Deciphering the role of fungal calmodulin gene using Host-mediated gene silencing approach in apple

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Abstract

Premature leaf fall caused by *Marssonina coronaria* is one of the most destructive diseases of apple in India. In this study, host induced gene silencing approach was exploited to develop resistance to this disease in an apple cultivar 'Red Chief'. Calmodulin gene (CaM) having its role in fungal differentiation, development and pathogenicity was selected as target. hpRNAi construct was prepared from the conserved off target free partial gene sequence of CaM and used for transformation trials. Upto 6% kanamycin resistant shoots were obtained on selective medium having 5–6 mg/l kan after 7 weeks of coculture. In PCR analysis of 13 RNAi putative transformants, 10 lines were found positive with CaM and nptII gene specific primers and six lines showed hybridization signal. Semi qRT-PCR revealed variable levels of transgene expression among RNAi lines which seems to be related to copy number of integrated gene. *In vitro* detached leaf assay revealed lesion development and disease progression in wild type after 5 dpi but not visible in five CaM RNAi lines. Microscopic examination of infected control leaves showed fully developed, septate mycelium, and conidia along with necrosis of whole tissue while three transformants showed reduced growth and differentiation of fungus and in rest three, hyphal development and necrosis were strongly restricted. We conclude that trafficking of dsRNA/ siRNA from apple plants to pathogen might have triggered the down regulation of fungal CaM gene which confirms that deciphering the role of CaM through HIGS lead to resistance to *Marssonina blotch* in apple.

Key Message

Reduction in fungal growth and differentiation in leaves of RNAi transformants of 'Red Chief' is associated with down regulation of fungal CaM gene expression as compared to wild type.

Introduction

M. coronaria (Ellis & J.J. Davis) also known as apple blotch fungus is a higher fungi i.e. Ascomycota that belongs to family Dermateaceae. *Marssonina blotch*, causing premature defoliation of leaves, is one of the most devastating diseases of apple in India and other apple producing countries (Lee et al. 2011). There are several reports demonstrating that this disease can cause serious defoliation and reduce tree growth, flowering, fruit quality, as well as yield (Harada et al. 1974; Lee et al. 2011; Sharma and Sharma 2006; Zhou et al. 2012). In HP, premature leaf fall is one of the serious concerns causing considerable losses of approximately 65 to 80% defoliation prior to harvest (Sharma and Verma 1999; Sharma and Sharma 2006). Most of the commercial apple cultivars are susceptible to this disease (Li et al. 2012). 'Red Chief' is one of these but an important spur cultivar recommended for commercial production in India. It is a limb sport of 'Starkrimson'. About 92.70% disease incidence and 49.45% disease intensity were observed in this cultivar in polyhouse conditions (Rather et al. 2017). Disease appears as small black lesions (early symptoms) on leaves which further coalesce, cause them to become yellow and lead to the premature leaf fall. In later stages, it also infects fruits making them unsuitable for sale thus leading to direct economic loss (Sharma and Gautam 1997). Characteristics of this fungus is the formation of an ascus containing ascospores which are released by sexual fruiting bodies (apothecia)

from overwintered leaves and dispersed by the wind. In case of asexual stage, acervuli develop at the beginning of the disease cycle after intensive rain periods in spring and early summer (Sharma et al. 2004) and conidia are released by acervuli. A penetration peg pierces the cuticle and epidermis after appressoria formation. Penetration through stomata has not yet been observed (Zhao et al. 2013). During infection, subcuticular hyphae develop between epidermal cells and cuticle. Mycelia grow in the intercellular space and attack the host cell wall. In India, protective fungicidal spray program is adopted every year to keep the disease under check (Kumar and Kumar 2014). The excessive use of fungicides, their adverse effects on the environment and human health, and development of resistant pathogen population becoming less sensitive towards agrochemicals (Yang et al. 2012) have been major concerns in recent years. Therefore, this phytopathogenic fungus now requires new methods of control which are environmentally benign.

In recent years, transgenic plants with improved resistance to biotic stress have been developed via RNAi technology. All the eukaryotes including plants exhibit RNA silencing machinery that plays an essential role in regulating various development processes as well as in providing defense from invading pathogens. RNAi technology can be employed to control the plant diseases based on the idea that post transcriptional gene silencing (PTGS) of target gene can be done by *in vitro* feeding of dsRNA in various plant pathogens like insects, fungi, and the resistance to infecting pathogens in host plants is conferred by the expression of such dsRNAs. This approach has been termed as Host Induced Gene Silencing (HIGS) and emerged as an alternative to plant protection because of its selectivity for desired target gene with less side effects as compared to the other biotechnological approaches.

This strategy involves the down regulation of one or more important pathogen genes that are required for growth, development and pathogenicity through the uptake of gene specific dsRNAs or siRNAs induced by transgenic plants during the course of infection (Ruiz Ferrer and Voinnet 2009). Huang et al. (2006) firstly applied this technology in Arabidopsis to target the vital genes of root-knot nematodes by expressing specific dsRNA in transgenic host plants. After that, HIGS has been successfully tested against a number of fungal pathogens which includes *Puccinia striiformis* and *P. tritricina* in wheat (Yin et al. 2009; Panwar et al. 2013), *Blumeria graminis* in barley and wheat (Nowara et al. 2010), *Fusarium graminearum* in barley (Koch et al. 2013), *F. oxysporum* in banana (Ghag et al. 2014) and *Bremia lactucae* in lettuce (Govindarajulu et al. 2015) to develop fungal resistant plants.

