-ml aliquot of freshly grown *Agrobacterium* culture was inoculated in 200 ml YEM medium containing [10 g l^{-1} mannitol, 10 g l^{-1} yeast extract, 5 g l^{-1} NaCl and 1.5% Bacto Agar (pH 7.0) supplemented with 50 mg l^{-1} rifampicin and 100 mg l^{-1} kanamycin] and incubated in orbital

shaker overnight at 220 rpm at 28 °C. The *Agrobacterium* cells containing the above plasmids were collected by centrifugation at 1751×*g* for 10 min, resuspended the pellet in 10 ml of sterile distilled water, pellet the cells again and finally resuspend the pellet in infiltration buffer containing (10 mM $MgCl_2$, 10 mM MES at pH 5.6, with 200 µM acetosyringone), bacterial culture was diluted to five different concentrations of OD_{600} 0.1, 0.25, 0.5, 0.75 and 1.0 and incubated for 2–4 h in room temperature.

All the experiments in this study were carried out on cotyledonary leaves of 10–12 day old cowpea seedlings. Agroinfiltration by employing both, syringe and vacuum was carried out separately. For syringe infiltration, bacterial suspensions were transferred to 2-ml needle-less syringe, syringe tip was placed on the abaxial surface of the cowpea cotyledonary leaf and pressure was applied. To evaluate vacuum infiltration, the cowpea cotyledonary leaves were pin pricked lightly without causing much damage, then the leaves were submerged in a 250 ml beaker with 150 ml of bacterial suspension (Supplementary Fig. 2) and vacuum in the range of 39.96–93.32 kPa was applied using a vacuum pump (IHVP, IVP-150, Genei, Bangalore, India) in pulses of 5 s to 5 min time intervals. Then the container was gradually allowed to return to the atmospheric pressure, the infiltrated leaves were washed with sterile distilled water and maintained at 26 ± 1 °C and 60% ± 1 relative humidity.

Purification of GBNV and inoculum preparation

Tomato plant parts exhibiting the GBNV disease symptoms of chlorotic and necrotic lesions were collected from the field and the virus was purified by re-inoculating and maintained on cowpea cv. C-152 grown under temperature and humidity controlled greenhouse conditions. Briefly, the tomato plant parts showing viral disease symptoms were harvested and macerated using sterile motor and pestle with chilled 0.05 M phosphate buffer containing (K_2HPO_4 and KH_2PO_4) of pH 7.0 with 0.1% β-mercaptoethanol, 0.2% sodium sulphite and a pinch of Celite 545. Carborundum 320 grit (as abrasive) was dusted on healthy cowpea cotyledons and the extract was applied directly on the adaxial surface of cotyledons by rubbing gently with uniform pressure and the inoculated leaves were washed with water and the seedlings were incubated in under controlled (at 26 ± 1 °C, 60% ± 1 RH) conditions. Cowpea plant samples showing GBNV symptoms were collected and sap extract was prepared as described above in phosphate buffer to inoculate to the previously (48 h) agroinfiltrated cowpea cotyledons. The inoculated cowpea plants were maintained at temperatures of 20 ± 1 °C, 26 ± 1 °C and 31 ± 1 °C and a uniform relative humidity of 60% ± 1 (Supplementary Fig. 2). The symptoms were recorded after 3 days post inoculation (dpi) to evaluate the virus infection. Three replicates (n = 15 plants/replicate) of each treatment and control were maintained.

Evaluation of transient expression by GUS Assay

The transient expression from pBI121 infiltrated cowpea plants was determined by GUS assay according to Jefferson [28]. At 8th day post infiltration, the cotyledonary leaf discs were submerged in staining solution containing 61 mM Na_2HPO_4, 39 mM NaH_2PO_4, 0.1% triton X-100, 10 mM EDTA, 0.3% H_2O_2 and 1.5 mM X-Gluc. The staining solution with leaf discs was placed in a vacuum chamber and vacuum was applied in five pulses of 30 s each and the discs incubated in same solution at 37 °C for overnight. The stained leaf discs were submerged in 95% ethanol overnight and then washed with 70% ethanol, until the chlorophyll discoloration was observed. The stained discs were placed on the glass slides and images were captured using Stemi 508 Stereo Microscope (ZEISS, Germany) at 2.5× magnification.

