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ORIGINAL ARTICLE

MYMIV-AC2 protein suppresses hairpin-induced gene silencing in *Nicotiana tabacum* cv. Xanthi

Jamilur Rahman · Neeti Sanan-Mishra · Sunil Kumar Mukherjee

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Abstract Construct encoding self-complementary 'hairpin' RNA (hpRNA) has been shown as an effective trigger of post-translational gene silencing. Gene silencing can be effectively inhibited by virus-encoded silencing suppressors that have been acquired evolutionarily to surmount silencing and allow infection. Here, we show that AC2, the silencing suppressor protein of *Mungbean Yellow Mosaic India Virus* (MYMIV), can inhibit the hpRNA-induced gene silencing *in planta*. *Phytoene desaturase* (*PDS*)-silenced transgenic lines were generated using *PDS*-hairpin gene-silencing construct in *Nicotiana*. The photobleach phenotype was observed in different T1 and T2 *PDS*-silenced lines indicating that the hairpin gene-silencing construct can efficiently down-regulate or silence the endogenous gene activity, and that the silencing is stably inherited over generations. The analysis of abundance of the *PDS* transcripts showed that the degree of silencing ranged from 20 to 70 % in different T0 and T1 transgenic lines. Upon introduction of MYMIV-AC2 in the silenced *PDS* transgenic lines, by genetic hybridization, the *PDS* expression was restored in F1 hybrids. The F1 lines (♀*PDS* X ♂MYMIV-AC2) showed the normal green phenotype instead of the photobleached phenotype indicating that introgression of MYMIV-AC2 in *PDS* silenced T0 lines, suppresses the hairpin gene-silencing. Our findings suggest that hpRNA vector can efficiently and stably induce gene silencing in plants and that MYMIV-AC2 can inhibit this silencing. This is the first report where we put forward that the dual system of hpRNA and MYMIV-AC2 suppressor can serve as an efficient switch for turning off and turning on gene expression, with potential applications in gene function analysis.

Keywords Gene silencing · Viral suppressor · hpRNA · *Phytoene desaturase* (*PDS*) · *Nicotiana*

List of abbreviations

CaMV	Cauliflower mosaic virus
hpRNA	Hairpin RNA
MYMIV	*Mungbean Yellow Mosaic India Virus*
PTGS	Post-translational gene silencing
RNAi	RNA interference
TGS	Transcriptional gene silencing

J. Rahman (✉) · N. Sanan-Mishra · S. K. Mukherjee
Plant Molecular Biology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110067, India
e-mail: jamilsau@gmail.com; rjamilur@ice.mpg.de

N. Sanan-Mishra
e-mail: neeti@icgeb.res.in

S. K. Mukherjee
e-mail: sunilmukherjee@hotmail.com; sunilm@icgeb.res.in

J. Rahman
Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka 1207, Bangladesh

Present Address:
J. Rahman
Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Straße 8, 07745 Jena, Germany

Introduction

Gene silencing, often referred to as RNA silencing or RNA interference, is an evolutionarily conserved mechanism that protects the host genome against invasive nucleic acids, such as viruses, transposons, and transgenes (Hannon 2002). Both post-transcriptional gene silencing (PTGS) and

transcriptional gene silencing (TGS) processes mediate effective defence mechanisms against invading genetic elements (Waterhouse et al. 2001). The PTGS mechanism detects double-stranded RNA (dsRNA) intermediates of the foreign nucleic acids and degrades them into 21- to 24-nucleotide small interfering RNAs (siRNAs). These small RNAs then act as sequence-based guides for specific protein complexes to target and destroy the homologous message-bearing nucleic acids. The dsRNA is thus an effective trigger of gene silencing which operates by sequence-specific RNA degradation (Waterhouse et al. 2001; Sharp 2001).

Introduction of hairpin RNA (hpRNA) can efficiently produce dsRNA in plants and animals. It has been demonstrated that gene constructs producing hpRNA are the most effective method for silencing gene expression in plants (Waterhouse et al. 1998; Smith et al. 2000). Many reports are now available for constructs specifically designed to deliver and express dsRNA in plants, in the form of hpRNA, eliciting a high degree and frequency of silencing of viral genes, transgenes, and endogenous genes (Waterhouse et al. 1998; Smith et al. 2000; Chuang and Meyerowitz 2000; Wang et al. 2000; Wesley et al. 2001). The phenotypic stability of the hpRNA-induced gene silencing over many generations has been considered as a crucial advantage in reliable application of gene silencing in plants and animals (Watson et al. 2005). Such hpRNA constructs have great potential as a tool for gene discovery and functional analysis (Wesley et al. 2001; Somerville 2000).

In plants, gene silencing also serves as an adaptive immune response that restricts accumulation or spread of invading viruses (Waterhouse et al. 2001). However, in what could be considered as an "evolutionary arms race", the viruses encode suppressors of silencing to inhibit the gene silencing pathway, allowing infections in plants. The viral suppressor is a protein or an RNA element that blocks silencing of viral nucleic acid sequences guided by small RNAs, resulting in more severe pathogenic symptoms and diseases (Rahman et al. 2012). The discovery of the viral silencing suppressor itself played an important role in establishing RNA silencing as a natural antiviral response (Li and Ding 2006). Up to now, more than 50 viral silencing suppressors have been characterized from plant viruses and a few of them are also known from insect and animal viruses. Most of the suppressors act by distinct mechanisms and at discrete steps in the RNA silencing pathway and show no sequence relatedness. The AC2 protein of *Mungbean yellow mosaic India virus* (MYMIV), a member of the Geminiviridae family, genus *Begomovirus,* is one of the viral suppressors of RNA silencing (Rahman et al. 2012; Karjee et al. 2008; Singh et al. 2007; Bisaro 2006; Trinks et al. 2005).

AC2 is a multifunctional protein in Begomoviruses as it functions both as a transactivator of viral transcription and as a suppressor of RNA silencing (Trinks et al. 2005). AC2 protein is about ~15 kDa in size with three conserved domains, namely, a basic N-terminal nuclear localization domain, a core DNA binding domain with non-classical Zn finger motif (C37X1C39X7C47X6H54X4H59C60) in the middle, and a C terminus acidic transactivation domain (Trinks et al. 2005). In addition to its core role in viral transcription, AC2 is also known as a strong viral pathogenicity factor. Recently, we demonstrated that MYMIV-AC2 can overcome transgene-mediated gene silencing and enhance the transgene expression *in planta* by enhancing the expression of transgenically silenced *GFP* and *TOPOII* lines (Rahman et al. 2012).

