Indian Journal of Biotechnology
Vol. 18, April 2019, pp 119-125

Designing of siRNAs for various target genes of *Cucumber mosaic virus* subgroup IB

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Received 13 July 2018; revised 10 March 2019; accepted 18 March 2019

Cucumber mosaic virus (CMV) is a major production constraint in black pepper causing stunted disease. Resistant varieties are unavailable and control measures are not effective till now. Recently, RNA interference (RNAi) is the most promising strategy for combating virus infection in plants but the effectiveness depends on sequence specificity between the transgene and the targeting virus. This study was undertaken to design the most suitable region for double stranded RNA synthesis with maximum specificity and minimized off–targets for all the five genes of CMV from black pepper. A 400 bp off–target minimized region identified from each of the five genes was subjected to sequence polymorphism study with selected CMV subgroup IB strains and common 'siRNAs' were designed *in silico*. As 3b gene had the least variations (of 17%) with four common and potential siRNAs designed from this region *in silico*, a hairpin construct was assembled using this region in *Agrobacterium* that can be used for developing black pepper resistant to selected CMV subgroup IB strains.

Key words: *Cucumber mosaic virus*, black pepper, RNA interference, siRNA designing, hairpin construct

Introduction

Black pepper is known as the king of spices, one of the precious spice in the world and has played a major role in India's international trade. From an economic point of view, pepper yield is often low and variable. Viral disease known as stunted disease contribute to low yields and reduced fruit quality. Diseased plants show mottling and mosaic on leaves. Stunting of whole plant is seen under severe cases, spike length is greatly reduced and spikes are poorly filled causing yield reduction. Association of *Cucumber mosaic virus* (CMV) with the disease is reported from India, China, Sri Lanka and Brazil[1]. CMV spreads non–persistently through aphids and infected stem cuttings. Besides black pepper, more than 1200 crops from 100 plant families are affected by this virus[2].

CMV is a plus sense RNA virus (Genus: *Cucumovirus*, Family: Bromoviridae) with three RNAs enclosed in separate particles. RNA1 codes for a single gene 1a whereas RNA2 and RNA3 code for two genes each. The genes 1a and 2a have role in replication[3], 2b gene acts as the silencing suppressor[4], 3a gene codes for the movement protein and 3b gene, the coat protein important in transmission of the virus by aphids[5]. CMV strains are classified into subgroup I and II, subgroup I is further subdivided into IA and IB based on serology, symptomology and hybridization studies[6]. Complete genome sequencing of black pepper isolate of CMV revealed that the isolate belongs to subgroup IB.

Chemicals to control viruses are lacking and resistant varieties are unavailable. Thus, developing pathogen derived resistance (PDR) is the best management strategy, for which selection of suitable transgene is important. Recent studies show that transgenes in the form of hairpin structures where sense and antisense strands of the same sequence separated by an intron, will trigger RNA silencing and confer systemic resistance against viral genomes[7]. The mechanism known as RNA interference (RNAi) has been successfully used for conferring resistance to pathogens in crops and also for improving the quality traits in crops. However, the effectiveness of the mechanism depends on the sequence similarity between the transgene and the viral genome[8]. Thus, sequence polymorphism in the target gene limits the application of RNAi to a great extent. Hence, this study was undertaken to identify the most appropriate region among five genes of the current isolate for

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dsRNA synthesis that has minimum off-targets and also to design the common (siRNAs) that can target all the selected CMV subgroup IB strains. Lastly, hairpin vector construct was developed in the *Agrobacterium* strain EHA105 using the appropriate sequence identified.

Materials and Methods

The 1a, 2a, 2b, 3a and 3b open reading frame (ORF) sequence of black pepper isolate of CMV, was used for the study. These sequences were subjected to dsCheck online software (http://dsCheck.RNA.jp/)[9] to select the specific region that will yield dsRNA. A 400 bp off-target minimized region was selected by the software from all the five genes. Corresponding sequences of 13 isolates of CMV subgroup IB for which complete genome sequence information was available were retrieved from National Center for Biotechnology Information (NCBI) (Table 1), and sequence polymorphism of these regions was then studied using the software DnaSP version 5.10[10]. The selected dsRNA sequences were then subjected to siDESIGN center (http://www.dharmacon.com), online software for identifying the common siRNAs present in these regions.