Evaluation of viral gene silencing by hpRNAi-MVR

Elisa

The viral load in the virus inoculated leaves (3dpi and 6dpi) was assessed by Double antibody coated-Enzyme linked immunosorbent assay (DAC-ELISA) employing GBNV specific antibodies [23]. Buffer infiltrated and virus infected plants served as positive controls, where buffer infiltrated mock inoculated (without virus) plants served as negative controls.

RT-PCR and qRT-PCR

To analyze the transient expression in infiltrated plants, 48 h after infiltration, the total RNA were isolated from the agroinfiltrated plants of buffer control (BC), pBI121 vector control (VC) and hpRNAi-MVR (MVR) using TriSure reagent, Bioline (Bioline Reagents Ltd., UK). DNA traces in the RNA were removed by treating with DNase I (Thermo Scientific, US) and then RNA was purified by using phenol/chloroform/isoamyl alcohol (25:24:1 v/v) and finally precipitated by sodium acetate and absolute alcohol. The quantity and quality of RNA were assessed using spectrophotometer, Spectra Max® Model No. M2 (Molecular Devices, CA, USA) and visualized by denatured 1.2% Agarose gel electrophoresis. First strand cDNA was synthesized by using 1.5 µg total RNA using Tetro cDNA synthesis kit, Bioline (Bioline Reagents Ltd., UK). To detect the transgene in infiltrated plants, NPT II (Neomycin phosphotransferase II) gene specific PCR primers were used to amplify a 410 bp fragment from both MVR and VC plant

samples. Actin gene Vu actin (*V. unguiculata* actin) was employed as an internal control. To assess the GBNV load 6 days post inoculation in the agroinfiltrated plants, total RNA was isolated from the cowpea cotyledons using TriSure reagent, Bioline (Bioline Reagents Ltd., UK), followed by DNase I treatment (Thermo Scientific, USA). The synthesized cDNA was used for PCR and qRT-PCR. The virus specific transcript accumulation in plants from MVR, VC, BC and Mock inoculations were verified by conventional PCR and agarose gel electrophoresis prior to qRT-PCR. QRT-PCR was carried out using Fast Start Essential DNA Green Master mix (Roche, Germany) using Light Cycler® 480 II (Roche, Germany). To evaluate the viral gene silencing qRT-PCR was carried out by relative quantification using 4 µl of diluted cDNA and the expression level was calculated using $2^{-\Delta\Delta CT}$ method [38]. To detect the GBNV expression from agroinfiltrated cowpea cotyledons, for PCR, qRT screen GBNV-N F and qRT screen GBNV-N R primers (these primers amplified a region different than that was used in hpRNAi-MVR) were used to amplify the GBNV nucleocapsid gene, and primers, qRT VuAct2 F and qRT VuAct2 R of cowpea actin gene were used to amplify VuActin2 gene as internal control (Supplementary Table 1). VuActin2 was used to normalize the expression of GBNV in the infected cowpea tissues. The conditions for amplification were as follows: 6 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 60 °C, 15 s at 72 °C and melting curve were analyzed from 60 to 97 °C with 0.1 °C steps.

Determination of chlorophyll content

The leaf chlorophyll content was determined from BC, VC and MVR vacuum infiltrated plants. 100 mg of leaf samples were ground with 80% acetone and made up to 10 ml, the content was incubated in refrigerator overnight. The solution was centrifuged at $2737\times g$ for 10 min and the supernatant was transferred to fresh tube and the absorbance were recorded at 663 and 645 nm using Spectra Max® Model No. M2 (Molecular Devices, CA, USA) [6].

Data analysis

One way ANOVA was used for statistical analysis, the significant value was considered ($P < 0.001$), and all statistical tests were carried out using GraphPad Prism 5.

Results

Parameters of agroinfiltration in cowpea

Vacuum-agroinfiltration was highly efficient than syringe infiltration, in vacuum infiltration extending the duration of vacuum pulses from 30 s to 1 min and 5 min period made no difference in infiltration efficiency. Of the different bacterial concentrations highest transformation was achieved with OD_{600} 0.5.