Gene constructs encoding hpRNA are highly effective at inducing the silencing of both endogenous genes and transgenes that are homologous to the silencing effectors. These have been effectively employed in conferring virus resistance in plants (Smith et al. 2000; Chuang and Meyerowitz 2000; Wang et al. 2000; Wesley et al. 2001; Levin et al. 2000; Sijen et al. 2001; Wang and Waterhouse 2004). They have also been shown to induce RNAi in animals (Wang and Waterhouse 2002). In the present study, we investigated whether the suppressor activity of MYMIV-AC2 can overcome the hpRNA-induced gene silencing *in planta*. We used the *phytoene desaturase* (*PDS*) gene as a target of hpRNA because its silencing would result in photobleached leaves, a readily visible phenotype. To investigate whether AC2 can overcome this silencing, MYMIV-AC2 was stably introduced in the hpRNA-induced *PDS*-silenced tobacco lines by a genetic hybridization approach. It was observed that *PDS* expression was stably restored in F1 hybrid populations. The results thus reveal the potential of MYMIV-AC2 to suppress hpRNA-induced gene silencing *in planta*.

Materials and methods

Construction of *PDS* hairpin loop RNA plant binary vector

For the construction of pHANNIBAL-*PDS* hairpin loop vector, sense strand (5′–3′) of *PDS* was cloned into *Xho*I and *Eco*RI sites using forward 5′GCCCTCGAGGGCACT CAACTTTATAAACC3′ and reverse 5′GGAATTCCTTC AGTTTTCTGTCAAACC3′ primers. The *PDS* antisense (3′–5′) strand was cloned into *Bam*HI and *Xba*I sites using forward 5′GCCTCTAGAGGCACTCAACTTTATAAAC C3′ and reverse 5′AGGGATCCCTTCAGTTTTCT3′ primers of pHANNIBAL vector (CSIRO, Australia). *PDS* gene was amplified from the cDNA of *Nicotiana tabacum*

cv. Xanthi by PCR using forward 5′CCGGATCCGGCAC TCAACTTT3′ and reverse 5′AGGGATCCCTTCAGTTT TCT3′ primers using Platinum Taq Polymerase (Invitrogen). The whole cassette of *PDS* hairpin loop (*PDS* sense–intron–*PDS* antisense) was sandwiched between CaMV35S promoter and OCS terminator. To construct the pART27-*PDS* hairpin loop plant binary vector, a ~4.0-kb-long cassette (CaMV35S–*PDS* sense–intron–*PDS* antisense–OCS terminator) was recovered from *PDS*-pHANNIBAL plasmid by *Not*I enzyme to mobilize the cassette into the pART27 plant vector. This pART27-*PDS*-hairpin loop recombinant vector was subsequently used for *Agrobacterium*-mediated genetic transformation to silence *PDS* in tobacco plant.

Agrobacterium-mediated tobacco transformation to silence *PDS*

Agrobacterium-mediated genetic transformation of tobacco was done by using the leaf disc method. The pART27-*PDS* hairpin loop plant binary plasmid vector was transformed into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium* cells containing the pART27-*PDS* hairpin RNAi construct were used for transformation of tobacco explants using the leaf disc transformation method (Horsch et al. 1985). The *Agrobacterium* culture was prepared in YEM broth at 28 °C and 200 rpm until the OD reached 0.6 at λ_{max} 600 nm for plant transformation. Sterile leaf discs of about 3- to 4-week-old WT tobacco were infiltrated with the *Agrobacterium* containing the pART27-*PDS* hairpin RNA construct and were kept for co-cultivation for 2–3 weeks. Following co-cultivation, the explants were selected on regeneration MS medium containing kanamycin (150 mg/L) and cefotaxime (250 mg/L). Calli appeared from the co-cultivated leaf discs in selection and regeneration MS medium within 2 weeks of co-cultivation, while the control leaf discs (without co-cultivation) died down within this period. Shoots started to regenerate from green calli within 3–4 weeks. Shoots measuring approximately 2.0 cm were transferred to half-MS (Murashige and Skoog 1962) medium containing antibiotic for rooting. The rooted seedlings were hardened in vermiculite before being transferred to the soil in earthen pots.

Plant materials and growth condition

Nicotiana tabacum cv. Xanthi wild-type, MYMIV-AC2 transgenic T2 lines and *PDS* silenced transgenic lines plants were grown and maintained at 26 °C, 16 h photoperiod, in a greenhouse. For germination of tobacco seeds, seeds were sterilized with 70 % ethanol for 1 min followed by thorough washes with water. Then, the seeds were kept in MS media containing appropriate antibiotics for 4 days in the dark, after which they were transferred to the light for further growth. Germinated seedlings were then transferred to vermiculite and then to soil pots for maintenance.

Self-fertilization and crossing experiment

Transgenic tobacco plants (AC2 lines and *PDS* silenced lines) were self-fertilized and only selected lines were advanced to the T2 generation. The selection of transgenic MYMIV-AC2 lines was based on phenotypic anomalies, where only the hypomorphic lines were advanced to T2 generation. Transgenically silenced *PDS* plants showing bleached phenotype were used in the subsequent crossing experiment. In the crossing experiment, transgenic *PDS* silenced T2 lines were used as recipient plants, whereas MYMIV-AC2 expressing T2 lines were used as donor plants. The F1 hybrid seeds were germinated in MS-medium containing kanamycin (250 mg/L), and subsequently the F1 population was grown and maintained in the greenhouse.

Genomic DNA isolation and genomic DNA PCR to screen transgenically silenced *PDS* lines

The genomic DNA of putative transgenically silenced *PDS* lines was isolated from the plant tissues using the standard CTAB-based genome-DNA extraction protocol (Sharma et al. 2002) and further used for molecular analysis. Transgenic analysis was carried out using promoter specific primer CaMV35S forward (5′AAGTTGACTGCCTGCAG GTC3′) and *PDS* sense strand reverse primer (5′AGGGAT CCCTTCAGTTTTCT3′).

RNA isolation, Northern blot and RT-PCR analysis

Total RNA was isolated using Trizol reagent (Invitrogen) from young leaves following the manufacturer's instructions. For northern blot analyses, 20 µg of total RNA was separated by electrophoresis on a 1 % formaldehyde agarose gel and blotted to a Hybond-N nylon membrane (Amersham Biosciences). The blots were hybridized with a [α-32P]dCTP-labeled *PDS* probe. For reverse transcriptase (RT), PCR 2 µg of DNase-treated (DNase I, amp grade; Invitrogen) total RNA was used to synthesize first strand cDNAs using SuperScript™ III Reverse Transcriptase (Invitrogen). These cDNA templates were used for PCR amplification of *PDS* with forward 5′ CCGGATCCGGCA CTCAACTTT3′ and reverse 5′AGGGATCCCTTCAGTTT TCT3′ primers. The PCR products were analyzed on a 1 % agarose gel. The relative intensities of the bands were estimated by densitometric intensity analysis using ImageJ software (http://rsbweb.nih.gov/ij/).