CMV coat protein 336 bp region starting from the 5′ end was selected for the preparation of hairpin construct. The various steps involved in the 3b hairpin construct preparation in cloning vector are shown in Figure 1. Viral coat protein gene in the sense and antisense orientation was first amplified from infected black pepper leaves using the primers AIB 211 and AIB 212 for sense; AIB212 and AIB 213 for antisense (Table 2). In order to amplify the sense fragment, the forward and reverse primers were flanked with restriction enzymes *Bam*H1 and *Nsi*1 respectively, followed by six bases overhangs for proper restriction. Similarly the antisense fragment was amplified by using the forward primer flanked with *Sac*1 and reverse primer with *Nsi*1. Phytoene desaturase sixth intron from tomato to be inserted between the sense and antisense fragments was amplified using the primers flanked with *Nsi*1. The sense PCR product was first restricted with *Nsi*1 for 1 h at 37°C, enzyme inactivated by incubating at 65°C for 15 min. Then *Bam*H1 was added and incubated for 45 min and the enzyme was inactivated by incubating at 80°C for 5 min. Similarly, antisense PCR product was restricted with *Nsi*1 followed by *Sac*1. Intron PCR product was restricted with *Nsi*1 alone. The cloning vector pTZ57R/T (Fermentas, USA) was also restricted with *Bam*H1 and *Sac*1 sequentially. The restricted sense, antisense and intron along with the double restricted pTZ57R/T vector were kept for ligation in the ratio 3 : 8 : 3 : 1 overnight. The ligated product was transformed into *Escherichia. coli* DH5α using InstaTA cloning kit (Fermentas, USA). The transformed colonies were screened by PCR using the intron primers AIB 214 and AIB 215 (Table 2). Plasmid was isolated from the selected colonies and confirmed by restriction analyses and sequencing. The hairpin construct was then released from the cloning vector and ligated into the binary vector pBI121 both restricted using *Bam*H1 and *Sac*1. This was then cloned into the *Agrobacterium* strain EHA105 by triparental mating using the helper plasmid pRK2014 and confirmed by PCR, restriction and sequencing. The schematic picture of the restriction of 3b hairpin

Table 1 — Details of isolates of selected *Cucumber mosaic virus* subgroup IB strains and their accession numbers used in the study

			GenBank Accession Number		
Isolate	Host	Country	RNA1	RNA2	RNA3
Black pepper	*Piper nigrum*	India	KU947029	KU947030	KU947031
WN1	*Piper nigrum*	China	KT004542	KT004543	KT004544
KO	*Capsicum annuum*	India	KM272277	KM27278	KM272275
ND	*Solanum lycopersicum*	India	GU111227	GU111228	GU111229
CLW2	*Cucumis sativus*	Malaysia	JN054636	JN54637	JN054635
HM3	*Lycopersicon esculentum*	Egypt	KT921314	KT921315	No Data
IX	*Lycopersicon esculentum*	Philippines	U20220	U20218	U20219
IA	No data	Indonesia	AB042292	AB042293	AB42294
Nt9	*Lycopersicon esculentum*	Taiwan	D28778	D28779	D28780
SD	*Nicotiana tabacum*	China	AF071551	D86330	AB008777
Tfn	*Solanum lycopersicum*	Italy	Y16924	Y16925	Y16926
Pl-1	*Solanum lycopersicum*	Spain	AM183114	AM183115	AM183116
Vir	*Capsicum annuum*	Italy	HE962478	HE962479	HE962480