Infection process in plants by fungal pathogens is highly specific and regulated. For RNAi induced gene silencing in fungi, vital genes which are involved in fungal differentiation, invasion, growth and pathogenicity are of greater interest. Therefore, the identification of these important genes is a prerequisite and the most crucial step for higher success rate of HIGS (Pareek and Rajam 2017). Calcium plays crucial and numerous roles in different fungi e.g. important for mycelial dimorphism in *Ceratocystis ulmi* (Muthukumar and Nickerson 1984), serves as a branching signal in *Fusarium graminearum* and *Neurospora crassa* (Robson et al. 1991; Reissig and Kinney 1983) and helps in appressorium formation in *Metarrhizium anisopliae* and *Colletotrichum trifolii* (Leger et al. 1990; Warwar and Dickman 1996). Calmodulin (CaM) is a highly conserved acidic protein present in all eukaryotic cells. Its primary

role is to serve as an intracellular Ca^{2+} receptor, participating in signaling pathways and cellular processes involving protein kinases, phosphatases, phosphodiesterases and cytoskeletal proteins (Carvalho et al. 2003), leading to proliferation, motility, and cell cycle progression. Ca^{2+} -mediated protein phosphorylation is one of the important mechanism in eukaryotic cells by which they transduce extracellular signals into intracellular responses. Carvalho et al. (2003) also described the participation of calmodulin gene in fungal differentiation and development of specialized structures (appressoria) in phyto and entomopathogenic fungi. Kim et al. (1998) explained the role of Ca^{2+} -calmodulin signalling in controlling spore germination and differentiation in *Colletotrichum gloeosporioides*. Despite these reports, CaM gene has not extensively been targeted for HIGS approach. However, these studies made us to believe that CaM gene could be a potential target to decipher its function using RNAi.

In recent studies in our laboratory (Chauhan et al. 2018), the calmodulin gene has been isolated from *Marssonina coronaria* and cloned in pCambia1300 vector. For cloning of hpMcCaM gene silencing cassette, 381 bp off targeted region was amplified from fungus and hpRNAi construct was developed. RNAi was used to validate the function of Mc-CaM gene in *M. coronaria* which showed morphological abnormalities in fungal transformants colonies and conidia production. Transformed conidia were unable to develop blotch in apple leaves after 15 days post infection as compared to the untransformed wild type (Chauhan 2019). Thus, the obtained results encouraged us to transform apple using HIGS approach with the expectation of enhancing fungal resistance following disruption of CaM gene of *M. coronaria*. Therefore, in the present investigation, we describe the preparation of silencing construct of hpMcCaM for host induced gene silencing (HIGS) in apple cultivar 'Red Chief' for which hpRNAi cassette was cloned in binary vector pRI 101-AN. Apple transgenic lines were generated which exhibited full/ partial resistance to pathogen. Single and double copy transgenic events were generated which are correlated to level of gene silencing.

Material And Methods

Construction of silencing binary vector having CaMcds in sense and antisense orientation

For apple transformation, pRI 101-AN vector of about 10.417 kb (Fig. 1) was used because it has all the desirable features for expression of a target gene in plants. First, restriction maps of selected DNA fragment was prepared by using NEB cutter V2.0 to identify the restriction sites in the gene for creation of restriction sites in primers to facilitate cloning into vector. The selected fragments were used for further primer designing manually using customized parameters (Sambrook et al. 1989) and were crosschecked with web based DNA Calculator software program (Sigma). Before cloning CaM partial cds in pRI 101-AN vector, 314 bp of CaM target gene sequences were amplified from cDNA of *M. coronaria* isolate Himachal Pradesh by using primers CaMS-F (Sal1)/CaMS-R (Kpn1) GGGTCGACCCCTCCAAGCTCGCAAAGAA/CCCGGTACCTCCACTGCCAGTAAACAGCTG and CaMAS-F (BamH1)/CaMAS-R (Kpn1) GGGCCGGATCCCCCTCCAAGCTCGCAAAG/ GGGCCGGTACCGCCGTGTGCGGGTTCGATT for sense and

antisense, respectively, having restriction sites specific to the vector to facilitate the cloning. The antisense fragment was designed to be 50 bp shorter than the sense. The amplified sense / antisense fragments were cloned into pTZ57R/T cloning vector. The positive clones of CaM were digested with *Sal* I & *Kpn*I and cloned into the *Sal* I & *Kpn*I restriction sites of pRI 101-AN vector individually, resulting in pRI 101-AN-CaM-S vector. Further, the positive clones of pTZ57R/T antisense of CaM was digested with *Kpn*I & *Bam*HI and cloned into the same sites of pRI 101-AN-CaM-S vector resulting in RNAi-pRI 101-AN-CaM vector.

The binary vector pRI 101-AN-CaMRNAi was mobilized into *Agrobacterium tumefaciens* EHA105 by freeze thaw method (Sambrook et al. 1989). These transformed colonies were checked by plasmid PCR with gene-specific primers. The positive ones were used for apple transformation.

Bacterial strain and construct

Agrobacterium tumefaciens strain EHA105 harbouring the plasmid pRI101-AN hpRNA:CaM, containing 381bp region of calmodulin gene in sense and antisense orientation under the control of CaMV 35S promoter and NptII gene as selection marker in plant under the control of NOS promoter and terminator was used for transformation trials. Cultures were maintained in Luria-Bertoni Agar (LBA) medium (Sambrook et al., 1989) containing kanamycin (50mg/l) and rifampicin (15mg/l), and incubated at 28°C.

Source of explant and in vitro cultivation

In vitro axillary shoots of apple cultivar 'Red Chief' were used as the source of leaf explants for transformation experiments. These axillary shoots were maintained in tissue culture laboratory of the Department of Biotechnology, Dr. YSP UHF, Nauni (H.P.) by regular monthly sub-culturing on multiplication MS (Murashige and Skoog 1962) medium supplemented with 0.8 mg/l benzyladenine (BA), 0.5 mg/l gibberellic acid (GA₃), 0.1 mg/l indole butyric acid (IBA) and solidified with 8 g/l agar (Kumari, 2018). All the cultures were grown in the incubation room at 25°C with a 16/8 (light/dark) photoperiod. Young, fully expanded leaves from four weeks old proliferating cultures were excised, wounded and cultured on recently established shoot regeneration medium containing 5mg/l BA and 0.5mg/l naphthalene acetic acid (NAA) for the control experiment (Kumar et al. 2021).

Cocultivation of leaf explants with Agrobacterium

Single bacterial colony was inoculated into 25 ml of LB broth containing the above mentioned antibiotics and grown at 28 °C and 200 rpm overnight. The culture was then pelleted at 8000 rpm for 5 min at 4°C, and dissolved in 25 ml MS basal liquid medium. 1 ml of this culture was mixed in 20 ml of same medium. For infection, 2 days precultured leaf explants were transferred into 10 ml of MS basal liquid medium containing 200 µl of bacterial suspension for 3–15 min with gentle pricking and shaking. Agroinfected leaves were dried by soaking in sterile filter paper and cocultivated in dark for 1–4 days on shoot regeneration medium (MS + 5 mg/l BA and 0.5 mg/l NAA). Further, in order to get putative shoots, 4 rounds of subculture after every fifteen days were carried out on the selective shoot regeneration medium containing 500mg/l cefotaxime to control the bacterial growth and 5-9mg/l kanamycin for shoot

selection. However, at each subculture, the concentration of cefotaxime was reduced to 200 mg/l. Putative RNAi transgenic shoots that emerged from leaf explants were excised and micropropagated on medium with 5 mg/l kanamycin and 100 mg/l cefotaxime. Shoot regeneration was measured as percent explants forming the shoots. Each regenerated shoot induced from a single explant was considered as a line.