RT-PCR analysis of agroinfiltrated plants

The presence of transgene in VC and MVR agroinfiltrated plants were confirmed by amplifying a 410 bp of NPT II gene by RT-PCR at 48 h of post vacuum infiltration. The presence of the band of the amplicon indicates presence of the transgene and in turn the successful agrobacterial infiltration and transfer of T-DNA in the plant cells, while no PCR amplification was observed in BC and NTC (no template control) (Fig. 1).

Determination of chlorophyll content in the agroinfiltrated tissues

Chlorophyll content in GBNV inoculated BC, VC and MVR agroinfiltrated plants was analyzed. The reduction in chlorophyll content was observed more in VC and BC than in MVR infiltrated plants, while consistently higher chlorophyll content was observed in hpRNAi-MVR OD_{600} 0.5 and 0.75 than the other concentrations. Total chlorophyll content was higher in MVR infiltrated and GBNV infected plants by 6.1, 20.1, 48.1, 50.2 and 50.1% in OD_{600} 0.1, 0.25, 0.5, 0.75 and 1.0 compared to the VC respectively, indicating that the plants exhibited lower level of viral infection due to hpRNAi-MVR silencing (Fig. 2).

GUS assay

The GUS assay results indicate that the higher staining was observed in OD_{600} 1.0 followed by 0.75 and 0.5 OD_{600} infiltrations and the higher intensity and extent of spread of staining indicating the higher transformation efficiency. In buffer control (BC) blue signals were not observed indicating that the GUS staining was due to the infiltration of the vector containing agrobacterial cells (Fig. 3).

Estimation of viral load by ELISA

The highest viral load was detected in GBNV infected Buffer control (BC) closely followed pBI Vector control (VC) infiltrated plants while lowest viral load was observed at OD_{600} 0.5 infiltrated plants. The viral titers in MVR OD_{600} 0.1, 0.25, 0.5, 0.75 and 1.0 infiltrated plants were 92.6, 54.7, 34.3, 34.4, 35.6% at 3 dpi, and 92.4, 53.2, 35.9, 35.92, 37.22% at 6 dpi respectively, compared to the VC. No significant differences were observed in viral titer of BC and VC (Figs. 4, 5).

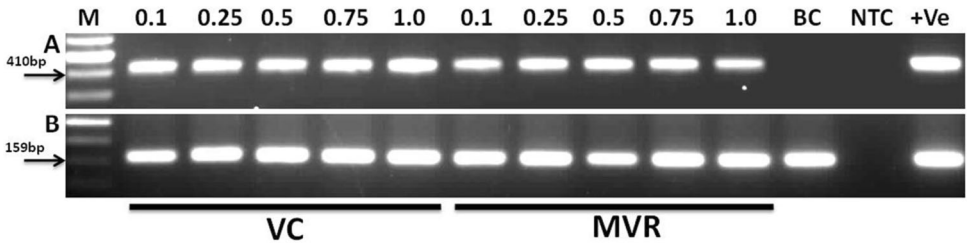

Fig. 1 RT-PCR analysis of Agroinfiltrated cowpea plants- (*a*) gene specific primers were used to PCR amplify the NPT-II gene to confirm the transient expression in agroinfiltrated cowpea plants; M- 100 bp ladder; VC- pBI121 vector control; MVR-hpRNAi-MVR; BC, buffer control; NTC, no template control; +Ve, positive control. (*b*) *Vigna unguiculata* actin2 (VuAct2) primers was used to amplify the actin gene in cowpea plants. M- 50 bp ladder