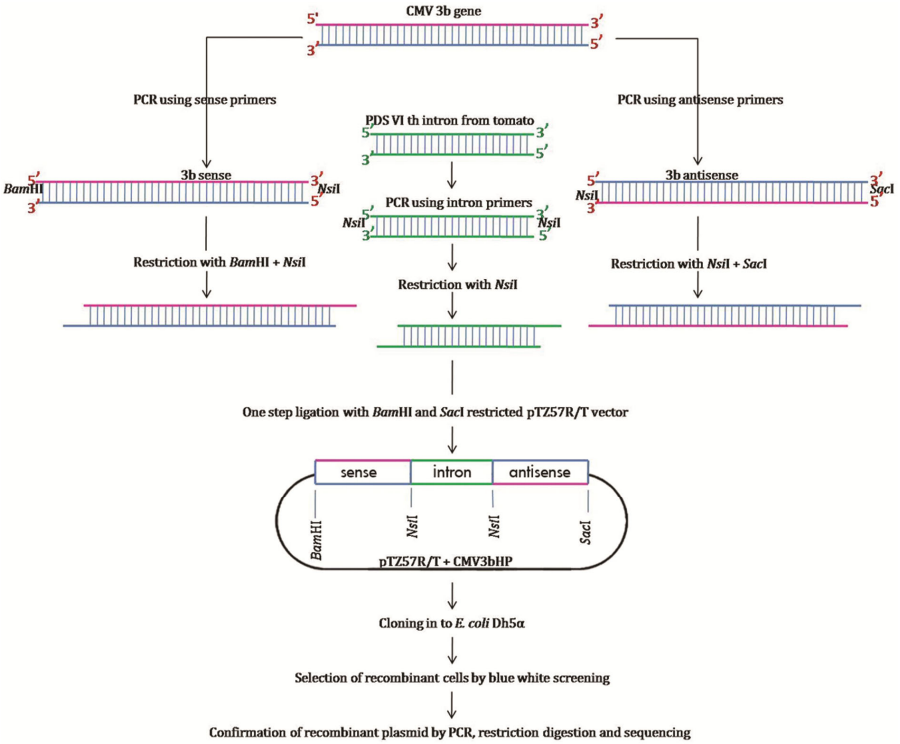

Fig. 1 — Schematic diagram showing different steps in 3b hairpin construct preparation in the cloning vector pTZ57R/T.

Table 2 — Details of primers used for hairpin construct preparation and confirmation

Primer	Sequence (5′……3′)	Amplicon size (bp)	Region	Remarks
AIB 211	AGGAGGGGATCCATGGACAAA TCTGAATCAA	349	Nucleotide (NT) 1–337 from 5′ end of CP gene.	For amplifying CMV CP in sense orientation
AIB 212	GGGGGCATGCATGATTAACTCAA TTTGAAT			
AIB 213	AGAAGGGAGCTCATGGACAAATCT GAATCAA	349 with AIB 212	NT 1–337 from 5′ end of CP gene.	For amplifying CMV CP in antisense orientation
AIB 214	GGCGGCATGCATCTGCAAATTATA TCAAAGA	236	NT 1–227 from phytoene desaturase 6th intron from tomato	For amplifying intron
AIB 215	AGGAGGATGCATGTGAGC TAATCACGAGTAA			
AIB 267	TAATCACGAGTAAATTTCTCCC	227	NT 1–227 from intron of coat protein hairpin construct	For amplifying intron without restriction enzymes
AIB 268	TGCACACAAAAGGTCCAATG			
AIB 267	TAATCACGAGTAAATTTCTCCC	800	NT from intron to the promoter region of coat protein hairpin construct	For amplifying the region from intron to promoter of transformed pBI121
AIB 108	TCCAACCACGTCTTCAAAGC			
AIB 258	CGAAACGCTGTTCGGCCTGTGG	898	*Agrobacterium* chromosomal virulence gene *ChvA*	For confirmation of *Agrobacterium* strain
AIB 259	GTTCAGCAGGCCGGCATCCTGG			
AIB 268	TGCACACAAAAGGTCCAATG	900	Region from the intron of the hairpin construct to the kanamycin gene	For confirmation of hairpin construct
AIB 269	ACCGCGCGCGATAATTTAT			

construct from the cloning vector pTZ57R/T, ligation into the binary vector pBI121 and mobilization into *Agrobacterium* strain EHA105 is shown in Figure 2.