Results

Isolation and cloning of partial *PDS* gene

The partial mRNA of *PDS* was amplified from the cDNA of *Nicotiana* (cv. Xanthi) using specific primers (Fig. 1a) to construct the *PDS*-hpRNA vector as described in "Materials and methods". It has been shown that a fragment as small as 98 bases is sufficient for effective gene silencing in hairpin vectors, such as the pHANNIBAL RNAi vector (Waterhouse and Helliwell 2003). The primers amplified a partial *PDS* cDNA of ~409 bp (Fig. 1b) which was then

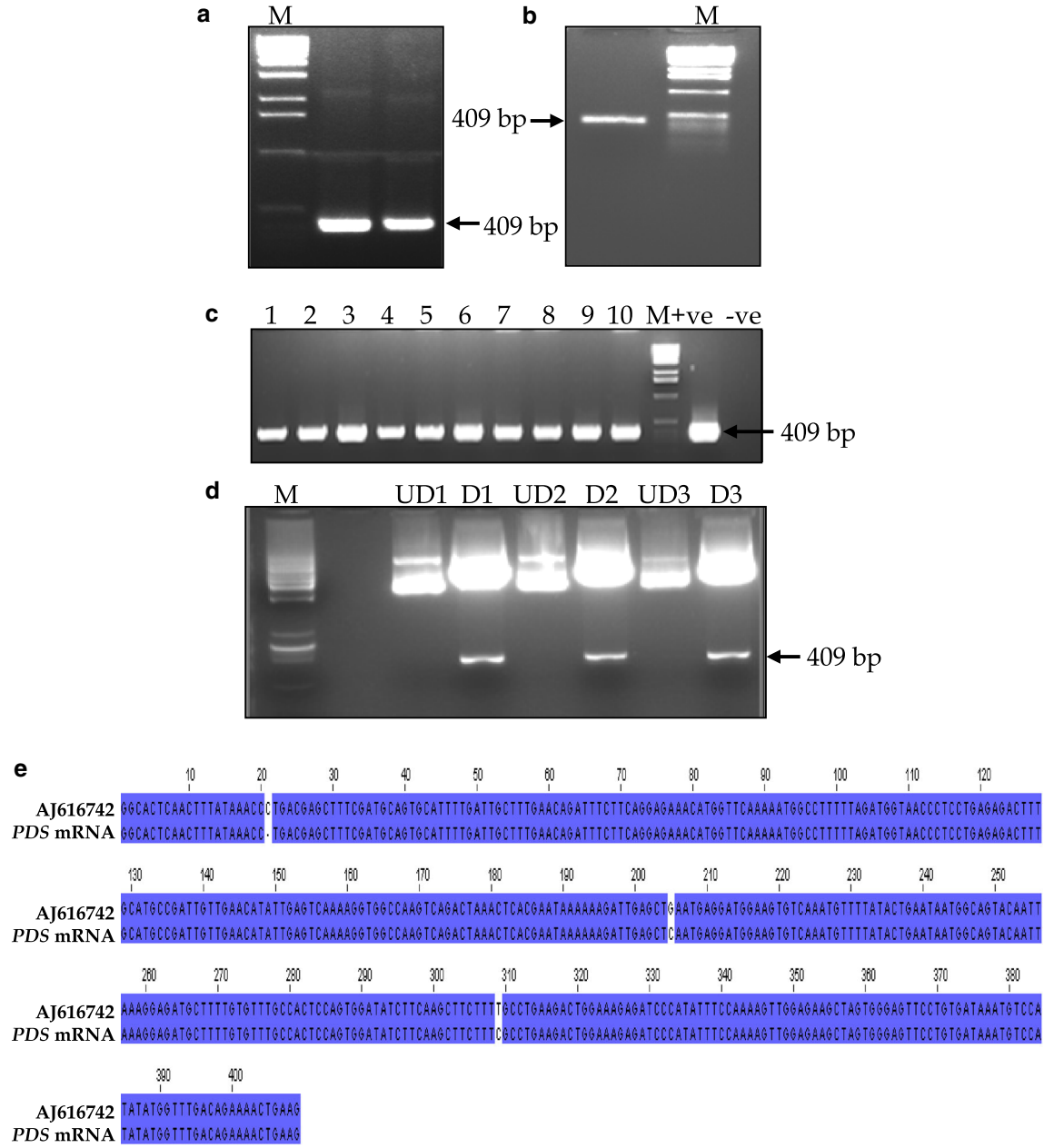


Fig. 1 Isolation and cloning of partial mRNA of *phytoene desaturase* (*PDS*) from *N. tabacum* cv. Xanthi. **a** Ethidium bromide-stained agarose gel showing amplifications of a 409-bp fragment of *PDS* from tobacco cDNA. *M* indicates the 1-kb DNA ladder. **b** 409-bp *PDS* DNA eluted from gel was checked and used for cloning into the TOPO-TA vector. **c** Ethidium bromide-stained agarose gel showing colony PCR of *PDS* cloned into the TOPO-TA vector with *PDS*-specific primers. *1–14* positive clones, *+ve* cDNA used as template in PCR as positive control and *–ve* water used as template in PCR as negative control **d** *Eco*RI digestion of three PCR positive clones showing the 409 bp DNA fragment. *D* digested and *UD* undigested plasmid DNA. **e** ClustalW alignment of nucleotide sequences of AJ616742 (NCBI submitted partial mRNA of *PDS*) with cloned putative partial *PDS* gene

cloned into a TOPO-TA vector (Fig. 1 c, d) and the positive clones were sequenced. The sequence of the cloned partial-*PDS* fragment was aligned with the sequence of NCBI submitted partial-*PDS* gene (NCBI Accession: AJ616742.1; Tao and Zhou 2004), and the ClustalW alignment showed that the sequence obtained from the clone matched to the extent of ~99 % (Fig. 1e). This *PDS* clone was subsequently used to construct the *PDS*-hpRNA vector.