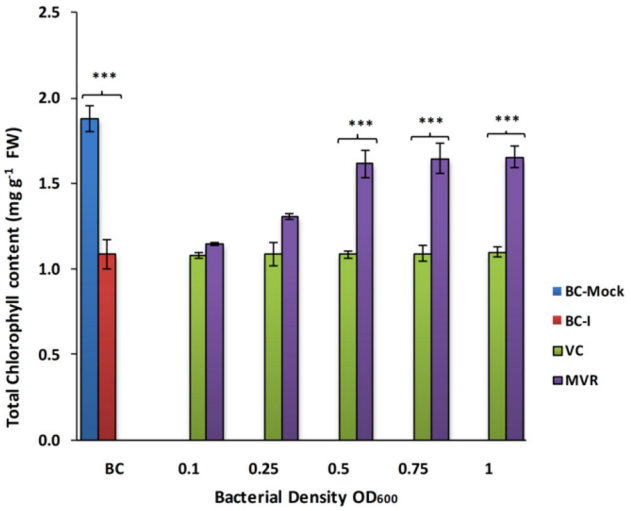

Fig. 2 Estimation of total chlorophyll content in Agroinfiltrated cowpea cotyledonary leaves. BC, buffer control; BC MOCK, buffer infiltrated Mock inoculated (without virus); BC-I, buffer infiltrated GBNV inoculated; VC- pBI121 binary vector infiltrated cowpea; MVR-hpRNAi-MVR infiltrated cowpea, the significance (***) was calculated by comparing VC versus MVR at each Bacterial concentration

Determination of GBNV silencing level by qRT PCR

To evaluate the silencing efficiency of hpRNAi-MVR construct in agroinfiltrated cowpea plants, transcript abundance of GBNV 'N' gene was evaluated by qRT-PCR. The results revealed significant viral gene silencing by the expressed hpRNAi-MVR in the infiltrated plants. A significant relative viral gene silencing of over 900 folds was observed in agrobacterial cell densities of OD_{600} 0.5 and above infiltrated plants compared to the vector control plants. These findings validated the visual observation of virus symptoms on the plants and also the intensity of GUS staining and viral load estimation by ELISA. The viral load was gradually decreased in MVR infiltrated OD_{600} 0.1, 0.25, 0.5, 0.75 and 1.0 of about 10.3, 124.8, 931, 979.4, 992 folds compared to VC Plants. There was no difference in N gene expression in BC and VC (Fig. 6).

Discussion

In this study, we used a hpRNAi-MVR vector developed earlier, which is targeted for silencing four viruses by targeting 7 genes that include viral silencing suppressors from 3 commonly infecting virus groups of commercially important solanaceous host crops, tomato and chilli. To study the efficiency and effectiveness of hpRNAi-MVR vector, we aimed to rapidly screen the hpRNAi-MVR vector against one of the target viruses, GBNV by agroinfiltration method using cowpea as an indicator host plant. Crops encounter diverse virus pathogens in the field, and the sequence specificity of RNAi constructs limits the wide range of virus resistance. This limitation could be overcome by employing chimeric hairpins or selection of sequences with consensus region among the target viruses [12]. Many viruses have evolved silencing suppressors which hinder the viral gene silencing by the host plant. Targeting single viral gene was not sufficient from the mixed virus infections in field condition, to overcome this problem targeting multiple gene segments of virus genes using hpRNA could therefore result in more robust virus resistance from broad range of virus infections [7, 67, 72, 73]. The potential of RNA interference in imparting plant virus resistance has been exploited in many crops [35]. In common bean, RNAi was employed to develop resistance against bean golden mosaic virus and these transgenic plants were commercially released for cultivation [2]. Virus resistance has been demonstrated in various plant species by using hpRNAi constructs [68, 69]. N gene and NSs genes of Tospovirus were targeted using hpRNAi constructs for induction of gene silencing to achieve Tospovirus resistance in tomato and tobacco [22, 39].

The cowpea cultivars C-152 and Pusa Komal are employed as the indicator hosts for WBNV (watermelon bud necrosis virus) and GBNV respectively [21, 25]. For rapid screening of hpRNAi constructs, transient expression by agroinfiltration is the method of choice, as it is fast and suitable for wide range of crops and is independent of in vitro regeneration protocols [18, 29, 56]. Transient expression was reported to be far more efficient than

