Common siRNAs for various target genes of the fruit borer, *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae)

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RNA interference (RNAi), a sequence-specific gene silencing mechanism, is emerging as an important tool in agriculture for the management of insect pests. Since sequence variation of the target genes in different populations of the target pest is the possible limiting factor in the application of RNAi in the field, a study was undertaken to elucidate the sequence polymorphism of five important genes (actin, glutathione-S-transferase, cytochrome P450, chymotrypsin and serine protease) from the fruit borer, *Helicoverpa armigera*. An off-target minimized region (500 bp) was identified for dsRNA synthesis from all the above sequences and the nucleotide variations in this region were analysed *in silico* to design common siRNAs for each of the target genes that could be further utilized for downstream applications.

Keywords: Gene silencing, *Helicoverpa armigera*, RNA interference, sequence polymorphism.

HELICOVERPA ARMIGERA is a highly polyphagous pest that inflicts serious damage to a wide spectrum of crops such as cotton, tomato, lady's finger, chickpea, pigeon pea, chilli, maize and sorghum worldwide[1]. The ability of *H. armigera* to thrive on diverse host plants is an adaptive advantage for its better survival in the ecosystem, which is achieved by its high mobility, fecundity and capacity to develop resistance to a wide spectrum of chemical insecticides[2]. In the past three decades, transgenic technology has been developed to generate insect-proof plants to reduce yield loss and pesticide utilization[3,4]. Engineering crop plants (e.g. cotton, pigeon pea, etc.) to express genes coding for insecticidal crystalline proteins from *Bacillus thuringiensis* (*Bt*) has achieved great field success[5], but the threat of accelerated development of resistance to *Bt* is looming large, which challenges the sustainability of *Bt*. In this context, there is an urgent need to look for an effective and safer alternative, where RNA interference (RNAi) comes handy. RNAi is a species – and sequence-specific gene silencing mechanism often more elaborate in the basal genomes. The observations by Fire *et al.*[6] that the exogenous application of dsRNA elicited RNAi in *Caenorhabditis elegans* made the scientists examine the potential of RNAi in various fields of research. In this regard the initial experiments on RNAi in insect pest management were not successful and consistent due to many factors and hence RNAi remained only as a laboratory tool in gene validation until recently. However, two recent papers have demonstrated the practical utility of RNAi in insect pest management[7,8]. This has rekindled the interest in RNAi in the management of various insect pests belonging to Lepidoptera, Coleoptera, Diptera, plant parasitic nematodes and Diamondback moth (*Plutella xylostella*)[9]. Utility of RNAi has also been demonstrated in functional genomics for improving various crop quality traits and conferring resistance to viruses, bacteria, fungi and plant parasitic nematodes[10]. The practical way of managing various pests in the field level lies in the delivery of dsRNA for a particular gene by *in planta* expression[7]. Among the various factors that limit the successful field application of RNAi is the sequence polymorphism of the target gene[11]. In the present study, we have analysed the sequence polymorphism of some important genes such as actin, glutathione-S-transferase (GST), cytochrome P450 (CYP9A14), chymotrypsin and serine protease from different populations of *H. armigera* collected from different cropping systems in India and its likely fallout in selecting a suitable region for dsRNA synthesis. For this purpose we employed the on-line dsRNA design tool, dsCheck, that provides off-target, minimized regions of approximately 500 bp for *H. armigera* BCRL strain and compared the variations with other populations for different genes. Further, we also employed the small interfering RNA (siRNA) design tool, siDESIGN, to identity a common siRNA sequence for all the genes from different populations of *H. armigera*.

Larvae were collected from different locations, viz. Wasim (Maharashtra), Akola (Maharashtra), Tandoor (Andhra Pradesh), Vadodhara (Gujarat), Yavatmal (Maharashtra), Wardha (Maharashtra), Bangalore 1 (Karnataka; NBAII) and Bangalore 2 (Karnataka; BCRL; Table 1).

Total RNA was isolated from individual larvae using RNAqueous (Ambion, USA) following the manufacturer's protocol. Individual larvae (300 mg) were crushed thoroughly in liquid nitrogen and 800 µl of lysis/binding buffer was added and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a fresh 2.0 ml tube with equal volume of 64% ethanol and transferred to elution columns, centrifuged at 6,000 rpm for 2 min and washed with wash solution provided in the kit. Excess wash solution was removed by centrifugation at 10,000 rpm for 2 min and total RNA was eluted in 50 µl of ellution buffer (pre-heated at 75°C) provided in the kit and stored at –20°C until further use.

