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In silico Designing of Effective and Specific dsRNAs and siRNAs for Post-transcriptional Silencing of Whitefly, *Bemisia tabaci* Genes with Minimized Off-target Effects

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ABSTRACT

Whitefly (*Bemisia tabaci*) is a phloem-sap sucking and the most invasive insect-pest which transmits several pathogenic viruses to the important crops worldwide causing a damage of billions of dollars per year. Novel, effective and eco-friendly strategies are urgently required for their management. RNA interference (RNAi) is a next generation bio-pesticide technology with high species-specificity and environmentally-friendly. The four whitefly vital genes, namely, *Vitellogenin receptor*, *Ryanodine receptor*, *Nicotinic acetylcholine receptor beta 1 subunit* and *Laccase 1* that are important for the survival and reproduction of whitefly were selected to design effective and specific dsRNAs and siRNAs for their knockdown via RNAi. BLAST homology search was performed to remove off-target effects on the coding sequences of whitefly selected genes sequences retrieved from the NCBI database. Potent siRNAs were designed using i-score designer. Mean free-energy (MFE) calculated using RNA fold server was used to identify sequences having lowest MFE and higher stability. The accessibility of mRNA target sites by siRNAs was predicted by Sirna algorithm of S fold web server. The siRNAs selected in this study were highly specific and efficient to silence whitefly genes with no or negligible off target effects.

Key words: Whitefly, management, RNAi, dsRNAs/siRNAs, off-target effects, *in silico* tools

INTRODUCTION

Whitefly (*Bemisia tabaci*) is a phloem sap sucking insect-pest of important food and fibre crops in the world and transmits several pathogenic viruses resulting in losses of several million dollars per year. It is listed among the world's most invasive species. These are controlled by use of chemically synthesized insecticides that are harmful to environment. However, these rapidly evolve resistance against conventional pesticides rendering their management difficult. RNA interference is an evolutionary conserved process of gene silencing where a target gene is silenced by degradation of its mRNA by introducing its complementary dsRNA. This process plays an essential role in identification of gene function and for the effective control of insect-pests and viruses. RNAi has been used for the management of whiteflies by decreasing expression of its vital genes thus reducing their fitness for adaptation. dsRNAs of whitefly genes have been injected into whitefly body or fed orally via artificial diet or sprayed exogenously on plants or injected or expressed in plants to reduce the targeted gene expression resulting into control of whiteflies (Grover *et al.*, 2018; Suhag *et al.*, 2021). dsRNAs are cleaved by specialized cytoplasmic RNase enzyme, dicer into small non-coding double stranded molecule called, siRNAs. The siRNAs are 21-23 nucleotides long and have two nucleotide overhangs at 3'end (Malik *et al.*, 2016). When siRNA interacts with RISC (RNA induced silencing complex), RISC is activated and it cleaves siRNA's passenger strand through its endonuclease component called argonaute2 (AGO2), but it does not cleave the guide strand which is kept intact with RISC. Consequently, the guide strand guides the active RISC towards its target mRNA resulting into binding of siRNA with the complimentary mRNA which in turn is cleaved

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by AGO2 and causing silencing of the targeted gene (Luo *et al.*, 2017). However, a potential risk of non-specific binding of siRNA to mRNA of non-target organisms including beneficial insects such as pollinators and natural predators leads to off-target silencing. To avoid off target effects, target gene selection and dsRNAs/siRNAs design are important deliberation. *In silico* sequence homologies search between all possible sRNA sequences and available genome sequences would help to identify specific sRNAs with no off-targets (Jaiwal *et al.*, 2020; Suhag *et al.*, 2021).