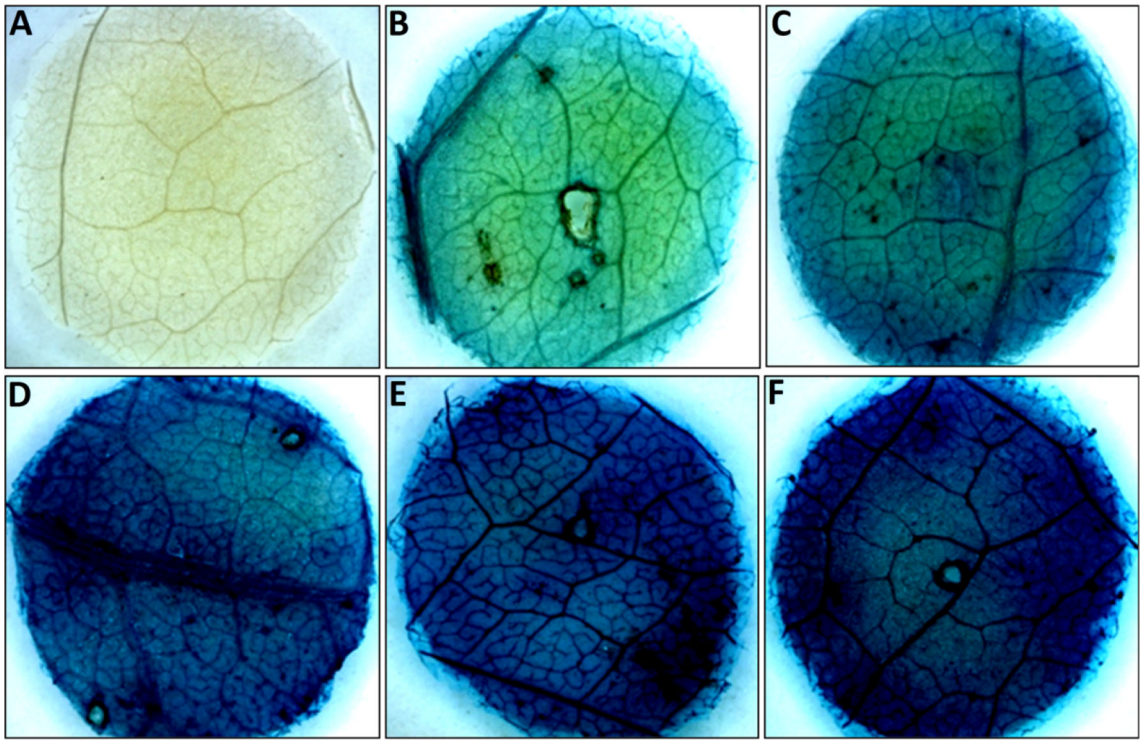

Fig. 3 GUS Assay: Optimization of vacuum agro-infiltration in cowpea cotyledons using pBI121 binary vector. **a** Buffer infiltrated; **b**–**f** agrobacterial concentrations for infiltration; **b** OD_{600} 0.1; **c** OD_{600} 0.25; **d** OD_{600} 0.5; **e** OD_{600} 0.75; **f** OD_{600} 1.0