Genomic DNA isolation and transgene integration in RNAi lines

Total genomic DNA was isolated from fresh leaves of untransformed control (wild type) and putative RNAi transgenic shoots using the method followed by Virseck Marn et al. (1999) and purified using RNase treatment. PCR confirmation of the transgene was done with nptII and CaM gene specific forward and reverse primers. The nptII primer sequences were (F) 5'-CTTCCCGCTTCAGTGACAAC-3'; (R) 5'-TTGGGTGGAGAGGCTATTCG-3' and CaM sequences were (F) 5'-ATTCTAGAGGCGCGCCCCCTCCAAGCTCGCAAAAG-3'; (R) 5'-GCGGATCCATTAAATTCCACTGCCAGTAAACAGCTG-3', respectively. The PCR reaction was run in Thermal Cycler (Eppendorf, Germany) and the reaction conditions were used as follows: denaturation initially at 95°C for 5 min, 35 cycles at 95°C for 30 sec, primer annealing for one min at 58°C, extension at 72 °C for 30 sec and a final extension at 72 °C for 8 min. The final PCR product was analysed on appropriate concentration of agarose gel (2%).

Southern blotting for transgenic RNAi lines

To ensure the T-DNA integration, 15ug of genomic DNA of each PCR positive RNAi transgenic line was restricted with EcoR1 enzyme and electrophoresed on 1% agarose gel, run overnight at 25–30 volts to get an evenly restricted gDNA. It was transferred by capillary action onto a nylon membrane (mdt, membrane technologies, India) in 2XSSC buffer and subjected to hybridization with 32P labelled nptII primers as described by Sambrook et al. (1989). Labelling of the probe was carried out using DecaLabel DNA Labeling Kit (Thermo Scientific). The pattern of hybridization was visualized in phosphorimager.

Semi-quantitative RT-PCR analysis

Transcriptional variation of CaM gene level was analyzed using semi qRT-PCR. Total RNA was isolated from young, fresh leaves of 40 days old cultures of untransformed control and putative RNAi transgenic shoots using SV Total RNA Isolation System (promega). First-strand cDNA was synthesized from 500 ng of the extracted RNA using High-Capacity cDNA Reverse First-strand cDNA Kit (Thermo scientific) according to the manufacturer's recommendations. Template RNA (500 ng), oligo (dt) primer and nuclease free water were added in appropriate volume in DEPC treated tube, centrifuged and incubated at 65°C for 5 min. Master mix was prepared by adding 5X reaction buffer, RiboLock RNase inhibitor, 10mM dNTP mix and RevertAID RT (200 µl) followed by addition of RNA samples into each reaction, centrifuged for proper mixing. Reverse transcription PCR was performed with the programme viz., step 1: 42°C for 60 mins, step 2 : 80 °C for 5 mins, step 3: 12° C for ∞. Apple actin gene (house keeping gene) was used as internal control. Mdo-Actin primers F-AACAGATGTGGATTGCAAAGGC and R-

GTGTAGATTCTTTGTTCCCCGC were designed from *Malus domestica* actin (LOC103424317) gene from GenBank (melting temperature 60–62°C) by using NCBI Primer-BLAST. Equal amounts (2 µl) of first-strand cDNA obtained from control and Southern positive RNAi transgenic lines were used as template for the PCR reactions. Primers for CaM gene for qPCR were F-GAGGTGTCCGGAGAGCTATG and R-ATCAGATGTGCGGGTTCGAT. Amplification was carried out with 35 cycles of 45 sec denaturation at 94°C, primer annealing at 61°C for actin and 57°C for CaM for 45 sec, extension at 60°C for 1 min and final extension for 7 min. The product was analysed on 2% agarose gel.

Detached leaf assay

In this experiment, fully expanded fresh leaves of *in vitro* grown Southern positive RNAi transformed lines and control shoots were cut gently (without wounding) and placed on the basal medium. Fungal spore suspension was prepared in sterile water with a final concentration of 10⁶ spores/ml from 15 days old fungal culture. 1µl of this spore suspension was inoculated on leaves of all types of plants. Petriplates were incubated at 22–25°C under high humidity (90–95%) and 16:8h light:dark photoperiod. Visual observations were taken after 5, 15 and 20 days interval for lesion/blotch like symptom development on inoculated leaves of RNAi transformants and compared with the wild type plants inoculated similarly. Water control was taken as negative control.

Microscopic examination

20 days after fungal infection, thin sections of infected leaves were examined under the microscope at 20X magnification using light microscope (Nikon Eclipse CI, Japan). Invasion, growth and differentiation of fungus was observed and photographed.

Rooting and hardening of RNAi transformants

For rooting, shoots were transferred to basal medium after 7 days of incubation in ½ strength liquid MS medium containing 0.3mg/l IBA. Any difference in root induction and rooting frequency in wild type and RNAi transformants were noted. After rooting, the plantlets were hardened in cocopeat and vermicompost mixture in ratio of 5:1.

Experimental design, data collection and statistical analysis

The experiments were conducted using completely randomized design (CRD) for single factor experiment with each treatment in triplicates. The data were subjected to analysis of variance and differences among mean values were carried out according to Duncan's Multiple Range Test $p < 0.05$ using SPSS software version 16.0 (SPSS Inc., Chicago, USA)

Results

Preparation of hpRNAi construct with CaM gene for apple transformation

The graphic representation of the cloning strategy to prepare the apple RNAi construct using pRI 101-AN vector is depicted in Fig. 2. The primer pair CaMS-F (*SaI*)/CaMS-R (*Kpn*1) having additional sequences of *SaI* in the forward primer and *Kpn*1 in the reverse primer resulted in the amplification of 381 bp from the fungus. Whereas, another primer pair CaMAS-F (*Bam*H1) / CaMAS-R (*Kpn*1) was able to amplify 330 bp of target antisense fragment of CaM gene. These amplicons were cloned into pTZ57R/T vector. Putative recombinant colonies were screened by colony PCR using CaM specific primers generating 381 bp and 330 bp amplicons for sense and antisense, respectively. The PCR positive clones were confirmed by digestion with *SaI* and *Kpn*1 for sense cloning and *Kpn*1 and *Bam*H1 for antisense, which released out ~ 381 bp sense and ~ 330 bp antisense fragment (Figs. 3a, b) The pTZ57R-CaM-S and pTZ57R-CaM-AS cloning was finally validated by sequencing.