Construction of *PDS* hairpin RNA plant binary vector

To develop the *PDS*-hpRNA vector, the pHANNIBAL RNAi vector backbone was used (Fig. 2a). *PDS* sense strand (5′-3′) was cloned using the *Xho*I and *Eco*RI sites in the pHANNIBAL vector. The clones were checked by colony PCR using *PDS*-specific primers (data not shown) and two PCR positive clones were confirmed by *Xho*I and

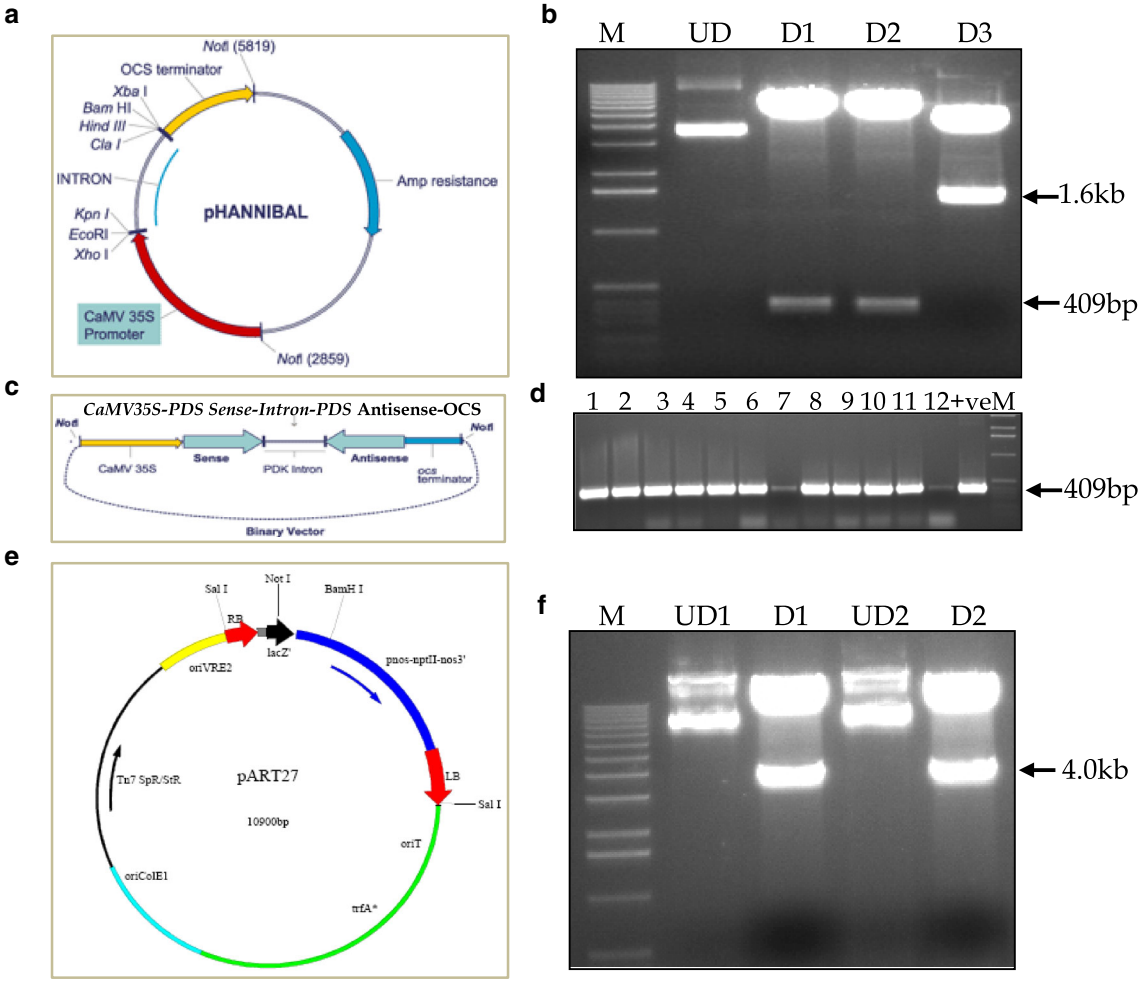


Fig. 2 Preparation of *PDS* hairpin RNAi binary construct to develop transgenically silenced *PDS* tobacco line. **a** Map of pHANNIBAL hairpin RNAi vector were used to prepare *PDS* hairpin RNAi construct. *PDS* sense strand has been cloned into *Xho*I and *Eco*RI sites, while *PDS* antisense has been cloned into *Bam*HI and *Xba*I sites. **b** Ethidium bromide-stained agarose gel shows restriction enzymes digestion analysis of *PDS*-pHANNIBAL RNAi construct. *Lane D1* shows 409-bp DNA fragment of sense strand after digestion with *Xho*I and *Eco*RI enzymes. *D2 lane* shows the DNA fragment of same length antisense strand digested with *Bam*HI and *Xba*I enzymes, while *lane D3* shows the DNA fragment of ~1.6-kb DNA (digestion with *Xho*I and *Xba*I enzymes) that contains sense (409 bp)–intron (800 bp)–antisense (409 bp) strands. *UD* and *M* represent the undigested plasmid and 1-kb DNA ladder, respectively. **c** Schematic representation of whole cassette of *PDS* hairpin loop (*PDS* sense–intron–*PDS* antisense) that is sandwiched between CaMV35 promoter and OCS terminator. This ~4.0-kb length of cassette (CaMV35S–sense–intron–antisense–OCS terminator) was recovered from the *PDS*-pHANNIBAL plasmid by *Not*I enzyme to mobilize the cassette into the pART27 vector (**d**), linearized by *Not*I enzyme. **e** Agarose gel showing colony PCR of *PDS* hairpin construct cloned into pART27 vector with *PDS* specific primers. *1–6* and *8–11* were positive clones, *+ve* (plasmid used as template in PCR) was positive control. **f** *Not*I restriction enzyme digestion of two PCR positive pART27-*PDS* hairpin loop clones shows ~4.0-kb DNA fragment of insert (CaMV35S–sense–intron–antisense–OCS terminator). *D* digested and *UD* undigested plasmids. This pART27–*PDS*-hairpin recombinant vector was subsequently used for *Agrobacterium*-mediated transformation to silence *PDS* in wild-type tobacco

*Eco*RI restriction enzyme digestion (Fig. 2b) of their plasmid DNA. After cloning the *PDS* sense strand into the pHANNIBAL vector, the *PDS* sense-pHANNIBAL construct was further used for cloning of the *PDS* antisense (3′-5′) strand. The *PDS* antisense strand was cloned in *Bam*HI and *Xba*I sites in the *PDS* sense-pHANNIBAL vector and the clones were confirmed by *Bam*HI and *Xba*I restriction enzymes digestion (Fig. 2b) of isolated plasmid DNA. To confirm the final *PDS*-pHANNIBAL hairpin vector, the clones were digested with *Xho*I and *Xba*I restriction enzymes. As expected, a DNA fragment of ~1.6 kb, containing *PDS*-sense (409 bp)–intron (800 bp)–*PDS*-antisense (409 bp), was observed after digestion (Fig. 2b, lane d3).