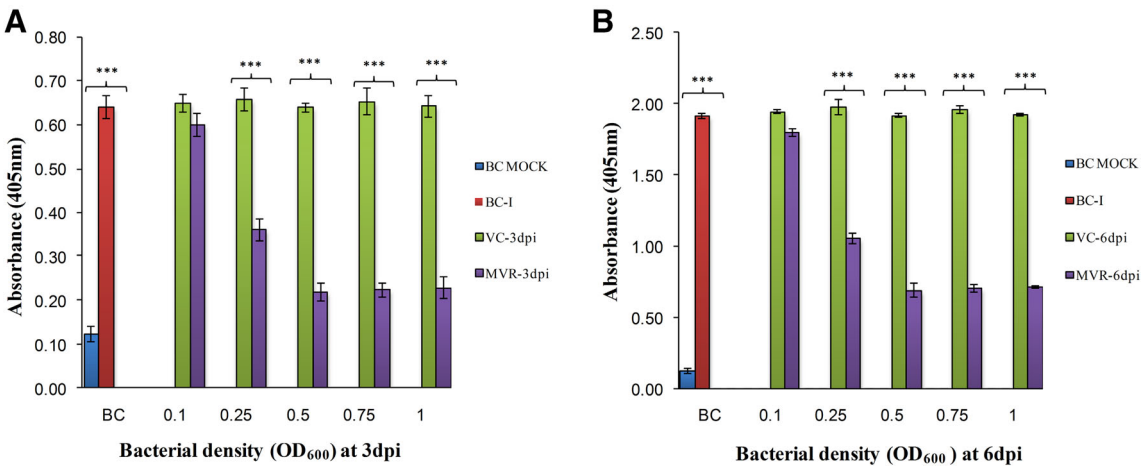

Fig. 4 Estimation of viral titer of GBNV in Agroinfiltrated cowpea by DAC-ELISA. **a**, **b** represents the GBNV viral load was determined at 3 and 6dpi of GBNV inoculation. BC, buffer control, BC MOCK, buffer infiltrated Mock inoculated (without virus); BC I, buffer infiltrated GBNV inoculated; VC- pBI121 binary vector infiltrated cowpea; MVR-hpRNAi-MVR infiltrated cowpea, the significance (***) was calculated by comparing VC versus MVR at each concentration

stable integration based expression [27]. The transient expression also offers several advantages like, assay speed, cost effectiveness and it is less laborious to screen the transgene construct without position effect bias [31].

For Agroinfiltration, advantages of syringe infiltration method were reported by a number of researchers [10, 18, 71], in this experiment, we have compared both the methods for agroinfiltration, the syringe-infiltration and vacuum-infiltrations. We observed that syringe mediated agroinfiltration was less effective compared to vacuum-infiltration, due to poor infiltration and spread of the bacterial suspension in cowpea seedlings. This may be due to the tender cotyledonary leaves and mechanical damage at the site of contact of the tip of syringe barrel [60]. The poor transformation from syringe infiltration may due to many factors viz., small aperture of stomata, spongy mesophyll