Further, pRI 101-AN plasmid vector was linearized with *SaI* and *Kpn*1 (Fig. 3c). The confirmed clone of pTZ57R-CaM-S was also digested with *SaI* and *Kpn*1 and ligated to this linearized backbone of the pRI 101-AN to develop pRI 101-AN-CaM-S clones. Cloning was confirmed by colony PCR and restriction digestion in similar manner generating 381 bp for sense in putative recombinants and the fallout (Fig. 3d). Subsequently, pRI 101-AN-CaM-S digested with the *Kpn*1 and *Bam*H1 resulted in linearized vector backbone for antisense cloning (Fig. 3e) which was further cloned to develop putative pRI 101-AN-CaMRNAi. Cloning was confirmed by colony PCR and digestion with *Kpn*1 and *Bam*H1 generating ~ 330 amplicons for antisense, in putative recombinants (Fig. 3f). The restriction digestion confirmation of the pRI 101-AN-CaMRNAi construct with *Bam*HI&*Kpn*1; *Bam*H1 & *Eco*R1 and *Nde*I & *Eco*R1 to release sense, antisense and sense + antisense, respectively is clear in Fig. 4. The results obtained from the mfold revealed the formation of stable hairpin loop dsRNA (Fig. 5).

After mobilization into *A. tumefaciens* EHA105, the plasmids of putatively transformed *A. tumefaciens* colonies were checked for the presence of CaMRNAi cassette by PCR using gene specific primers which corresponded to ~ 381 bp and 330 bp for CaM sense and antisense respectively (Fig. 6a,b). The plasmid PCR positive clones were used for apple transformations in this study.

Optimization of infection and co-cultivation time

It was observed that 20.7%, 45.9%, 61.0%, 79.7%, leaf explants got infected using 3,5,7 and 9 mins infection time respectively. Highest frequency (92%) of explant infection was obtained after 2 days co-cultivation period when 2 days precultured leaves were infected for 11 mins (Table 1). Whereas, further increase in infection time (13 & 15 mins) led to 98.8 and 100% explant infection even after one day of cocultivation. But excess bacterial overgrowth was found over the leaf explants which made difficult to blot these as they stick to blotting sheets. Moreover, yellowing of leaf explants was also observed. Therefore, 11 min infection time with two days co-cultivation period were found effective for transformation trials.

Table 1

Effect of Agrobacterium infection time duration on leaf explants of apple cultivar 'Red Chief'

Effect of Agrobacterium infection time duration on leaf explants of apple cultivar 'Red Chief'			
S.No.	Infection time duration (min)	Explants infected Mean $\pm$ SE	Explants regenerated Mean $\pm$ SE
1	0	0 $\pm$ 0.0 ^a	92.5 $\pm$ 0.17 ^a
2	3	20.7 $\pm$ 0.17 ^b	0 $\pm$ 0.0 ^b
3	5	45.9 $\pm$ 0.59 ^c	2.1 $\pm$ 0.05 ^c
4	7	61.0 $\pm$ 0.14 ^d	3.7 $\pm$ 0.11 ^d
5	9	79.7 $\pm$ 0.17 ^e	5.8 $\pm$ 0.11 ^e
6	11	92.2 $\pm$ 0.11 ^f	9.8 $\pm$ 0.11 ^f
7	13	98.8 $\pm$ 0.11 ^g	0.0 $\pm$ 0.0 ^b
8	15	100 $\pm$ 0.0 ^h	0.0 $\pm$ 0.0 ^b

The data were statistically analyzed using Duncan's multiple range test (DMRT). For each variable studied, experiments were repeated three times with 30 explants. Data represent the mean $\pm$ standard error (SE) of three experiments. In the same column, significant differences according to the least significant difference (LSD) at the $P < 0.5$ level are indicated by different letters.

Selection of the putative transformed shoots

After co-cultivation in dark, 500mg/l cef was found effective to inhibit the Agrobacterium excess growth in the present study. In order to reduce the combined stress of both the antibiotics on transformed leaf explants, the concentration of cef was reduced gradually after every 15 days interval from 500 to 200 mg/l. It was observed that with kan at 5–6 mg/l, callus was induced three weeks after cocultivation. Frequency of putative transformed leaf explants regenerating direct shoots were 6% and 3.6% on 5 and 6 mg/l kan respectively on decreased cef level (200 mg/l) seven weeks after cocultivation (Table 2, Fig. 7c-e). Whereas untransformed control shoots regenerated normally after five weeks of culture on medium without antibiotics (Fig. 7a). Very less callus induction and shoot regeneration (1.6%) were observed eight weeks after co-cultivation on 7 mg/l kan (Fig. 7e). It has further been observed that only two albino shoots were obtained on medium with 8 mg/l kan (Table 2). At a little higher dose of 9 mg/l kan with 500 mg/l cef, there was no callus induction and leaves started turning yellow one week after coculture. After shifting these explants to reduced dose of cef (200 mg/l), no callus induction and regeneration were observed and progressive leaf necrosis was developed. Untransformed leaves were unable to regenerate and became necrotic and dead on medium supplemented with similar doses of antibiotics (Fig. 7b).

Table 2

Regeneration of putative transgenic shoots on selective medium with kanamycin and cefotaxime

S.No.	Antibiotic		Frequency of explants regenerated Mean $\pm$ SE	Number of shoots per explant	Shoot type
	kanamycin(mg/l)	cefotaxime (mg/l)			
1	0	0	92.5 $\pm$ 0.17 ^a	3–4	Direct
2	5	200	6.0 $\pm$ 0.11 ^b	1–2	Direct
3	6	200	3.6 $\pm$ 0.23 ^c	1–2	Direct
4	7	200	1.6 $\pm$ 0.23 ^d	1	Direct
5	8	200	0.8 $\pm$ 0.11 ^e	1	Direct and albino
6	9	200	0.0 $\pm$ 0.00 ^f	-	-

The data were statistically analyzed using Duncan's multiple range test (DMRT). For each variable studied, experiments were repeated three times. Data represent the mean $\pm$ standard error (SE) of three experiments. In the same column, significant differences according to the least significant difference (LSD) at the $P < 0.5$ level are indicated by different letters.