Results and Discussion

Previous studies show that the ideal size of dsRNA for RNAi vector for controlling plant viruses is 300-800 nt. Thus, a 400 bp region was selected to

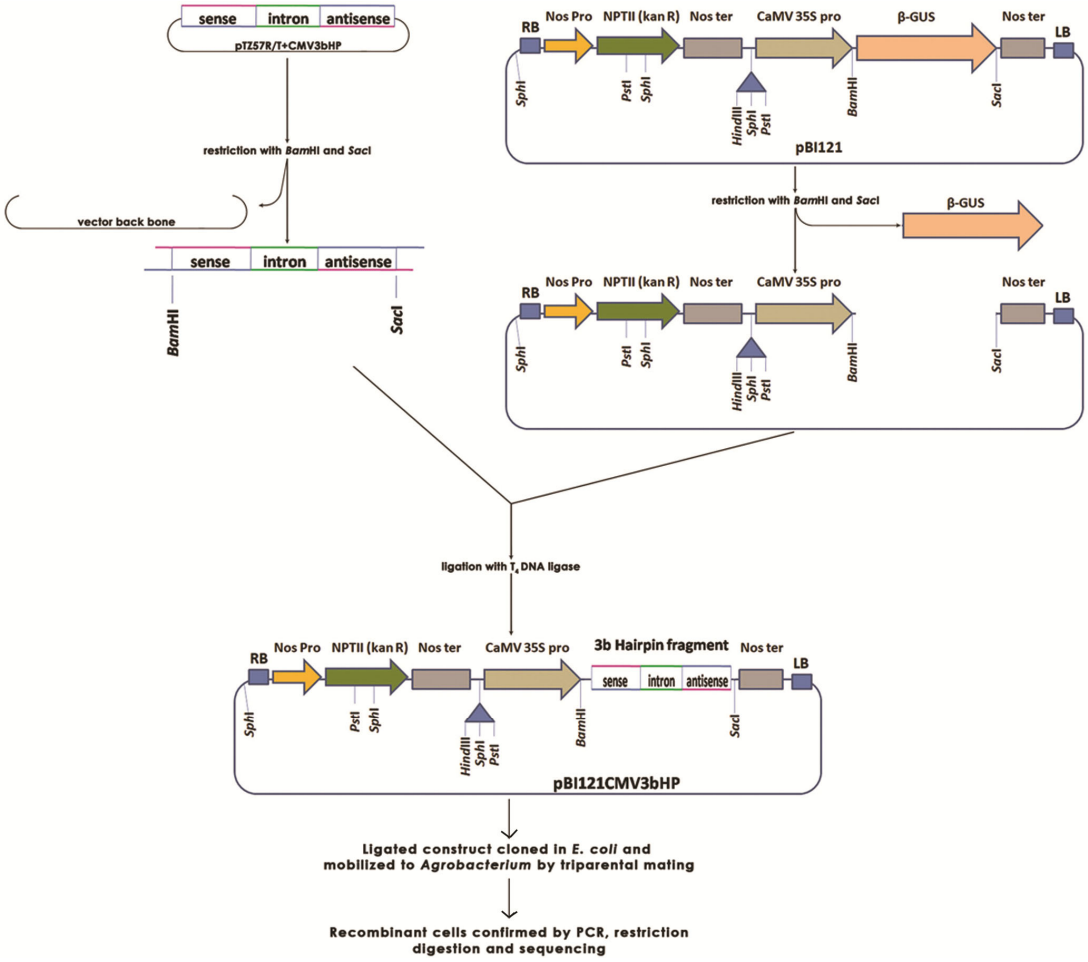

Fig. 2 – Schematic diagram of the 3b hairpin construct preparation in the binary vector pBI121.

minimize the off-targets, for all the five genes of black pepper CMV except the 2b gene where the complete 336 bp region was selected for dsRNA synthesis. Analysis of these regions by dsCheck revealed that the sequences from 2326-2702 bp of 1a gene, 600-999 bp of 2a gene, 1-337 bp of 2b gene, 92-491 bp of 3a gene and 28-427 bp of 3b gene were the region most suitable for dsRNA synthesis. Comparison of these regions with thirteen selected CMV subgroup IB strains by DnaSP showed a sequence polymorphism of 25, 30, 32, 21 and 17% for 1a, 2a, 2b, 3a and 3b genes, respectively (Table 3). Analysis by the siDesign software revealed many siRNAs from all the five genes of black pepper CMV which were uncommon among the different isolates except the four designed from the 3b gene (Table 4). These common siRNAs can target all the subgroup IB strains used in the study. As 3b gene is the most conserved with minimum sequence variations and as common siRNAs were recorded from the 3b gene, this region was taken for the hairpin vector construction.