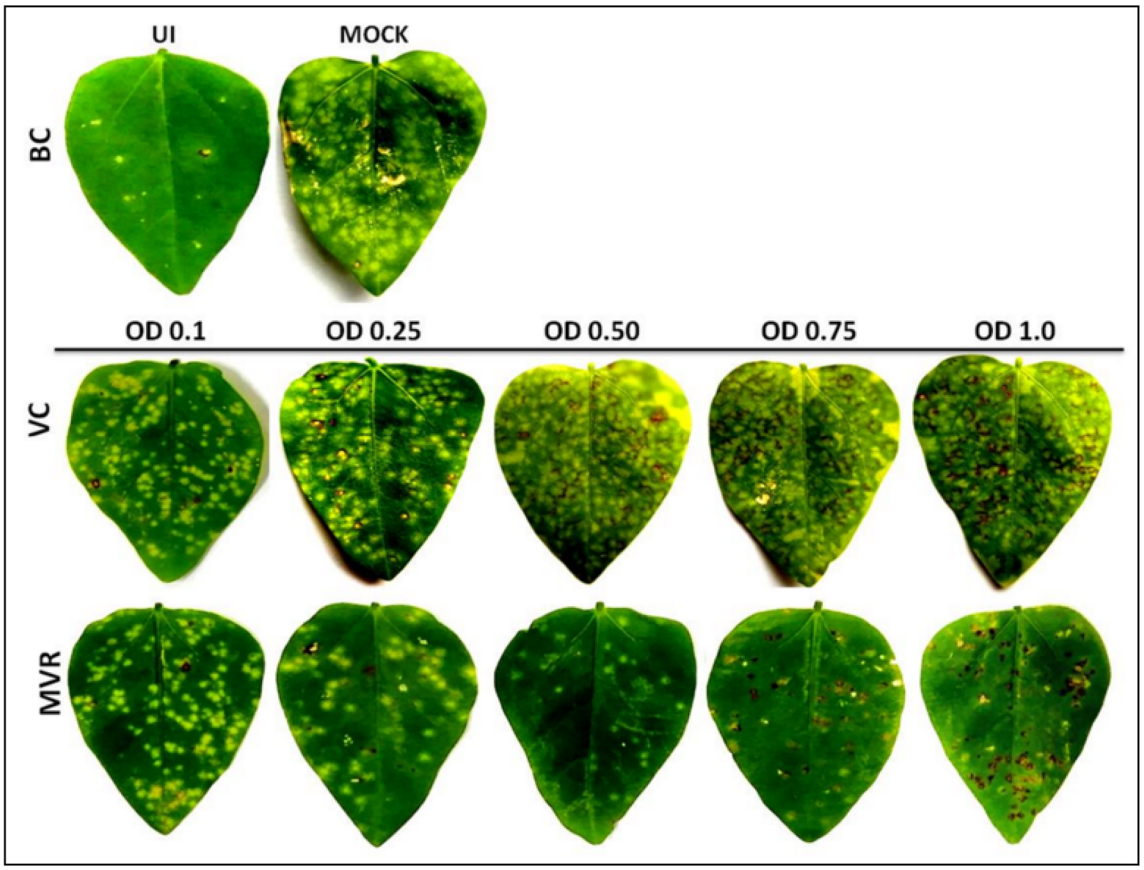

Fig. 5 Optimization of different bacterial concentrations of MVR and VC by agroinfiltration to evaluate the induction of GBNV silencing. BC, buffer control; UI, un inoculated buffer infiltrated plants; MOCK- GBNV was inoculated in buffer infiltrated plants; VC- pBI121 vector infiltrated in cowpea with different bacterial concentrations; MVR- hpRNAi-MVR infiltrated in cowpea with bacterial concentrations; OD_{600}-0.1, OD_{600}-0.25, OD_{600}-0.5, OD_{600}-0.75, OD_{600}-1.0-indicates that the concentration of Agrobacterium at OD_{600} infiltrated in cowpea

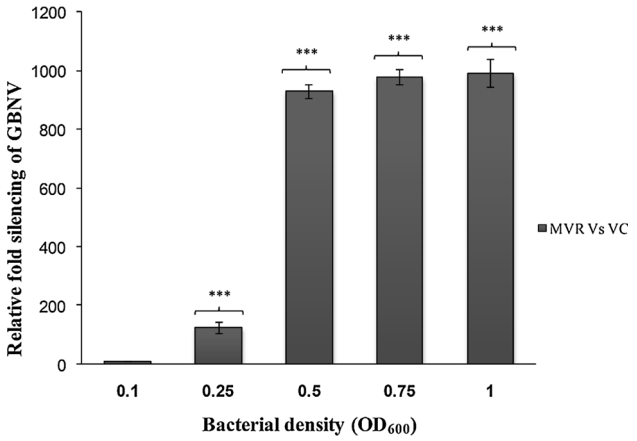

Fig. 6 The relative accumulation of GBNV at different bacterial concentrations in agroinfiltrated cowpea was determined by qRT-PCR. The transcripts of the N gene were normalized to Vu actin gene. The comparison was made between VC and MVR at each bacterial concentration, the significance (***) was determined by comparing OD_{600} 0.1 to the other bacterial concentrations

cells, high concentration of palisade and tissue fragility leads to high mechanical damage at injected area [33, 57, 65]. Due to this we have employed and optimized the vacuum infiltration in terms of number of pulses and bacterial cell densities, we have observed that three 30 s of vacuum pulses at 66.6 kPa vacuum are optimum for agroinfiltration of cowpea tissues and increased duration/vacuum leads to increased tissue damage (Visual assessment, data not shown).

To assess the transient agroinfiltration from different bacterial concentrations GUS assay was employed using pBI121 as vector control in cowpea. We have observed, strong and uniform intensity of GUS staining in OD_{600} of 0.5 and higher bacterial concentrations, and we have found that OD_{600} of 0.5 to be optimum as the higher bacterial densities lead to tissue wilting and tissue necrosis while the transient transformation was lower at lower (< 0.5) bacterial densities. In detached leaves of *Anthurium andraeanum* highest transformation efficiency of OD_{600} 0.8, was observed in [20]. Transient expression by *Agrobacterium* infiltration in lettuce, tomato and

Arabidopsis was optimized [70], they have observed higher transformation efficiencies at 0.4–0.5 OD_{600}.