To prepare the *PDS* hairpin RNA construct, we used pART27 vector (Fig. 2d). The *PDS* hairpin cassette (CaMV35S –*PDS* sense–Intron–*PDS* antisense–OCS terminator; Fig. 2c) was fished out from the *PDS*-pHANNIBAL construct by *Not*I restriction enzyme and cloned into the pART27 plant transformation vector, linearized by the *Not*I enzyme. A ~4.0-kb DNA fragment of *PDS* hairpin cassette (Fig. 2c) released by *Not*I digestion of the plasmid DNA confirmed the positive clones (Fig. 2f). The recombinant pART27-*PDS* hairpin plant construct was used for *Agrobacterium*-mediated transformation in tobacco to silence the *PDS* gene.

Development of *PDS*-silenced transgenic tobacco

PDS-silenced transgenic tobacco lines were developed by *Agrobacterium*-mediated genetic transformation in wild-type (WT) tobacco (cultivar Xanthi). Interestingly, we found some photo-bleached shoots regenerated from the calli of co-cultivated leaf discs within 3–4 weeks (Fig. 3a, b). About 50 full-grown bleached or partially bleached (Fig. 3b, c) shoots were obtained and transferred onto rooting medium. From these, 45 shoots gave rise to profuse roots in rooting medium. We observed a range of photo-bleaching phenotype, from high to medium, in the leaf area of regenerated plantlets (Fig. 3d–i). It was also observed that the putative transgenic lines were dwarf compared to WT control regenerated plants (Fig. 3g, i). The *PDS* hairpin construct, transformed into tobacco, possibly triggered the silencing of the endogenous *PDS*-mRNA so that the formation of *PDS* was disturbed in T0 lines. Therefore, it is logical to assume that the observed photo-bleaching and dwarf phenotypes in *PDS*-silenced T0 lines were due to the disturbance of carotenoid and gibberellic acid biosynthesis pathways.

The dwarf and bleached regenerated plantlets with roots were subsequently transferred to vermiculite and then soil for further maintenance. Interesting, we observed that, after transferring the photo-bleached shoots in the vermiculite in the greenhouse, the bleaching phenotype in the *PDS*-silenced T0 transgenic lines persisted, though it was reduced as compared to that observed in the regeneration and rooting medium. Besides the observed photo-bleached phenotype, all the lines showed lean and feeble growth as compared to that of WT (Fig. 3j, k) in the greenhouse.

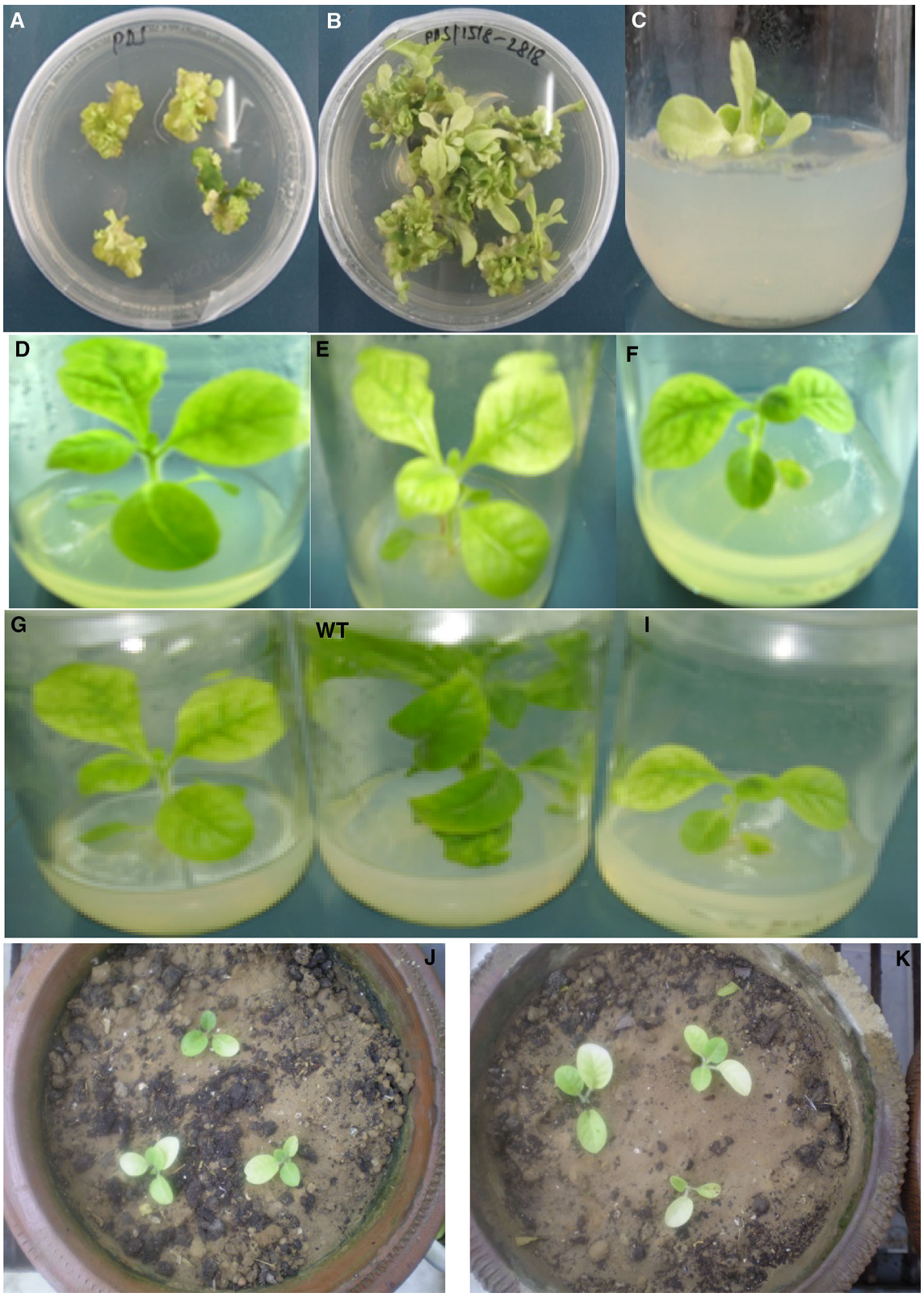
Fig. 3 Development of *PDS*-silenced transgenic tobacco after transformation with *Agrobacterium* containing *PDS* hairpin RNAi binary construct. **a** Bleached shoots regeneration from the calli of co-cultivated leaf discs within 3–4 weeks. **b** Regenerated bleached shoots in Petri plate. **c** Bleached shoots transferred to medium for rooting. **d–f** Three independent photo-bleached regenerated plantlets with roots. A range, from high to medium, of photo-bleached phenotype observed in the leaf area of regenerated plantlets. **g, i** Two putative *PDS*-silenced transgenic plantlets compared with WT (wild-type). Dwarf and photo-bleached phenotypes observed in transgenics compared to WT. **j, k** Six independent silenced or partially silenced transgenic *PDS* T1 lines showing photobleaching and less vigorous phenotypes