Table 3 — dsRNA region selected for different genes of *Cucumber mosaic virus*

Gene	Accession No	Region selected	Sequence polymorphism (%)	Common siRNAs
1a	KU947029	2328–2726	25	–
2a		660–1059	30	–
2b	KU947030	1–336	32	–
3a		172–571	21	–
3b	KU947031	77–476	17	4

Polymerase chain reaction (PCR) of the hairpin construct assembled in both the cloning and binary vector gave specific products (Fig. 3 & 4). Double restriction using *BamH*1 and *Sac*1 released the 1 kb hairpin construct and restriction using *Nsi*1 released the 227 bp intron. Sequencing showed that the total hairpin construct is 1 kb including the restriction enzyme sites where the intron of 227 bp is flanked in

Table 4 — Common siRNA and target positions in the 400 bp dsRNA selected region of 3b gene of *Cucumber mosaic virus*

Region	Sense strand (sequence)	Start position	GC%	Score
3b	GAGTTAATCCTTTGCCGAA	220	42	79
	AGTTAATCCTTTGCCGAAA	221	37	75
	GTTAATCCTTTGCCGAAAT	222	37	74
	CGAGTTAATCCTTTGCCGA	219	47	68

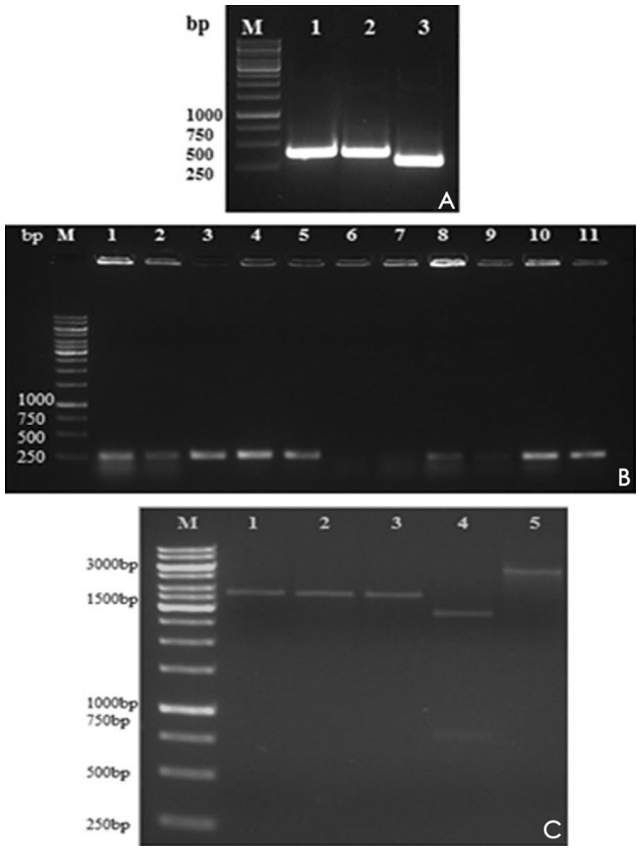

Fig. 3 – Confirmation of 3b hairpin construct in cloning vector pTZ57R/T in *E. coli*, (A) amplification of 3b sense construct using primers AIB 211 and AIB 212 (Lane 1: product size 350 bp), antisense using primers AIB 212 and AIB 213 (Lane 2: product size 350 bp) and intron using primers AIB 214 and AIB 215 (Lane 3: product size 240 bp) from phytoene desaturase 6th intron from tomato, (B) colony PCR using intron primers AIB 214 and AIB 215 to screen the transformed colonies; Lane 1-11 represents different colonies, (C) Restriction analyses of hairpin construct where Lane 1, 2 and 3 represents single restriction of plasmid using *Bam*H1, *Sac*1 and *Nsi*1 respectively. Lane 4: double restriction of plasmid using *Bam*H1 and *Sac*1 releasing the 1 kb hairpin construct and Lane 5: unrestricted plasmid; Lane M: 1 kb ladder.

between the sense and antisense strands of 337 bp each. Intron was in the sense orientation (Fig. 5).