In the present trial, 16 independent transformation experiments (events) on 'Red Chief' apple were performed with a total of 640 explants. Among them, five events producing no putative transgenic line showed 31% failure. If two shoots arose from one wounded site, only one was cloned and counted as separate line. A total of 22 putative RNAi transgenic shoots were recovered, some of which were isolated for further studies (Table 3, Fig. 8a,b). Overall, 3.43% putative transformants raised while actual transformation efficiency has been found to be 1.56% (Table 4). The selected transgenic lines were micro propagated on medium containing 6 mg/l kan with 100mg/l cef (Fig. 8c,d). Each line was maintained individually in cultures. Multiples shoots were obtained on all putative lines after four weeks (Fig. 8e). Wild type (control) and putative transgenic shoots were morphologically similar showing equal multiplication rates. Whereas, albino putative shoots obtained on 8 mg/l kan were unable to multiply and became necrotic after one week of multiplication.

Table 3
Data showing number of RNAi putative shoots and their molecular analysis

RNAi events	Number of leaf explants producing kan resistant shoots	Total number of RNAi putative shoots	Type of shoots	Number of PCR positive lines		Southern blot analysis with 32p-nptII primers		Semiq-RT-PCR band intensity
				With CaM primers	With nptII primers	Number of positive lines	Gene copy number	
RNAi – 1a,b	2	2	Direct	2	2	-	-	-
RNAi- 2a,b,c,d	2	4	Direct	2	2	1	1	++
RNAi – 3	0	0	-	0	0	-	-	-
RNAi – 4a,b	1	1	Direct	1	1	1	1	++
RNAi – 5	1	0	Albino	0	0	-	-	-
RNAi – 6	0	0	-	0	0	-	-	-
RNAi – 7a,b	2	4	Direct	1	2	1	2	+++
RNAi – 8a,b	1	2	Direct	1	2	1	2	+++
RNAi – 9	0	0	-	0	0	-	-	-
RNAi – 10a,b	1	1	Albino	0	0	-	-	-
RNAi – 11a,b	2	2	Direct	1	2	1	2	+++
RNAi – 12	0	0	-	0	0	-	-	-
RNAi – 13a,b	1	2	Direct	0	0	-	-	-
RNAi – 14	0	0	-	0	0	-	-	-

*Total number of explants culture 45– 50 per event.

+ Low band intensity; ++ Average band intensity; +++ High band intensity

RNAi events	Number of leaf explants producing kan resistant shoots	Total number of RNAi putative shoots	Type of shoots	Number of PCR positive lines		Southern blot analysis with 32p-nptII primers		Semiq-RT-PCR band intensity
				With CaM primers	With nptII primers	Number of positive lines	Gene copy number	
RNAi – 15a,b	2	2	Direct	1	1	1	1	+
RNAi – 16a,b	2	2	Direct	1	1	-	-	-
Total	17	22		10	10	6		
*Total number of explants culture 45– 50 per event.								
+ Low band intensity; ++ Average band intensity; +++ High band intensity								

Table 4
Transformation efficiency

Total number of inoculated leaves	Total number of putative shoots obtained	Putative transformation frequency	Number of lines checked	Number of PCR positive RNAi lines	Actual transformation frequency
640	22	3.43	13	10	1.56

Transgene integration in putative RNAi transformants

A total of 13 putative RNAi transformants from independent events were taken individually for DNA isolation. PCR was run with DNA of RNAi putative shoots, untransformed shoots as negative control and plasmid as positive control. PCR analysis revealed that 10 out of 13 kan resistant putative RNAi lines and positive control (plasmid DNA) contained CaM gene specific sense, antisense and kan fragments of 381bp and 225 bp amplicons respectively (Fig. 9a,b) while no amplification was observed in water control. It confirms the integration of McCaM hp-RNAi construct into transformed lines.

For Southern blotting, the genomic DNA from PCR positive lines and untransformed control were digested with EcoR1 enzyme overnight at 37°C. The analysis revealed that RNAi lines RNAi-2a, RNAi-4 and RNAi-15 contained one copy of transgene whereas, RNAi-7b, RNAi-8a and RNAi-E11b showed two copies integration pattern. Rest of the lines did not show any hybridization signal. No hybridization signal was detected in wild type control plant (Fig. 10).

For semi qPCR, a number of PCR cycles ranging from 16–36 were tested with CaM gene specific and actin primers. It was observed that the intensity of calmodulin band (381 bp) increased upto 34 cycles and no further increase beyond these cycles which showed that PCR had reached a plateau state. Similarly, increased intensity of Mdo-actin band (110bp) was observed upto 32 cycles. To demonstrate the level of expression of the CaM gene in the developed RNAi transformants of apple cultivar ‘Red Chief’,

six PCR and Southern positive CaM-RNAi lines (RNAi-2a, RNAi-4, RNAi-15 RNAi-7b, RNAi-8a, RNAi-11b) were examined. The same level of expression of actin gene was observed in untransformed control and RNAi transformed lines. Whereas, different RNAi lines showed variable levels of transgene expression with CaM gene specific primers. CaM RNAi apple lines RNAi-11b, RNAi-8a and RNAi-7b showed high level of expression as compared to RNAi-2a, RNAi-4 and RNAi-15 which showed low expression (Fig. 11a,b). No expression was obtained in untransformed control.

In vitro **detached leaf assay**

Visual observations revealed that five days after inoculation (5dpi), the size of lesion gradually increased on wild type leaves which further increased upto 15 days showing progression in infection. Whereas, negligible increase in lesion development was observed after 5dpi to 20 dpi in RNAi transformants (Fig. 12). The lesion with irregular size and shape was marked by brown-black colour. No symptoms were observed on RNAi transformants and water treated controls. Small black dots of acervuli were also visible on lesions of infected wild type leaves while such structures were not present on the transgenics except one line RNAi-15 (Fig. 12). It has further been observed that fungal inoculum on leaves of RNAi-7b, RNAi-8a, RNAi-11b lines started disappearing with increase in the time of inoculation and marked by the absence or very less pigmentation (brown black) on leaves (Fig. 12).