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Table 1. Collection of samples and their locations in India

Target gene	Place of collection	State	NCBI accession
Actin	Wardha cotton	Maharashtra	HM629436
	Vadodhara pigeon pea	Gujarat	HM629437
	Tandoor cotton	Andhra Pradesh	HM629438
	Yavatmal cotton	Maharashtra	HM629439
	Wasim cotton	Maharashtra	HM629440
	Akola chickpea	Maharashtra	HM629441
	Bangalore NBAII	Karnataka	HM629442
	Bangalore BCRL	Karnataka	HM629443
Cytochrome CYP9A14	Wardha cotton	Maharashtra	HM209432
	Vadodhara pigeon pea	Gujarat	HM209436
	Tandoor cotton	Andhra Pradesh	HM209435
	Yavatmal cotton	Maharashtra	HM209433
	Wasim cotton	Maharashtra	HM209434
	Akola chickpea	Maharashtra	HM209437
	Bangalore NBAII	Karnataka	GU323799
	Bangalore BCRL	Karnataka	HM209438
Chymotrypsine	Wardha cotton	Maharashtra	HM209419
	Vadodhara pigeon pea	Gujarat	HM209420
	Tandoor cotton	Andhra Pradesh	HM209421
	Bangalore NBAII	Karnataka	GU323796
	Bangalore BCRL	Karnataka	HM209422
Glutathione-S-transferase (GST)	Wardha cotton	Maharashtra	HM209430
	Vadodhara pigeon pea	Gujarat	–
	Tandoor cotton	Andhra Pradesh	HM209428
	Yavatmal cotton	Maharashtra	HM209429
	Wasim cotton	Maharashtra	HM209427
	Akola chickpea	Maharashtra	HM209431
	Bangalore NBAII	Karnataka	–
	Bangalore BCRL	Karnataka	–
Serine protease	Wardha cotton	Maharashtra	–
	Vadodhara pigeon pea	Gujarat	HM209424
	Tandoor cotton	Andhra Pradesh	HM209423
	Yavatmal cotton	Maharashtra	HM209425
	Bangalore NBAII	Karnataka	–
	Bangalore BCRL	Karnataka	HM209426

Primers for the above-mentioned genes were designed by employing IDT on-line software using the sequence information for the available accesssions in NCBI for *H. armigera* (Table 2).

About 1 µg of RNA was used for first-strand cDNA synthesis using RETROscript (Ambion, USA) according to the manufacturer's protocol. The RNA with oligodT primer was mixed with 2.0 µl of nuclease free water and heated to 75°C for 3 min, and 10× RT buffer (2.0 µl), 2.5 mM each of dNTP mix (4.0 µl), 1.0 µl of RNAase inhibitor (10 units/µl) and 1.0 µl of MMLV-reverse transcriptase (100 units/µl) were added, mixed and briefly centrifuged. The final 20 µl of reaction mixture was kept at 44°C for 90 min and the RT enzyme was heat-inactivated at 92°C for 10 min. The cDNA thus generated was used for PCR amplification using gene-specific primers.

The cDNA was diluted with nuclease free water (1 : 3) and used for PCR amplification employing gene specific primers. Briefly, 5 µl of 10× bufffer, 2.5 mM each of dNTP mix (2.0 µl), 10 pmol each of forward and reverse primers (2.0 µl), template cDNA (3.0 µl) and *Taq* polymerase 1.0 µl (3U; Takara, USA). The components were mixed with 35 µl of nuclease free water and PCR amplified with the following parameters; initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 45 s and extention at 72°C for 1 min and final extention was done at 72°C for 20 min. The PCR-amplified product was electrophoresed in 1.2% agarose gel, stained with ethidium bromide and visualized under UV transilluminator.