Screening and analysis of hairpin-induced silenced *PDS* lines

The putative *PDS*-silenced T0 transgenics were confirmed by molecular analysis. To confirm the presence of the *PDS*-hpRNA construct in the photo-bleached transgenic lines, PCR analysis was performed using CaMV35S forward and *PDS* reverse primers. gDNA from the transformed and WT lines was isolated and used as template in PCR reaction. Amplification of ~0.8-kb fragments from T0 tobacco lines 1, 2, 4, and 5 confirmed their transgenic nature, while no amplification was found from WT and line #3 (Fig. 4). The PCR-positive and photo-bleached T0 lines were subsequently advanced to T1 (Fig. 3j, k) level and used for further experimentation.

To analyze the abundance of *PDS* transcripts in the different T0 transgenic lines, northern analysis was performed using total RNA isolated from different *PDS*-silenced T0 transgenic lines as well as WT tobacco. The analysis revealed that 30–70 % of *PDS* transcripts were silenced in different T0 transgenic lines compared to WT (Fig. 5a).

The T0 lines (lines 1–7, 10, 11, 1,2 and 14) which showed enhanced *PDS* silencing were self-fertilized and advanced to T1 level. The T1 lines which showed bleached or partial bleached phenotype were further analyzed to check for the reduced levels of *PDS* transcript through RT-PCR technique before using them in the crossing experiment. Our data suggest that silencing of *PDS* was stably transmitted to the T1 level and that the silencing ranged from 20 to 60 % in different T1 lines compared to WT (Fig. 5b). Among the T1 progenies of lines 1, 2, 4, 5, 6, and 7, the plants which exhibited better silencing were selected for subsequent crossing experiments.


A
B
C
D
E
F
G
WT
I
J
K

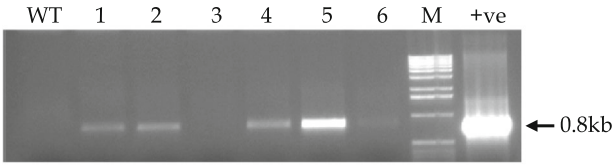


Fig. 4 Screening of transgenically silenced *PDS* T0 lines. PCR of genomic DNA isolated from different T0 lines (lines 1–6) and from the control (WT) line using CaMV35S forward and *PDS* sense strand reverse primers; 0.8-kb amplification of PCR bands from lines 1, 2, 4, and 5 confirmed these transgenic tobacco T0 lines, while no amplification was observed in WT. *+ve* represents TOPO-*PDS* plasmid used as template in PCR and *M* indicates the 1-kb DNA ladder

Reversal of *PDS* silencing in F1 (♀*PDS* X ♂MYMIV-AC2) hybrids

To know whether the suppression activity of MYMIV-AC2 can induce the *PDS* expression from its silenced state, MYMIV-AC2 was introduced in the background of hpRNA-silenced *PDS* lines. Six different MYMIV-AC2-expressing transgenic T2 plants, that had been developed earlier in our laboratory (Rahman et al. 2012), were used as male parents (♂), while six different *PDS* hpRNA silenced transgenic T1 plants were used as the female parents (♀) to generate the cross-combinations. The self-hybridized *PDS* silenced lines (T1) and AC2 expressing lines were used as controls.

The F1 (♀*PDS* X ♂AC2) hybrids were analyzed to check for the induction of *PDS* expression in F1 hybrids. It was observed that all the F1 seedlings showed the normal green phenotype (Fig. 6c–e), while the selfed *PDS*-silenced line exhibited the photo-bleached phenotype (Fig. 6b). The reversal of *PDS* expression in the F1 hybrids was further confirmed by molecular analysis (Fig. 6f, upper panel: F1 lines: 3, 5, 9, 13, 20), where an appreciable amount of *PDS*-mRNA was detected. Next, we investigated whether the reversion of *PDS* in the F1 lines (Fig. 6c–e) was due to suppression activity of AC2, and the presence of the AC2 transcripts was also analzsed. The MYMIV-AC2 transcripts level corroborated well with the *PDS* expression profile. MYMIV-AC2 transcripts were present in all the *PDS*-expressing F1 lines (Fig. 6f, middle panel: F1 lines: 3, 5, 9, 13, 20) but were absent in the *PDS*-silenced T1 line (Fig. 6f, middle panel). This indicates that, after introgression of MYMIV-AC2 suppressor in the *PDS*-silenced