Microscopic examination

Upon microscopic examination, it has been found that fully developed, septate and brown colored mycelium were visible at the site of infection of wild type leaves. Developing bicelled conidia were also seen (Fig. 13a) with necrosis of whole infected leaf tissue. Evaluation of effect of gene silencing on the growth of fungal mycelia in RNAi transformants showed that there was significant arrest of mycelial growth and conidia formation as compared to wild type. No hyphae, mycelium and conidia formation were observed in RNAi-7b, RNAi-8a and RNAi-11b lines (Fig. 13d,e,f). Fungus failed to invade the tissues because epidermis seems to be unaffected and therefore remained intact. Green tissues were visible, only small patch of necrosis was seen in these lines. Whereas, in RNAi-2a transformants, fungus was unable to colonise in the host leaf tissues (Fig. 13b), because no mycelial growth was found and hyphae failed to differentiate into conidiophores. Necrosis was found on upper portion of tissue. However, remnants of hypha were seen at the epidermis. In RNAi-4 line, less branched mycelium with deformed and reduced conidia formation were found (Fig. 13c). Only small area of tissue turned necrotic and rest remained green. There was only one line, RNAi-15 where fungus was able to colonize in leaf tissue because mycelial growth and conidia were observed, however, the mycelia were translucent, reduced in number, not fully developed and conidia less in number as compared to the wild type tissues (Fig. 13g).

Rooting and hardening

All six positive McCaM RNAi lines did not show any detectable variations with respect to morphology and growth. Root initiation in control plants was observed after two weeks when shifted to basal medium (Fig. 14a). Whereas, in RNAi transgenic plants, it was observed one week later (Fig. 14b). Plantlets were kept for hardening in glass house (Fig. 15).

Discussion

In the course of present investigation, a few important factors have been considered and modified to get putative shoots and improved transformation efficiency with *A. tumefaciens* strain EHA105. Two days preculturing, 11 min infection and 2 days co-cultivation duration had a positive impact on transformation. Optimal infection time and co-cultivation conditions are the most crucial factors required for the induction of virulence, which also improves the transformation efficiency (Kumar et al. 2010). Our results are consistent with other reports on fruit trees that the infection time varied from 5–20 min during genetic transformation experiments (Zhang et al. 2006; Liu and Pijut 2010; Sgamma et al. 2015). In apple cv. Jonagold, infection time of 1 min were found optimum (De Bondt et al. 1994) and in rootstocks Malling 26 and Malling 7 infection time of 5 and 15 mins respectively (Maheshwaran et al. 1992; Sharma et al. 2017). In a recent study done in our laboratory on same cultivar, maximum infection efficiency was obtained when leaf explants were infected for 7 min with *Agrobacterium* strain GV101 (Kumar 2020). Therefore, it is suggested that strain selection is an important criteria in apple transformation studies, because efficiency of transformation is influenced by the strain of *A. tumefaciens* and genotype (De Bondt et al. 1994).

The regeneration protocol via direct organogenesis as developed previously (Kumar et al. 2020), was applied for transformation of apple cultivar 'Red Chief'. It has been seen that with the increase in concentration of kan for selection of transformants, there was a gradual decline in regeneration efficiency as well as number of regenerated shoots per explant. *Agrobacterium* contamination was not found throughout the selection process after cef has been reduced to 200mg/l. Thus, it has been demonstrated that two weeks exposure of leaf explants to high level of cef on the efficient elimination of *A. tumefaciens* from leaves, followed by long term exposure to gradually decreased concentration of cef and low doses of kan for the generation of putative RNAi transformants were found the most effective. The incorporation of antibiotics for elimination of bacteria and selection of putative transformants generally results in the production of weak and albino shoots (Teixeira da Silva 2003). Kan at particular concentration blocks the ribosomal RNA synthesis in plastids resulting in no synthesis of chlorophyll. The lethal dose of kan is very much genotypic and explants type dependent (Haddadi et al. 2015). In the present study, 8mg/l kanamycin was found to be lethal for explant survival and inhibited shoot induction. The present results are supported by the previous published data which revealed that transformation rate in apple genotypes has remained low which was below 2% on a per explant basis and 10% in exceptional cases (Malnoy et al. 2008). Improved transformation efficiency observed in wounded leaves in the present work was supported by Norelli et al. (1996) who found that leaf wounding by crushing with forceps significantly increased the transformation as compared with cut leaves. In a recent report, agrofiltration in apple cultivars was found significantly more efficient than wounding with scalpel, to increase the transformation rates to 0.9–8.6% (Chevreau et al. 2019). However in their protocol, transformed shoots were obtained after 6 months while in the present case, these were obtained in only two months.

In the presently developed RNAi construct, for hairpin loop formation, antisense stretch was 50 bp shorter than the sense stretch, such that after transcription, the antisense RNA folds back and complements with

sense RNA to form dsRNA molecules with loop remained in between (Gupta et al. 2013). Initial screening of transformants by PCR using primers specific to npt11 and CaM genes revealed the presence of transgenes. Southern blot method has been routinely used in plant transformation experiments to confirm the integration and determine the copy number of transgene. Integration of single copy transgene was found to be a prerequisite for the stable expression of the transgene in transformed plants. Several copies of the transgene integration into the genome of transformed cell can lead to epigenetic transgene inactivation. Whereas, the increased transgene activity was observed in the presence of two or more transgene copies (Schubert et al. 2004; Marenkova and Deineko 2006). However, a low number of transgene copies in a plant chromosome showed lower incidence of instability (Jones 2005).

Before proceeding with semi-qPCR, it was necessary to analyze the number of PCR amplification cycles to be performed. Because the beginning of PCR cycle is characterized by exponential increase in the target molecule but in the later stages, the amplification efficiency of PCR decreases due to the reduced polymerase activity and limitation of other reagents in the reaction leading to no amplification. Selection of internal control in qRT-PCR is an indispensable step to achieve reliable gene expression. There are several studies which showed that even the widely used reference genes were not stable under different experimental conditions (Nicot et al. 2005; Marino et al. 2008). Therefore, the evaluation of reference genes to ensure their stability of expression during experimental conditions is necessary to obtain accurate results (Lee et al. 2010; Hong et al. 2008). In the present investigation, we assessed the gene expression of apple actin gene as internal control (housekeeping gene). Invariant expression of *M. domestica* actin gene has been exploited by a number of workers for normalizing qRT-PCR reactions during gene expression studies in apple (Espley et al. 2009; Janssen et al. 2008; Zhang et al. 2018).