The amplicons were purified through Nucleospin Extract II (MN kit, Germany), according to the manufacturer's protocol. The eluted PCR fragments were ligated with PTZ57R/T TA cloning vector using T4 DNA ligase enzyme (Fermentas TA cloning kit, USA). For transformation, 5 µl of ligation mixture was added to competent DH5α cells and the cells were plated on LB agar plates containing ampicilin (50 µg/ml), 40 µl of 100 mM IPTG and 40 µl of X-Gal stock solution (20 mg/ml) per plate. The plates were incubated at 37°C for 16–18 h. The recombinant clones were selected by blue–white selection

Table 2. Primer design

Target gene	Primer ID	Sequence (5′…3′)	PCR product size (bp)	NCBI accession no.
Actin	Act F Act R	atgtgcgacgaggaagttgct ttagaagcacttcctgtggacg	1131	X97615
GST	GST F GST R	atgccgaaagcagtattctact ttaaaacaatgcttcaggggc	621	EF591059
Cytochrome P450	cyp F cyp R	atgatagccctactatggctggcg ttactggcgcagcttgacccta	1592	AY487948
Chymtrypsin	chy F chy R	atgaagttcgtggcactgacact ttagagctggccgttgatcca	894	EU325550
Serine protease	SP F SP R	atgcgtttccttgctctgct ttatgcgttagatgagatccagga	777	Y12283

followed by screening for recombinant plasmid in the agarose gel and finally employing PCR amplification using gene-specific primers and sequencing. Sequence analysis was carried out employing the on-line software BioEdit and submitted to NCBI (Table 1).

The gene sequences were analysed using BioEdit CLUSTAL X (version 1.8) and the specific sequences were submitted to NCBI. The final gene-specific sequences were subjected to dsCheck on-line software (http://dsCheck.RNAi.jp/) to select the specific region to synthesize the dsRNA. A 500 bp off-target minimized region was selected by employing dsCheck on-line software for all five genes, viz. actin, GST, cytochrome P450, chymotrypsin and serine protease. These regions were compared for other populations of the study and variations were recorded. Further, the selected dsRNA sequences were subjected to siDESIGN center (http://www.dharmacon.com), a web-based on-line software system for computing highly effective siRNA sequences with maximum target specificity.

Utility of RNAi in the management of insect pests has been demonstrated in various species of Lepidoptera[12], as well as those belonging to the other orders, viz. Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera and Orthoptera[13]. In addition to insects, RNAi-based approach has also been attempted in the management of plant parasitic nematodes[14–16] and other plant pathogens such as viruses[17] and fungi[18]. RNAi was observed in nematodes reared on the virus-infected plants[14,15]. For sap-sucking insects, RNAi was successfully applied by feeding dsRNA with sugar solution[19–21]. Application of conventional insecticides poses the twin problem of insecticide resistance, and severely affecting the ecosystem and non-target organisms like parasitoids, predators and pollinators. RNAi is also a convenient platform for functional genomics of insects[22]. Since RNAi is a sequence-specific mechanism, it could pave the way for a paradigm shift in designing futuristic, species-specific and eco-friendly pest management strategies. Various factors like, concentration of dsRNA[23,24], nucleotide sequence[25], length of the dsRNA fragment[7,26–29], persistence of the silencing effect[24,30] and life-stage of the target organism[25,31] influence the silencing effect of RNAi. More importantly, the sequence polymorphism of the target gene is one of the crucial factors limiting the widespread application of RNAi on different ecotypes of target insects[11]. Variations in the sequences have a bearing on the primary siRNAs generated in the cytoplasm of the target insects and this in turn affects the mRNA cleavage mediated by the RNA Induced Silencing Complex (RISC), may be due to failure of RISC loading on target mRNA. Longer dsRNA could result in off-target effects. This has been demonstrated in the triatomid bug, *Rhodnius prolixus*; together with the targeted nitroporin 2, two other highly homologous nitroporin genes were silenced[25]. Similarly, vacuolar H^+ ATPase dsRNA of the Colorado potato beetle (*Leptinotarsa decemlineata*) also silenced the ortholog gene in Western corn rootworm, *Diabrotica virgifera virgifera*, although at higher dsRNA concentration compared to the Colorado potato beetle[8]. Hence the selection of an off-target minimized sequence is highly essential in efficient application of dsRNA-mediated RNAi in target insects. The present study demonstrates sequence polymorphism of five target genes, viz. actin, GST, cytochrome P450, chymotrypsin and serine protease from different populations of *H. armigera* and their possible influence on the selection of regions for dsRNA/siRNA synthesis. The gene-specific primers amplified the expected amplicon size for the various target genes; actin (1131 bp), GST (621 bp), cytochrome P450 (1592 bp), chymotrypsin (894 bp) and serine protease (777 bp). This is in agreement with results of earlier workers[32–36] for the above genes. Sequence comparison of various target genes exhibited a total variation of 35 nucleotides (3.09%) for actin, 60 nucleotides (9.66%) for GST, 64 nucleotides (8.11%) for cytochrome P450, 36 nucleotides (4.02%) for chymotrypsin and 119 nucleotides (15.31%) for serine protease. In RNAi-mediated insect-pest management, it is essential to deliver the dsRNA to the target organism through various modes such as spray or by transgenic plants expressing dsRNA. Even though it is possible to express dsRNA for the full length of the target genes, it is not favoured as it might result in a pleiotropic effect. Appropriate length of the dsRNA is a determinant of uptake and silencing