CMV is one of the devastating viruses infecting many crops including Indian long pepper and betelvine[11] which are related species of black pepper. Control measures mainly involve use of chemicals to kill the viral vectors. None of the varieties or cultivars of black pepper are resistant to CMV. Thus, engineering PDR is the best alternative and coat protein is the most widely used gene. Due to the lack of specificity and as protein mediated resistance is overcome by high dose of the inoculum, recently, the most preferred and successful approach for PDR is RNAi[12].

RNAi is a natural phenomenon of RNA degradation activated by sequence specific RNAs. The mechanism is triggered by short interfering RNAs processed from the mRNAs arising from the transgenes. Length of the transgene used is very important for a successful RNAi. Though there are reports showing that sequences of 23-60 nt induces effective RNA silencing, transgenes with more than 100 nt is required for an effective RNAi and usually 300-800 nt region is preferred for controlling plant viruses. In the current study we have used the online software dsCheck to select a 400 bp region of the different genes of the present isolate with minimum off-targets. The sequence variations in the above selected regions were then compared among different CMV subgroup IB strains. Among the different genes, 3b had the lowest and 2b had the highest sequence variations of 17% and 32%, respectively. The results were consistent with the earlier reports where the level of gene conservation was highest in 3b gene and least in 2b gene[1,2,13]. In some cases siRNAs were found to be more effective compared to the long dsRNA region with high sequence polymorphism[14]. So the 400 bp selected regions were further subjected to another online software system siDesign common and potential siRNAs with minimum off–targets were generated only from the 3b gene. The off-targets of these siRNAs were not checked against the black pepper genome as complete genome sequence of black pepper is not available so far.

Finally, a hairpin construct of 3b gene having the maximum common siRNAs identified was developed in the plant transformation vector pBI121. This can be used for the transformation of black pepper and will confer resistance to CMV by generating siRNAs targeting many CMV subgroup IB strains. Isolation