In the present study, a correlation between copy number and transgene expression was observed in transformed lines. These results are in line with Southern blotting results, as RNAi lines of events RNAi-11b, RNAi-8a and RNAi-7b containing two copies of transgene showed similar level of expression (increased) as compared to RNAi-2a, RNAi-4 and RNAi-15 lines having single copy of gene which showed low transgene expression. In earlier studies a controversial correlation between transgene copy number and expression of target gene has been reported by several authors. Some reported a positive correlation whereas other found no or negative correlation (Schubert et al. 2004). Positive correlation between transgene copy number and expression was reported in potato and tobacco (Stockhaus et al. 1987), potato (Hobbs et al. 1993), rice (Ku et al. 1999), rapeseed (Tang et al. 2003) etc. On the other hand, many transformants containing multiple copies of an introduced gene showed low levels of transgene expression or silencing in tobacco (Hobbs et al. 1993), petunia (Jorgensen et al. 1996) and citrus (Cervera et al. 2000). In case of apple cultivar 'Pinova', no correlation between the gene copy number and expression was observed by Flachowsky et al. (2008).

In vitro detached leaf assay is rapid screening method to investigate the host resistance/susceptibility to pathogens with the advantage of mass selection of resistant plants, avoiding field-related problems like lack of uniformity of infection and environmental limitations (Sinclair and Dhingra 1995) and used in a number of crops (Arraiano et al. 2001; Jia et al. 2003). In the present study, gradual disappearance of

inoculum and brown black colour in RNAi transformants may be due to the reduction in synthesis of melanin (pigment). Reduction in pigmentation and conidia due to the silencing of CaM gene has been confirmed by Chauhan (2019). Previous reports have suggested the role of acyl transferase and BRM2 genes in *Colletotrichum falcatum* and *Alternaria alternata* respectively in melanin biosynthesis, the disruption of which affected the size and production of conidia, septal number etc (Kawamura et al. 1999; Scindiya et al. 2018). Gachomo et al. (2010) indicated that melanization of appressoria is a crucial factor for pathogenicity of Marssonina species, and defective appressoria development and loss of pathogenicity in alpha alpha plants due to silencing of CaM gene was revealed by Warwar et al. (2000).

Absence of mycelium and conidia in RNAi lines may be due to the reason that there was no hyphae which release enzymes to absorb nutrients from the host adding new vegetative growth (mycelium). The present observations are in line with the earlier studies conducted in our laboratory on *M. coronaria* which proved that very less conidia treated with synthetic CaM dsRNA molecules could be able to generate *in vitro* as well as not able to cause disease at 7 dpi (days post inoculation) on leaves of apple cv. Straking Delicious (Chauhan 2019). Hence, the present results clearly demonstrated that silencing of CaM gene has inhibited the growth of fungus and pathogenicity in three lines (RNAi-7b, RNAi-8a and RNAi-11b).

It is clear that inhibition/ significant reduction of hyphae, mycelial and conidial growth and defected septa were distinctly different among RNAi lines and seemed to be greatly dependent on the integration and expression of CaM gene in this study. The difference in fungal growth and differentiation among RNAi CaM lines could be attributed to variable downregulation of the target gene. It may be assigned to differential abundance of the introduced dsRNA which is determined by site of integration and dosage effect of the introduced genes (Kerschen et al. 2004). It is demonstrated here that taking up of CaM gene specific dsRNA/ siRNA from RNAi lines have down regulated the target gene transcript thereby confirming the down regulation efficiency of the introduced fragment. These results are supported by Reddy and Rajam (2016) who confirmed that the ingestion of *Helicoverpa armigera* chitinase gene specific dsRNA/ siRNA downregulated the target gene specific transcripts to its functional threshold level in RNAi lines of tobacco and tomato, which resulted in interruption in its biological function. Hence, the silencing of CaM gene adversely affected the growth and differentiation of *M. coronaria* in the present study.

After comparing the detached leaf assay results with Southern and semi q-RT PCR analysis, it can be established that the gene copy number as well as expression in the transformed lines are related to silencing of CaM gene which in turn reduced the growth, differentiation and virulence of *M. coronaria* and ultimately provides resistance to apple cultivar 'Red Chief'. RNAi transgenic lines RNAi- 11b, RNAi- 8a and RNAi-7b having two copy number of CaM gene did not show lesion development on leaves 15 dpi as compared to RNAi-2a, RNAi-4 and RNAi-15 containing single copy of gene.

Recent studies have demonstrated the transfer of dsRNA/ siRNA from plants to various pathogens in number of plant systems (Koch and Kogel 2014) and confirm the effectiveness of HIGS to protect the plants against pathogenic fungi, bacteria, nematodes and insects. Homology based gene silencing induced by transgene, dsRNA or antisense has been demonstrated successfully in various plants against

several species pathogenic fungi including *Neurospora crassa*, *Magnaporthe oryzae*, *Venturia inaequalis* and *Cladosporium fulvum* (Goldoni et al. 2004; Kadotani et al. 2003; Fitzgerarld et al. 2004; Hamada and Spanu 1998). In another study, transgenic barley plants carrying hairpin RNAi cassette targeting the Avra10 gene of fungus *Blumeria graminis* showed a significant reduction in fungal infection as compared to control plants suggesting the trafficking of dsRNA/ siRNA from host into pathogen triggering the silencing of fungal gene (Nowara et al. 2010).

The results established during the present study showed that partial as well as complete resistance against *M. coronaria* has been achieved. However the partial silencing observed for target CaM gene was expected because usually RNAi results in downregulation rather than complete silencing of gene/genes (Pasquinelli 2012). Observation of detached leaf assay confirmed the role of CaM gene in pathogenicity of *M. coronaria* and symptom appearance. It has been suggested that plant resistance against fungal pathogens depends upon the inhibition of development of infection structures like appressoria or infection cushion and disease severity correlates with the size and number of infection structures (Zhang et al. 2016), as they are involved in disease progression. The present results are further supported by Warwar et al. (2000) that CaM gene of *Colletotrichum trifolii* is highly expressed during germination of conidia and development of appressoria which confirm a possible role of CaM gene in appressorium formation. Therefore, the present findings prove that it is possible to downregulate/ silent pathogenicity genes *M. coronaria* using HIGS-RNAi.