Table 3. Nucleotide variations in the 500 bp dsRNA region for various populations of *Helicoverpa armigera*

Gene	Location/host crop	697	698	699	704	718	719	720	727	729	814	815	816	817	818	835	836	837	898
Actin	Bangalore BCRL	A	G	C	G	A	G	C	C	G	G	C	G	A	A	A	C	C	C
441–940	Wardha	G	C	G	C	.	.	.	.	.	A	G	C	T	G	G	T	G	A
	Vododhara	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Tandoor	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Yavatmal	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Wasim	.	.	.	.	C	C	G	T	T	.	.	.	.	.	.	.	.	.
	Akola	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Bangalore NBAII	A	G	C	G	.	.	.	.	.	G	C	G	A	A	A	C	C	C

Gene	Location/host crop	237	252	255	267	282	291	326	369	411	438	444	450	465	511	555	579	591	640
Chymotrypsin	Bangalore BCRL	C	T	G	T	T	C	A	T	C	T	T	T	G	C	C	C	C	T
157–656	Wardha	T	C	.	.	C	.	.	A	T	.	.	.	A	.	T	G	.	A
	Vododhara	.	.	.	A	.	.	G	.	.	C	C	C	G	.	C	C	.	.
	Tandoor	.	.	.	A	.	.	.	.	.	.	C	C	G	.	C	C	.	.
	Bangalore NBAII	.	.	A	.	.	T	.	.	.	T	.	.	.	T	C	C	T	.

Gene	Location/host crop	73	124	129	132	136	144	163	177	186	207	210	219	234	237	240	255	258	273	285	293	314	315	327	336	345	350
GST	Bangalore BCRL	G	C	T	T	C	C	G	T	A	C	G	G	A	G	T	G	A	C	T	A	C	A	T	G	G	T
45–544	Wardha	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	A	A	A
	Vododhara	.	.	.	.	T	.	.	C	.	.	.	.	G	C	C	A	G	.	.	.	.	.	T	G	.	.
	Tandoor	.	.	.	.	T	T	A	C	G	T	C	.	G	C	C	A	G	.	T	.	.	G	T	.	.	.
	Yavatmal	.	G	A	.	.	.	.	.	.	.	.	.	G	C	C	A	G	T	.	G	.	G	T	G	G	.
	Wasim	A	.	.	.	.	T	.	.	.	.	.	.	G	C	C	A	G	.	.	.	G	G	T	.	.	.
	Akola	.	.	C	.	.	.	.	.	.	T	C	.	.	.	.	A	G	T	.	.	G	.	T	.	.	.
	Bangalore NBAII	.	.	.	T	.	.	.	C	.	T	C	T	.	.	.	.	.	.	.	.	.	G	T	.	.	.