In the present study, *in silico* approach has been used to design effective and specific dsRNAs and siRNAs for the post transcriptional silencing of four whitefly vital genes, *Laccase 1*, *Vitellogenin receptor, Rynodine receptor* and *Nicotinic acetylcholine* receptor β-subunit for their control with minimal off-target effects. These whitefly genes play essential roles in their survival and adaptation on host plant. Laccases are polyphenol oxidases found in insects to oxidize secondary plant compounds involved in plant defence (Zhang *et al.*, 2018; Nitnavare *et al.*, 2021). One of the isoforms of laccase, *Lac1* is involved in iron homeostasis, oxidation of toxic compounds in the diet and immune defence of insects. Down regulation of *Lac1* gene is expected to be lethal for the insect as it might decrease the adaptability of whiteflies on host plant. Vitellogenin (Vg) is involved in the development of insect's oocytes and embryo. It is produced in fat body of insect, moves through haemolymph and accumulates in oocytes through mediation of vitellogenin receptors (VgR) present on the surface of oocytes (Upadhyay *et al.*, 2016). These proteins are important for fecundity, and therefore can be a potential RNAi target for insect control in crop plants. The third essential gene to be targeted is ryanodine receptor gene. Ryanodine receptors (RyRs) are Ca^{2+} channels present on endo (sarco) plasmic reticulum of muscle and neuron cells to regulate the release of calcium from intracellular stores (Li *et al.*, 2015). Down regulation of these receptors in *Bemisia tabaci* is expected to be lethal by evoking typical symptoms including failure of body contraction, feeding cessation, paralysis and subsequent mortality. The fourth gene to be targeted is the nicotinic acetylcholine receptor gene. Nicotinic acetylcholine receptors (nAChRs), the excitatory neurotransmitter receptors, are the major target for several insecticides (Somers *et al.*, 2018). Silencing these receptors through RNAi may prove an ideal approach to control whiteflies. Realising the importance of these four genes, the potent dsRNAs and siRNAs to knockdown the whitefly vital genes, *Laccase 1, Vitellogenin receptor, Rynodine receptor* and *Nicotinic acetylcholine receptor β-subunit* have been designed in the present research to effectively control whiteflies with no or minimal off-target effects.

MATERIALS AND METHODS

The coding sequences of the four selected genes of *B. tabaci* biotype, namely, *Laccase* 1, *Vitellogenin receptor, Rynodine receptor* and *Nicotinic acetylcholine receptor* β-subunit were retrieved in FASTA format from the nucleotide database of National Centre for Biotechnological Information (NCBI) (*http://www.ncbi.nlm.nih.gov/*). The information regarding these genes is presented in Table 1.

dsRNAs in each of whitefly gene were designed by identifying a continuous stretch of 100 or more than 100 nucleotides free of any off-target effects. The whitefly gene regions predicted for dsRNAs were: (1) *B. tabaci* vitellogenin receptor gene (*BtVgR*): 105 bp (Accession no. HM017828.2; from 2080 to 2184); (2) *Ryanodine receptor gene* (*BtRyR*) : 171 bp (Accession no. KY244091.1; from 250 to 420); (3) *Nicotinic acetylcholine receptor* beta1 gene (*BtnAChRB1*) : 195 bp (Accession no. KX602254.1; from 198 to 392) and (4) Laccase gene (*BtLAC1*) : 198 nucleotides (Accession no. KY643659.1; from 754 to 951).

The coding sequences of all the four selected genes were divided in 21 nucleotide long sequences. Thereafter, BLAST homology search was performed for each 21 nt long sequence. To avoid any off-target effect, only those sequences were selected which did not show any homology to any important gene sequence of non-target organisms. The sequences which showed more than 75% similarity or more than 16 base pair matched with non-target organisms were rejected.

A general criterion followed during siRNA designing was:

1. siRNA sequence length should be 21-23 nt long.
2. Avoid regions within 50-100 base pairs

of the start as well as the termination codon.
3. Intron region should be avoided.
4. Avoid the stretches with repeats of four or more nucleotides i. e. AAAA, GGGG.
5. GC content should be between 30-60%.
6. Avoid those sequences which showed similarity to other non-target genes.
7. Low complex sequences and repeats should be avoided.
8. Single Nucleotide Polymorphism (SNP) sites should be avoided.

The server "i-Score Designer" was used to calculate nine different algorithms (Amarzguioui, Ui-Tei, Takasaki, Hsieh, i-Score, s-Biopredsi, DSIR, Reynolds and Katoh) for predicting the most potential siRNA in dsRNA region (Verma *et al.*, 2020; Rahman *et al.,* 2021). This approach enhanced the specificity and efficacy of siRNA to silence the target gene. To predict active siRNAs, i-Score used a simple algorithm called inhibitory-Score (i-Score). Score was calculated for any siRNA by second generation algorithm like DSIR, s-Biopredsi and i-Score giving the indication of percentage reduction in the expression of gene by that specific siRNA. For example, if any siRNA had i-Score 66.2, it meant according to i-Score algorithm, that siRNA will reduce the expression of that gene by 66.2%.

A continuous stretch of more than hundred nucleotides which showed no homology to any other important genes in other organisms was selected to predict active siRNAs but to ensure complete removal of any off-target side effect, i-Score designer predicted siRNAs were again subjected to BLASTn search. The candidate siRNAs showing any similarity to any important non-target organism were discarded. DNA/RNA GC Content Calculator Endmemo (*http://www.endmemo.com/bio/gc.php*) software was used to calculate the GC% of selected siRNA. Only those siRNAs were considered whose GC content was between 30-60%.