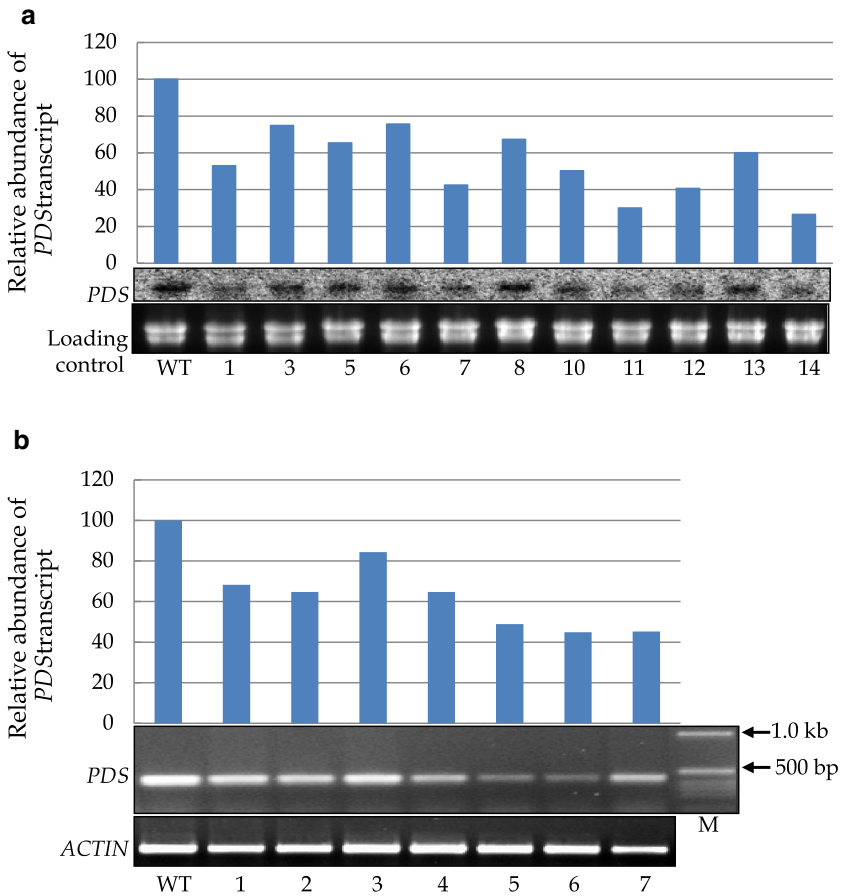


Fig. 5 *PDS* transcripts abundance analysis in T0 and T1 transgenic tobacco lines. The relative abundance of *PDS*-RNA. The RNA level of the WT has been assigned as 100 % and all other values have been calculated relatively. All analysis of **a** and **b** were performed with ImageJ software. *M* indicates 1-kb DNA ladder. **a** Northern blot analysis showing the relative changes in abundance of *PDS* transcripts in different T0 transgenics (*lines 1–14*). Histogram data provide the relative densitometric intensity of the signal normalized against the intensity of the 28S rRNA from EtBr strained blots for the corresponding lane. **b** RT-PCR analysis showing the relative changes in abundance of *PDS* transcript in different T1 lines (*lines 1–7*). Histogram data provide the relative intensity of the signal as deduced by comparing the intensity to that of *ACTIN* for the corresponding lane

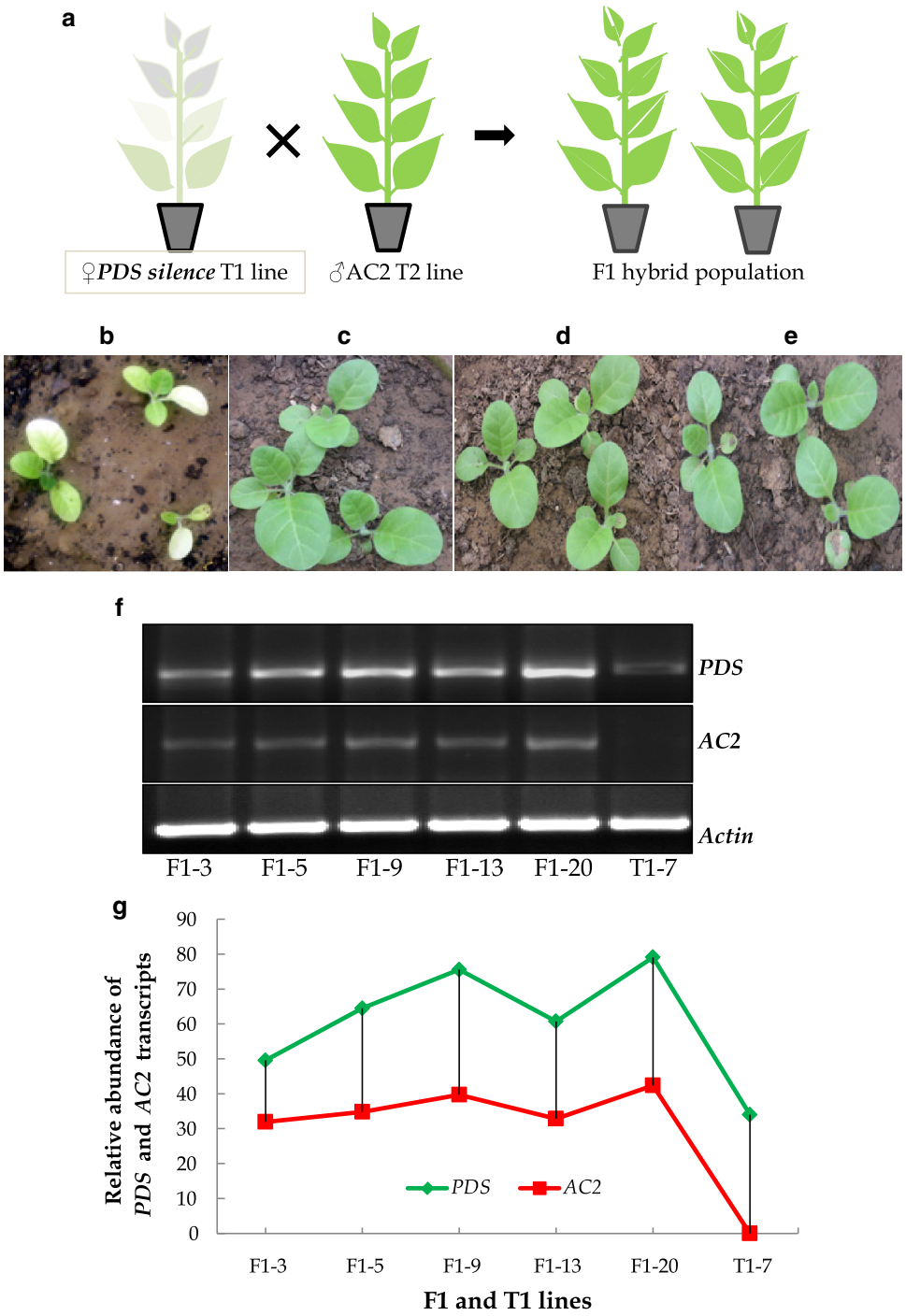


Fig. 6 Analysis of F1 (♀*PDS* X ♂MYMIV-AC2) hybrids. **a** Scheme of crossing. *PDS*-silenced T2 line was selected as the female (♀) parent and AC2 expressing T2 lines as the male (♂) parent to generate F1 hybrids. **b** hpRNA-silenced *PDS* lines showing the photo-bleached phenotype. **c**–**e** The F1 (♀*PDS*×♂AC2) hybrids showing the normal green phenotype due to reversal of *PDS* expression after introgression of MYMIV-AC2 suppressor in the *PDS*-silenced background. **f** RT-PCR analysis of *PDS* and AC2 transcripts abundance in different F1 hybrid lines (F1-3, F1-5, F1-9,F1-13, F1-20) and *PDS*-silenced T1-7 line. ACTIN was used as loading control (*bottom*). **g** The line chart shows correlation of transcripts abundance of *PDS* and AC2 in different F1 hybrids lines. The abundance of *PDS* and AC2 transcripts is presented after normalisation by comparing the ratio of densitometric intensity of the *PDS* and AC2 bands to that of *ACTIN* bands (as loading control). Densitometric intensity was analyzed with ImageJ software. The trend lines of the *PDS* and AC2 transcripts obtained display the positive correlation between the transcript levels in different hybrids lines, as discussed in the text

background, the hpRNA-induced silencing of *PDS* is suppressed. The disappearance of the photo-bleached phenotype in F1 hybrids indicates that suppressor activity of MYMIV-AC2 is capable of blocking the RNAi effect that was induced by the *PDS* hpRNA vector.