366	372	390	395	408	411	420	421	423	436	438	445	462	469	471	472	474	480	508	516	519	552	554
A	G	C	T	C	T	T	A	A	G	G	G	C	G	C	C	G	C	A	C	C	C	T
T	.	.	.	.	C	C	.	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.
A	.	.	.	.	.	T	.	.	.	A	A	.	A	.	.	.	.	A	T	T	.	.
.	A	T	C	.	.	T	G	.	.	.	.	.	.	.	.	.	.	A	.	.	.	C
A	.	.	.	.	T	T	.	.	.	.	.	.	.	G	T	C	.	A	.	.	G	.
A	.	.	.	T	T	.	.	.	A	.	.	T	A	.	.	.	.	A	.	.	.	.
A	.	A	.	.	T	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Gene	Location/Host crop	165	168	171	175	183	189	190	192	206	207	230	231	237	246	250	261	266	273	276	279	291	294
CYP9A14	Bangalore BCRL	C	C	C	T	G	T	T	G	T	G	A	C	G	A	C	G	A	A	C	G	C	C
160–659	Wardha	.	T	.	.	.	.	C	T	.	.	.	.	.	.	.	.	G	.	.	.	.	.
	Vododhara	.	C	T	C	.	.	.	.	C	A	.	.	A	T	.	.	.	.	.	.	T	.
	Tandoor	.	C	.	.	.	.	.	C	.	.	.	T	.	T	T	.	.	.	.	.	T	T
	Yavatmal	.	C	T	.	.	.	.	.	.	.	.	.	A	T	.	.	.	.	.	.	T	.
	Wasim	T	C	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	C	G	A	T	T
	Akola	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.
	Bangalore NBAII	.	C	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	T	T

(*Contd*)

Table 3. (*Contd*)

306	318	336	339	358	366	377	387	388
C	T	G	C	T	A	T	C	T
.	.	.	.	C	G	A	T	.
.	C	C	.	T	.	.	.	.
T	.	.	.	T	.	.	.	.
.	C	C	.	T	.	.	.	.
.	.	.	T	T	.	.	C	C
.	.	.	.	.	.	.	.	.
.	.	.	.	T	.	.	.	.

405	407	434	438	468	482	491	504	507	528	543	552	567	576	579	588	594	600	603	606	627	642	645
G	T	T	C	C	T	A	A	A	G	G	T	A	T	C	A	A	C	C	G	C	T	C
A	.	.	T	T	.	.	.	.	A	C	C	C	C	.	.	T	T	T	.	T	.	.
G	.	C	.	C	.	.	G	G	.	G	T	.	.	.	G	.	.	.	A	C	A	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
G	.	.	.	C	.	G	G	G	.	G	T	.	.	.	G	.	.	.	A	C	A	.
G	.	.	.	C	C	.	G	G	.	G	T	.	.	T	.	A	.	.	.	.	.	C
.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
G	.	.	.	C	.	.	G	G	.	G	T	.	.	.	G	.	.	.	A	C	A	.

Gene	Location/host crop	192	208	217	223	229	252	255	259	260	262	263	265	272	277	285	288	298	301	302	308	309	310	313
SP	Bangalore BCRL	T	T	T	A	T	G	G	C	T	C	A	C	C	G	T	T	C	G	C	A	C	A	A
191–690	Wardha	.	.	.	T	C	C	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.	.
	Vododhara	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	G
	Tandoor	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Yavatmal	C	.	C	.	G	G	T	T	C	A	G	T	.	.	.	.	.	.	.	.	.	.	.
	Bangalore NBAII	.	C	.	A	T	G	T	.	C	.	.	.	T	A	A	.	T	G	C	C	A	T	.

321	325	326	327	335	337	338	340
C	A	T	A	A	G	A	C
.	.	.	.	.	.	.	.
.	G	.	.	.	.	.	.
.	.	.	.	.	.	.	.
T	.	.	.	.	.	.	.
.	.	A	C	G	A	C	A

346	347	352	354	356	358	361	364	383	384	385	394	395	397	402	404	406	411	415	417	418	422	424	425	438	440	442	444	450
C	A	C	T	G	C	C	T	C	T	C	C	A	T	G	G	T	T	T	G	C	T	C	C	G	C	G	C	G
.	.	.	.	.	T	T	G	.	.	.	.	C	A	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.
.	.	.	.	.	C	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	T	.
.	.	.	.	.	.	.	.	.	A	T	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	A
A	C	A	C	C	G	C	T	T	.	.	A	A	.	C	C	C	.	C	C	T	G	G	C	C	T	A	A	.