Expression of symptoms and virus accumulation is dependent on the temperature of incubation, in our study, for GBNV infection a temperature of 31 ± 1 °C was found to be favorable compared to 20 ± 1 °C and 26 ± 1 °C. Similarly, higher temperatures of 30 °C and 25 °C favored increased expression of GBNV symptoms locally and systemically than lower temperatures of 20 °C and 15 °C and no symptoms were observed at 15 °C [59]. Another study revealed that, transient agroinfiltration was more efficient at a temperature of 25 °C and the temperatures above or below this affected the systemic spread of the silencing signal derived from agroinfiltration [50].

The antisera used for DAC-ELISA can efficiently detect the GBNV group in leguminous and solanaceous host like cowpea, tomato, soybean, mungbean and urdbean crops [12, 23, 26]. The viral titer of GBNV was significantly lower in MVR infiltrated plants than controls and specifically lower viral load was observed at OD_{600} of 0.5, 0.75 and 1.0.

Viral infection has affected the leaf chlorophyll content due to the formation of chlorotic spots and yellowing. The retention of chlorophyll in hpRNAi-MVR plants indicates an increased GBNV silencing and lower virus replication in hpRNAi-MVR infiltrated plants. The total chlorophyll was gradually decreased due to yellow vein mosaic virus in mesta (kenaf) plants [9] and *sporadic tungro virus* disease in susceptible rice variety [24]. A reduction of chlorophyll content in host plants infected with different viruses on *Cucurbita pepo, Abelmoschus esculentus* and *Glycine max* [52] and also reported that the reduced chlorophyll content was observed in leaves of soybean infected with yellow mosaic virus [13].

In this study, the qRT-PCR analysis was performed to assess the extent of silencing of viral transcripts by the hpRNAi-MVR. Accumulation of viral transcripts was higher in VC, without any silencing while MVR infiltrations at 0.5 OD_{600} and above had drastically lower viral transcripts exhibiting over 900 folds silencing. These results reinforced that, the transient expression of hpRNAi-MVR construct in cowpea has imparted resistance to GBNV. Agroinfiltration studies have helped in identifying and selection of the effective fragment of the viral genome for dsRNA formation in RNAi mediated virus resistance in a bipartite Geminivirus, african cassava mosaic virus (ACMV). Selection of the viral silencing suppressor AC2 provided highest level of resistance to ACMV [49]. These results are in agreement with our studies, where we have targeted three viral silencing suppressors and obtained high level of virus resistance. In transgenic wheat expressing hpRNAi constructs targeting coat protein genes of triticum mosaic virus (TriMV) and wheat streak mosaic virus (WSMV) have exhibited stable virus resistance, in T6 generation plants, a 20 fold reduced viral titer was observed compared to control plants [54]. In developing a screen for the short hpRNAi construct expressing short consensus sequences of nucleocapsid (N) gene from three viruses, viz. groundnut ring spot virus (GRSV), tomato spotted wilt virus (TSWV) and tomato chlorotic spot virus (TCSV). The tobacco plants transiently expressing short hpRNAi have exhibited a resistance phenotype and the nucleocapsid gene transcripts were relatively lower in pNhpRNA expressing *Nicotiana benthamiana*. [16]. The transgenic soybean plants expressing *HC-Pro* hpRNAi constructs were resistant to soybean mosaic virus (SMV) and no viral RNA of (*HC-Pro*, *CP* and *CI*) was detected in these plants, while the viral RNA accumulation observed in SMV susceptible soybean lines [32]. Thus, all these reports indicate the potential of RNA interference in combating virus diseases and our method optimizes the screening process to help select the efficient RNAi constructs used to impart virus resistance.

While developing RNAi mediated virus resistant plants, it is essential to evaluate the RNAi constructs prior to stable transformation. To facilitate the evaluation of the effectiveness of the hpRNAi vectors we have optimized a simple method of transient expression using vacuum infiltration. The hpRNAi-MVR vector infiltrated cowpea plants were inoculated with GBNV to analyse the level of infection. We have optimized agroinfiltration by employing vacuum infiltration at three 30 s pulses of 66.6 kPa and Agrobacterial OD_{600} of 0.5. Viral load was reduced to the extent of about 900 folds compared to the vector control indicating efficient viral gene silencing by the infiltrated hpRNAi-MVR. Thus we have demonstrated an optimized protocol to screen hairpin RNAi constructs against Tospoviruses.

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