Tm (melting temperature) of the seed region (position 2-8 from 5' end) of siRNA was shown to be below 21.5°C. All the siRNAs with Tm either equal to or below 21.5°C were selected. The Tm of seed region was calculated by the OligoCalc tool (*http://biotools.nubic.northwestern.edu/OligoCalc.html*) using:

$$Tm=\{(1000\ H)/(A+S+R\ln\ (CT/4))\}-273.15+16.6\log[Na^+]$$

Here, R the gas constant (1.987 cal/deg./mol), Na^+ concentration of sodium fixed at 100 mM, A helix initiation constant (-10.8), S the sum of the nearest neighbour entropy change, H the sum of the nearest neighbour enthalpy change (kcal/mol).

For the prediction of efficient siRNAs, the selected siRNAs were further screened by secondary structure and G-value prediction. The G-value of the most stable secondary structure of siRNA strand was computed according to the RNA mfold algorithm by using i-Score designer tool (*https://www.med.nagoya-u.ac.jp/neurogenetics/i_Score/i_score.html*). The secondary structure and free-energy of folding of the selected siRNAs were calculated by using RNAfold web server (*http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi; Trotta, 2014*).

The accessibility of the siRNAs to their target sites and duplex thermodynamics were predicted using the Sirna module and other proposed rules of the Sfold server. The other parameters like probability profile of a given stretch of nucleotides in the target mRNA to remain single stranded and loop specific profiles were used to evaluate approachability of target site for siRNA.

RESULTS AND DISCUSSION

Plants and animals tolerate dsRNAs of >200 bp and process them to generate a pool of siRNAs to improve RNAi efficiency but simultaneously their small size and the conservation of genes across different organisms result in the off-target effects (Suhag *et al.*, 2021). In this study, short dsRNAs/siRNAs were designed for the maximum RNAi effects on the four whitefly vital genes, namely *Laccase 1*, *Vitellogenin receptor, Ryanodine receptor* and *Nicotinic acetylcholine receptor* β-subunit with the minimum off-target effects. The effective siRNAs were designed by comprehensively applying empirical guidelines available for their design and mRNA target accessibility for silencing whitefly genes. The coding sequences of the selected genes were retrieved from the NCBI. In each target gene

Table 1. Details of the four *B. tabaci* genes selected as the potential targets for RNAi

S. No.	Target gene	Accession number	Gene length (nt)	Predicted dsRNAs within the gene free from off target effects	Nucleotide sequence of predicted dsRNAs
1.	*Vitellogenin* receptor	HM017828.2	5742	105 bp (from 2080 to 2184)	TATTGGTGCGACAAAGAGGCCG GCACTGTAGAAGTGTTCAGTTT CTTCTCTCACAGAAGGAAGTTG TTGCTCCGGGAATTTGACGATG AAAAACCTTTCGCCATG
2.	*Ryanodine* receptor	KY244091.1	15369	171 bp (from 250 to 420)	TCTGAACAGGGCAAAGGAACTG GCTCTGGTCATCGAACTTTACT GTATGGAAATGCAATTCTGCTT CGACATCAAAATAGTGATATGT ATCTCGCCTGTCTTTCAACTAG CTCTTCTAATGACAAACTCTCAT TTGATGTTGGTTTGCAAGAACA TTCACAAGGTGAAGCG
3.	*Nicotinic acetylcholine* receptor β 1 subunit	KX602254.1	1597	195 bp (from 198 to 392)	GGCTGGCTTTCGTTCAACTTAT TAATGTGAATGAGAAGAATCAA ATTATGAAATCAAACGTCTGGT TGCGACTGGTATGGAATGATTA CCAACTTCGATGGGATGAAACA GATTACGGAGGGATAGGTGTTT TGCGATTACCACCGGATAAAGT GTGGAAACCTGATATTGTTCTT TTCAACAATGCCGATGGTA
4.	*Laccase* 1 (LAC1)	KY643659.1	2733	198 nucleotides (from 754 to 951)	GGCAAAGGACGCTACAAGAGTG GTAACGCTCACGAGGCCCTCAA GACGCCTCTTGCGATTTTCAAC GTGAAAAAAGGTGAAAAATACA GATTCCGATTGATAAATGCAGG ATTCCTCAACTGCCCAATCGAAA TGTCTATTGATAATCACACAATC ACTGTCATCAATAGCGACGGCG GTGACATCGAACCGGAAGAA