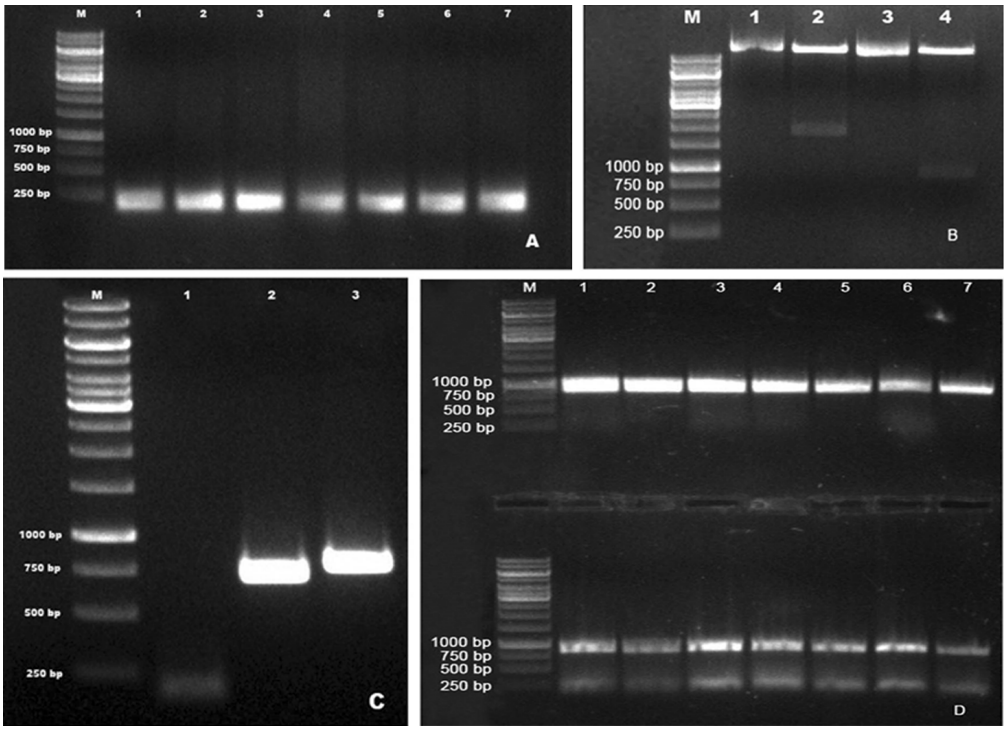

Fig. 4 – Confirmation of coat protein hairpin construct in binary vector pBI121 in *E. coli* and *Agrobacterium*. (A) Colony PCR using intron primers AIB 267 and AIB 268 to screen the transformed colonies giving approx. 240 bp product. Lane 1-7 represents different colonies. (B) Restriction analyses of hairpin construct where Lane 1 represent unrestricted pBI121 plasmid, Lane 2 represents pBI121 plasmid double restricted with *Bam*H1 and *Sac*1 releasing the 2 kb Gus gene, Lane 3 represents transformed pBI121 plasmid unrestricted and Lane 4 represents the transformed plasmid double restricted using *Bam*H1 and *Sac*1 releasing the 1 kb coat protein hairpin construct. (C) PCR confirmation of transformed plasmid where Lane 1 shows PCR using primers AIB 267 and AIB 268 giving approx. 240 bp product, Lane 2 is PCR done using primers AIB 267 and AIB108 giving 800 bp product and Lane 3 is PCR done using primers AIB 268 and AIB 269 giving 900 bp product. (D) PCR done for confirmation of coat protein hairpin construct in *Agrobacterium* where top wells show the amplification of different colonies using the primers AIB268 and AIB 269 giving 900 bp product and down wells show the amplification of colonies using the primers AIB258 and AIB259 giving 898 bp product. Lane M is 1 kb ladder. Details of primers is given in Table 2.

ggatccATGGACAAATCTGAATCAACCAGTGCTGGTCGTAACCGTCGGCGTCGTCCGCGTCGCGGTTCCCGCTCC
GCCTCCTCCTCTGCGGATGCTACATTTAGAGTCCTGTCGCAACATCTTTCGCGACTCATCAAGACGTTAGCAGCT
GGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGTTGTAAACCTGGATACACGTTCACCTCGATT
ACCCTGAAGCTACCAAAAATAGACCAAGGGTCTTATTATGGCAAAAGGTTGTTACTTCCTGATTCAGTCACTGAG
TTCATAAGAAGCTTGTTTCGCGCATTCAAATTCAAGTTAATCatgcatGTGAGCTAATCACGAGTAAATTTCTCC
CTCTTGTAGTTATTTTGTTAAACTTCCCTAATAAGCTGTAAAGTTGATTAGAATTCTAAAAAAAAAATCTGTAAA
ATTGATAATTCAATCACACCTATGGGACTTTACTAACCTTAAAAGAGCATAAAAGTTCATTACTTCTTCATTGGA
CCTTTTGTGTGCAGCTAAAATATTAAATTCTTTGATATAATTTGCAGatgcatGATTAACTCGAATTTGAATGCG
CGAAACAAGCTTCTTATCGAACTCAGTGACTGAATCAGGAAGTAACAACCTTTTGCCATAATAAGACCCTTGGTC
TATTTTTGGTAGCTTCAGGGTAATCGAGGTGAACGTGTATCCAGGTTTACAACGCTCACTACCCACAAAGGTTGG
GTGGTTAATAGTAGGACGACCAGCTGCTAACGTCTTGATGAGTCGCGAAAGATGTTGCGACAGGACTCTAAATGT
AGCATCCGCACAGGAGGAGGCGGAGCGGGAACCGCGACGCGGACTACCCCGACGGTTACGACCAGCACTGGTTGA
TTTAGATTAGTCCCTgagctc

Fig. 5 – Complete sequence of hairpin construct comprising 337 bp each of sense and antisense strands of 3b gene separated by 227 bp PDS intron. GGATCC is *Bam*HI, ATGCAT is *Nsi*I and GAGCTC is *Sac*I.

and sequencing of siRNAs generated in the transgenic resistant black pepper plants is needed for further confirmation of these siRNAs predicted *in silico*.

Acknowledgements

Authors thank Distributed Information Sub Centre and Director ICAR–Indian Institute of Spices Research, Kozhikode, Kerala for the facilities. Senior

author (Revathy K A) is thankful to the Department of Science and Technology, WOS–A, Government of India for the funding and Dr. M. Manamohan, Principal Scientist, ICAR–Indian Institute of Horticultural Research, Bengaluru for the technical guidance and facilities extended for the hairpin vector construct preparation.

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