Conclusion

It is concluded that *Agrobacterium* mediated transformation method in apple cultivar 'Red Chief' using *A. tumefaciens* strain EHA105 containing RNAi construct targeting CaM gene of *Marssonina coronaria* has been standardized for the first time. Six RNAi transformants obtained showed that the reduction in growth and differentiation of fungus in leaf tissues are associated with down regulation of fungal CaM gene expression as compared to wild type thus confirming the production of CaM-siRNAs in transformed apple cultivar Red Chief. Further, insertion of two copies of CaM gene in three RNAi transgenic lines may have more potential to make siRNAs in comparison to single copy as evidenced by the complete failure of fungal colonization in host tissues of these lines. Hence, deciphering the role of CaM gene using hpRNAi construct through Host Induced Gene Silencing approach has provided partial/ full resistance to Marssonina blotch in RNAi transgenic lines of apple cultivar 'Red Chief'. It is recommended that this approach can be useful to prevent the virulence of *M. coronaria* fungus during primary infection. Since the pathogen overwinters on infected fallen leaves, if this technology goes well it can reduce the inoculum resulting in less secondary infection.

Declarations

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Author contribution statement

MM conceived and designed the research plan. AC conducted cloning work and prepared construct. SV conducted transformation trials, molecular work and performed data analysis. SV and MM wrote the manuscript. All authors have reviewed and approved the final manuscript.

Potential conflicts of interest

The authors have no conflicting interests, and all authors have approved the manuscript and agree with its submission to Plant Cell, Tissue and Organ Culture.

Compliance with ethical standards

The authors declare that the study was carried out following accepted professional conduct. However no ethical approval was needed for the study as it did not involve the use of animals or human objects.

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Figures

Figure 1

Vector used for preparation of RNAi construct

Figure 2

Cloning strategy to prepare hpRNAi construct pRI 101-AN-CaMRNAi

Figure 3

Cloning of CaM in sense and antisense orientation in binary vector pRI-101-AN a Confirmation of CaM cloning in sense in pTZ57R vector by restriction digestion, UC- uncut pTZ57R-CaM-S, C1, C2 & C3- digested pTZ57R-CALM-S recombinant clones b Confirmation of CaM cloning in antisense orientation in pTZ57R vector by restriction digestion. UC- uncut pTZ57R-CaM-AS, C1, C2 & C3- digested pTZ57R-CaM-AS recombinant clones c Linearization of pRI-101-AN by restriction digestion for vector backbone. UC- uncut pRI-101-AN, C1 & C2- digested pRI-101-AN d Confirmation of CaM cloning in sense orientation in pRI-101-AN by restriction digestion. UC- uncut pRI 101-AN-CaM-S, C1- digested pRI 101-AN-CaM-S putative clone e Linearization of the confirmed pRI-101-AN-CaM-S for vector backbone. UC- uncut pRI 101-AN-CaM-S, C1- digested pRI 101-AN-CaM-S f Confirmation of CaM cloning in antisense orientation in pRI-101-AN-CaM-S by restriction digestion. M-1kb DNA marker, UC- uncut pRI 101-AN-CaM-AS, C1 & C2- digested pRI 101-AN-CaM-AS putative clones

Figure 4

Confirmation of CaM in pRI-101-AN. Release of CaM sense, antisense and full RNAi cassette by restriction digestion with Sal 1&Kpn 1 (SC) Kpn1 &BamH1 (ASC); EcoR1 &NdeI, (FCC),M- 1 kb DNA ladder, UCC-uncut pRI-101-AN-CaMRNAi

Figure 5

RNA folding prediction as generated by mfold. CaM RNAi cassette with dG = -429.30
 (<http://unafold.rna.albany.edu>)

Figure 6

Confirmation of RNAi - pRI 101-AN-CALM into *A. tumefaciens* GV3101 & EHA 105 using plasmid PCR with gene specific primers a sense b anti sense, M- 1 kb DNA ladder, G1, G2, G3, ...& 1-5; E1, E2, E3...& 6-9 GV3101 and EHA105 *A. tumefaciens* clones respectively, B-water control

Figure 7

a Control leaf explants with shoots on SRM without antibiotics b Untransformed leaf explants on SRM having 200mg/l cef + 5mg/l kan c-e Transformed explants on SRM having 200mg/l cef + 5-7mg/l kan showing putative RNAi shoots

Figure 8

a Direct putative shoots obtained after transformation b-c Putative RNAi -11b and RNAi -8a kept for multiplication d 4 weeks old multiplied CaM RNAi lines

Figure 9

Confirmation of putative transgenic lines with CaM a and nptII b gene specific primers a Lane 1-13 putative transgenic lines: RNAi-1, RNAi-1c, RNAi-2c, RNAi-2a, RNAi-5, RNAi-4, RNAi-7b, RNAi-7d, RNAi-8a, RNAi-10a, RNAi-11b, RNAi-15, RNAi-16 b PCR positive transgenic lines: 1-4,RNAi-1, RNAi-1c, RNAi-2c, RNAi-

2a, Lan6&7- RNAi-4, RNAi-7b, Lane-9,11,12,13-RNAi-8a, RNAi-11b, RNAi- 15, RNAi-16, M-1Kb DNA marker;
W- water control ; P-positive control

Figure 10

Southern blot analysis of 10 RNAi transgenic plants, WT- Wild Type, B- Blank 1-10 transgenic plants RNAi-1, RNAi-1c, RNAi-2, RNAi-2a, RNAi-4, RNAi-7b, RNAi-8a, RNAi-11b, RNAi-15, RNAi-16

Figure 11

a Amplification of CaM gene at 164bp b Amplification of actin gene in six RNAi transgenic lines at 110 bp, M- 100bp ladder, W- water control, WT-wild type, P- positive control

Figure 12

In vitro pathogenicity assay of RNAi-transgenic lines and wild type

Figure 13

a-g Microscopic examination at 20X of leaf cells of wild type (a) and transformants (b-g) 20 days after fungal inoculation

a

b

Figure 14

a In vitro rooting of wild type b RNAi transgenic lines

Figure 15

Hardening of transgenic lines