452	462	468	483	484	486	519	520	525	526	527	528	530	531	532	544	557	576	579	582	587	588	639	645	651	669	672
C	C	A	C	G	T	C	G	G	A	G	A	G	T	A	A	A	T	C	C	C	T	T	C	C	C	C
.	T	C	T	T	C	T	C	C	G	T	G	C	C	.	.	.	C	T	.	A	C	A	.	T	.	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	.
.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
T	.	.	C	.	.	.	.	.	.	.	.	.	.	T	T	G	T	.	.	.	.	T	.	.	.	.
.	C	A	C	G	T	C	G	G	A	G	A	G	T	.	.	.	T	C	T	C	T	T	T	.	.	T

Table 4. Common siRNA and target positions in the 500 bp dsRNA selected region

Gene	Location	Sense strand sequence	Region	Start position	GC %	Score
Actin (441–940)	Wardha	GTAAAGATCTGTATGCGAA	ORF	432	37	87
	Vododhara	GTAAAGATCTGTATGCGAA	ORF	432	37	87
	Tandoor	GTAAAGATCTGTATGCGAA	ORF	432	37	87
	Yavatmal	GTAAAGATCTGTATGCGAA	ORF	432	37	87
	Wasim	GTAAAGATCTGTATGCGAA	ORF	432	37	87
	Akola	GTAAAGATCTGTATGCGAA	ORF	432	37	87
	Bangalore NBAII	GTAAAGATCTGTATGCGAA	ORF	432	37	87
	Bangalore BCRL	GTAAAGATCTGTATGCGAA	ORF	432	37	87
GST (45–544)	Wardha	AGACATACGCTTTGAACGA	ORF	46	42	85
	Vododhara	AGACATACGCTTTGAACGA	ORF	46	42	85
	Tandoor	AGACATACGCTTTGAACGA	ORF	46	42	85
	Yavatmal	AGACATACGCTTTGAACGA	ORF	46	42	85
	Wasim	AGACATACGCTTTGAACGA	ORF	46	42	85
	Akola	AGACATACGCTTTGAACGA	ORF	46	42	85
	Bangalore NBAII	AGACATACGCTTTGAACGA	ORF	46	42	85
	Bangalore BCRL	AGACATACGCTTTGAACGA	ORF	46	42	85
Cytochrome CYP9A14 (160–659)	Wardha	GCTCCAAGATGAAGGCGAT	ORF	254	53	83
	Vododhara	GCTCCAAGATGAAGGCGAT	ORF	254	53	83
	Tandoor	GCTCCAAGATGAAGGCGAT	ORF	254	53	83
	Yavatmal	GCTCCAAGATGAAGGCGAT	ORF	254	53	83
	Wasim	GCTCCAAGATGAAGGCGAT	ORF	254	53	83
	Akola	GCTCCAAGATGAAGGCGAT	ORF	254	53	83
	Bangalore NBAII	GCTCCAAGATGAAGGCGAT	ORF	254	53	83
	Bangalore BCRL	GCTCCAAGATGAAGGCGAT	ORF	254	53	83
Chymotrypsin (157–656)	Wardha	GCAGCCAGATCAACGAGAA	ORF	365	53	76
	Vadodhara	GCAGCCAGATCAACGAGAA	ORF	365	53	76
	Tandoor	GCAGCCAGATCAACGAGAA	ORF	365	53	76
	Bangalore NBAII	GCAGCCAGATCAACGAGAA	ORF	365	53	76
	Bangalore BCRL	GCAGCCAGATCAACGAGAA	ORF	365	53	76
Serine protease (191–690)	Wardha	ACTCAAGGACCATGGACAA	ORF	140	47	85
	Vadodhara	ACTCAAGGACCATGGACAA	ORF	140	47	85
	Tandoor	ACTCAAGGACCATGGACAA	ORF	140	47	85
	Yavatmal	ACTCAAGGACCATGGACAA	ORF	140	47	85
	Bangalore NBAII	ACTCAAGGACCATGGACAA	ORF	140	47	85
	Bangalore BCRL	ACTCAGGACCAATGGAACA	ORF	140	47	80