A total of ~100 F1 seedlings obtained from 6 crosses were checked for *PDS* expression. We found ~29 % of F1 lines showed green, while ~45 % of F1 lines showed partial green or partial bleached (data not shown) phenotype indicating that a range of *PDS* reversion occurred in different F1 seedlings, while the mother *PDS*-silenced T1 lines appeared as the photo-bleached phenotype. Next, to study a correlation between the expression profiles of *PDS* and AC2 in F1 hybrids, we quantified the band intensity of *PDS* and AC2 transcripts shown in Fig. 6f and prepared a line chart (Fig. 6g). In general, we found that high AC2 expression led to high *PDS* expression (as evident in Fig. 6g, F1 lines 9 and 20), while low AC2

expression caused low *PDS* expression (as evident in Fig. 6g, F1 lines 3 and 13), indicating a correlation between reversion of *PDS* and MYMIV-AC2 expression in F1 hybrids. The lowest level of *PDS* expression was observed in the silenced T1 line where no AC2 expression was observed (Fig. 6f, g, lane T1-7). The trend lines of *PDS* and AC2 transcripts obtained display a positive correlation between the transcript levels in different hybrids lines (Fig. 6g). The green phenotype observed in the leaves of F1 hybrid lines indicated the reversal of *PDS* expression following the introduction of the MYMIV-AC2 suppressor in the *PDS*-silenced background. In the previous study, we found that introduction of MYMIV-AC2 in the silenced transgenic lines showed a high level expression of transgenes as well as a positive correlation between reversion of the silenced transgene and MYMIV-AC2 expression in F1 hybrid population and double transgenic lines (Rahman et al. 2012).

Discussion

RNA interference (RNAi) has been extensively used in various species to suppress the function of an endogenous gene and is becoming a common tool for the functional analysis of the genome (Hannon 2002). In plants, PTGS is triggered by small RNAs that are produced via a mechanism involving the production of dsRNA or self-complementary hpRNA (Waterhouse et al. 2001; Matzke et al. 2001; Vance and Vaucheret 2001). The constructs which would lead to the expression of such transcripts are thus an efficient way of inducing targeted gene silencing (Waterhouse et al. 1998). Transforming plants with hpRNA constructs typically generates a series of independent lines that have varying phenotypes and degrees of target mRNA reduction (Helliwell et al. 2002). In the present study, the endogenous *PDS* gene of tobacco was silenced by using the hpRNA construct. Stable transformation of this construct resulted in a range of photo-bleached *PDS*-silenced progenies in the T0 generation (Fig. 3). The molecular analysis revealed 30–70 % silencing of *PDS* transcripts in the T0 lines (Fig. 5a).

It has been reported earlier that the transformation of plants with hpRNA constructs leads to stable silencing that is inherited from generation to generation, thereby enabling the continued study of silencing phenomena (Waterhouse and Helliwell 2003). For example, the silencing of *Arabidopsis fatty acid desaturase2* (*FAD2*) gene by a hpRNA has been maintained for five generations and has been found to be consistent in its effectiveness (Stoutjesdijk et al. 2002). In the present study, it was also found that the *PDS* silencing was efficiently propagated to the T1 generation and the silencing level remained at 30–70 % (Fig. 5b). Therefore, our results also support that the hairpin gene-silencing constructs can efficiently down-regulate or silence the endogene activity, and that such silencing is also stably inherited over generations.

The length of the target sequence used in the hpRNA construct strongly influences the effective silencing and sometimes has a role in stable and efficient gene silencing in plants and animals. Silencing appears to be most efficient when sequences of more than 300 base pairs are used in hpRNA constructs to target the transcripts (Wang and Waterhouse 2002). However, fragments as small as 98 bases have been shown to be sufficient for effective gene silencing in hairpin vectors, such as pHANNIBAL RNA vector (Waterhouse and Helliwell 2003). It has been suggested that the presence of an intron in the hpRNA vector may enhance its silencing efficiency (Wang and Waterhouse 2002), although the exact role of introns in gene silencing remains to be answered. Interestingly, the hpRNA transgene with a sequence stretch of only 19 base pairs identical to the target gene was also shown to be effective for gene silencing (Wang and Waterhouse 2002). In the present investigation, a 409-bp-long stretch of target *PDS* gene was used to construct the *PDS*-hpRNA vector. Transformation of this hpRNA vector in *Nicotiana* produced a stable and efficient silencing of endogenous *PDS* gene in T0 lines (Fig. 3, 5). Our study also demonstrated that the hairpin loop-induced silencing was stably inherited in the T1 generation.

Viruses encode proteins that have acquired the activity of suppressing small RNA-based gene silencing. This is normally used during infection to overcome the silencing-based first line of host plant defense. Different suppressors such as HC-Pro of *Potyviruses*, P19 of *Tombusviruses*, 2b of *Cucumoviruses*, AC2 of *Begomovirus*, etc. have been shown to suppress gene silencing, although the mechanisms of their action are still under investigation. This study is the first report on the involvement of AC2 in suppressing the hpRNA-induced gene silencing. On the introduction of the MYMIV-AC2 suppressor in the hpRNA-induced *PDS*-silenced background by crossing between *PDS*-silenced (♀) and MYMIV-AC2 (♂) lines we observed a reversal of the photo-bleached *PDS* phenotype in the F1 hybrid population (Fig. 6). The observed normal green phenotype in the F1 lines was comparable to that of WT indicating that the *PDS* silencing was blocked by the suppression activity of MYMIV-AC2.

Conclusion

Virus-encoded silencing suppressors have the potential to overcome post-transcriptional gene silencing. Each suppressor acts by distinct mechanisms and at discrete steps in

the RNA silencing pathway. The findings obtained in this study suggest that MYMIV-AC2 can inhibit hpRNA-induced silencing. Our earlier studies have shown that MYMIV-AC2 can overcome transgene-mediated gene silencing to enhance its expression *in planta* (Rahman et al. 2012). Taken together, the results indicate that MYMIV-AC2 can be used efficiently to suppress both transgene-induced and hpRNA-induced siRNA-mediated gene silencing. This is the first report on the involvement of AC2 in suppressing hpRNA-induced gene silencing. We suggest that the dual system of hpRNA and MYMIV-AC2 suppressors can serve as an efficient switch for turning off and turning on gene expression, with potential applications in gene function analysis.

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Conflict of interest The authors declare that they have no competing interests.

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