efficiency in various organisms[7] and cell lines[26]. In feeding experiments, most sequences range between 300 and 520 bp for insect pests[13]. In the case of S2 cells, Saleh *et al.*[26] reported that the length of the dsRNA should be minimally 211 bp. The ideal size of dsRNA for designing the plant modular vector is 300–800 nt for controlling the plant viruses[37]. High percentage (90%) of gene silencing was observed when a 504 bp target sequence was selected (*Apis mellifera*, vitellogenin) by Nunes and Simões[38], with 50–70% (*Reticulitermes flavipes*, caste regulatory hexamerin storage protein) and 60% of gene silencing (*R. flavipes*, cellulose enzyme) with 500 bp target sequence lengths[39]. Therefore, it is necessary to select an appropriate off-target minimized region for various genes. Therefore, we have chosen 500 bp length for various target genes for area-wide application of RNAi-mediated management of insect pests such as *H. armigera*. It was also important to find out a conserved region in the target genes among various populations of *H. armigera*. We selected a 500 bp off-target, minimized dsRNA region using the on-line software dsChek[40] for Bangalore-2(BCRL) strains with the following regions: 441–940 bp (actin), 45–544 bp (GST), 160–659 bp (cytochrome P450), 157–656 bp (chymotrypsin) and 191–690 bp (serine protease), and the same selected region of dsRNA was compared with the other strains selected in the study. Upon comparison using software BioEdit CLUSTAL X (version 1.8), the following number of changes were observed in nucleotide positions (Table 3): 18 (actin – 3.6%), 49 (GST – 9.8%), 54 (cytochrome P450 – 10.8%), 18 (chymotrypsin – 3.6%) and 88 (serine protease – 17.6%). We have observed sequence variations in the 500 bp off target minimized regions for various target genes. Therefore, employing this long dsRNA would be less effective as compared to the common siRNAs. In a previous study siRNAs have been found to be effective in

silencing acetylcholine esterase of *H. armigera*[28]. The choice to use siRNA was based on its success in clinical research[27,29]. Hence we further subjected the selected off-target, minimized 500 bp dsRNA to a another web-based on-line software, systems siDESIGN (http://www.dharmacon.com, ON-TARGET*plus*), using the target sequence to find out common siRNAs across the populations considered in the study. This produced both common and uncommon siRNAs from all locations, except one, in the case of serine protease (Bangalore 2; Table 4). The common siRNAs recorded for a specific gene sequence of *H. armigera* can be applied to other populations effectively for a novel pest management strategy as concluded by the presence of common *in silico*-generated siRNAs across all the locations, but this has to be further confirmed by insect bioassays.

We have analysed the sequence variation in the off-target minimized 500 bp dsRNA region for various genes for different populations of *H. armigera*. *In silico* analysis revealed that both common and uncommon siRNAs are produced for each target gene in different populations of *H. armigera*. Since RNAi is a sequence-specific degradation mechanism, it is crucial to select a highly conserved, off-target, minimized sequence. Therefore, selection of regions in the target genes from various conspecific populations of *H. armigera* that produce common siRNAs is important for effective silencing of target genes with no off target effect. Here the demonstrated strategy may be useful for off-target, minimized, effective gene silencing of different populations and can be applied to any species for RNAi-mediated, off-target minimized, effective gene silencing. However, the particular length of dsRNA synthesized for a particular population has to be validated on other populations of *H. armigera* by bioassay studies.

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Consequences of underestimating ancient deforestation in South India for global assessments of climatic change

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Land-cover changes occurring before 1800 are often ignored in the estimation of CO_2 emissions, probably because they are poorly documented in most tropical countries. India appears to be an exception to this rule. It was possible to reconstitute the main stages of the land-cover history for a large region of South India, and therefore to retrace the dynamics of CO_2 emissions during nearly 1000 years. It was then possible to demonstrate that 25% of the total emissions occurred before 1800, and are mistakenly considered as more recent emissions.

Keywords: Ancient deforestation, carbon dioxide emissions, climate change, global assessments, land cover.

IN 2004, deforestation and biomass decomposition accounted for 17.3% of the total 49 Pg of CO_2-equivalent anthropogenic emissions of greenhouse gases (GHGs)[1], while fossil fuel consumption represented 56.6%. Global estimates of recent emissions are computed from country-level inventories following IPCC guidelines[2,3]. For past emissions, ancient land-cover changes are either neglected[4] or introduced through a historical frame[5,6]. In this communication, we assess the consequences of not considering ancient land-cover changes when estimating GHG anthropogenic emissions for a large study area located in South India (Figure 1), focusing on soil organic carbon (SOC) which represents the largest terrestrial C pool[7].

In 2001, the Intergovernmental Panel on Climate Change (IPCC)[4] calculations for land-use (LU) change related emissions amounted to 121 PgC for the period 1850–1990, with LU changes obtained by the difference between potential[8] and current[9] vegetation maps. Potential vegetation was represented by the 1850 land cover, with the implicit hypothesis that no significant deforestation occurred before that date, and that GHGs emissions due to land-use/land-cover changes had started at about the same time as those due to fossil-fuel consumption.

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