sequence, a 100 or more nucleotides long stretch of the coding region free from the homology with off-target organisms was identified using NCBI BLAST homology search engine. The details of the four target genes of *B. tabaci* are shown in Table 1. The coding sequence of each gene was first divided into 21 nucleotides long sequences. Each sequence then was subjected to sequence homology tool, BLAST search. Sequences which showed similarity of more than 16 nucleotides to any important gene of non-target organisms were further evaluated for the similarity in the seed region. The sequences with similarity in the seed region along with the homologous region of non-target transcript in mRNA were rejected. Thereafter, the regions which were 100 or >100 nucleotides long and free from off-target effects were selected to design dsRNAs. This region was then fed to i-Score designer to score effective siRNAs present in a dsRNA.The i-Score designer tool works on i-Score (inhibitory-score) algorithm (Verma *et al.*, 2020; Rahman *et al.*, 2021).

A list of potential siRNA sequences of all the four target genes predicted by i-Score designer tool are shown in Table 2. The details of all the parameters of siRNAs predicted by i-Score designer are given in Table 3. The designed siRNAs showed a strong correlation between the nucleotide sequence features and their silencing efficacy. Further, the potent siRNAs exhibited more sequence-based features at specific base positions as per the guidelines for the effective silencing of target gene. Fourteen siRNA candidates scored more than 75% (Table 3). The length of siRNAs derived from long dsRNAs varied with species. The dicer processes dsRNAs into 19 to 23 nucleotides long sequences with two nucleotides overhanged at 3' end. Twenty-five to twenty-seven nucleotides long sequences

Table 2. List of the potential siRNA sequences of *B. tabaci*

S. No.	Gene name	Sequence	Length	Location of siRNA within gene	GC (%)	ΔG of most stable 2° Structure	Secondary structure
1.	*Vitellogenin* receptor	GGCACTGTAGAAGTGTTCAGT	21	2101-2121	47.6	-4.8	1a
2.	*Vitellogenin* receptor	CTGTAGAAGTGTTCAGTTTCT	21	2105-2125	38	0.2	1b
3.	*Vitellogenin* receptor	GGAAGTTGTTGCTCCGGGAAT	21	2138-2158	52.4	-1.5	1c
4.	*Vitellogenin* receptor	CCGGGAATTTGACGATGAAAA	21	2151-2171	42.8	0.2	1d
5.	*Vitellogenin* receptor	CGGGAATTTGACGATGAAAAA	21	2152-2172	38.1	0.5	1e
6.	*Vitellogenin* receptor	GGGAATTTGACGATGAAAAAC	21	2153-2173	38.1	1.9	1f
7.	*Vitellogenin* receptor	GGAATTTGACGATGAAAAACC	21	2154-2174	38.1	1.8	1g
8.	*Ryanodine* receptor	CGAACTTTACTGTATGGAAAT	21	283-303	33.3	0.7	2a
9.	*Ryanodine* receptor	GAACTTTACTGTATGGAAATG	21	284-304	33.3	0.7	2b
10.	*Ryanodine* receptor	CTGCTTCGACATCAAAATAGT	21	310-330	38.1	2	2c
11.	*Ryanodine* receptor	GCTTCGACATCAAAATAGTGA	21	312-332	38.1	1.9	2d
12.	*Ryanodine* receptor	CGACATCAAAATAGTGATATG	21	316-336	33.3	-1	2e
13.	*Ryanodine* receptor	ATTTGATGTTGGTTTGCAAGA	21	381-401	33.3	-0.6	2f
14.	*Ryanodine* receptor	AGAACATTCACAAGGTGAAGC	21	399-419	42.9	-1.7	2g
15.	*Nicotinic acetylcholine* receptor beta1 subunit	GGCTTTCGTTCAACTTATTAA	21	202-222	33.3	1.6	3a
16.	*Nicotinic acetylcholine* receptor beta1 subunit	GAAGAATCAAATTATGAAATC	21	232-252	23.8	0.2	3b
17.	*Nicotinic acetylcholine* receptor beta1 subunit	GGTTGCGACTGGTATGGAATG	21	261-281	52.4	0.2	3c
18.	*Nicotinic acetylcholine* receptor beta1 subunit	GGTATGGAATGATTACCAACT	21	271-291	38.1	-3.2	3d
19.	*Nicotinic acetylcholine* receptor beta1 subunit	GATGGGATGAAACAGATTACG	21	294-314	42.9	0.5	3e
20.	*Laccase* 1 (LAC1)	CGATTTTCAACGTGAAAAAAG	21	809-829	33.3	-1.9	4a
21.	*Laccase* 1 (LAC1)	CGTGAAAAAAGGTGAAAAATA	21	819-839	28.6	3.6	4b
22.	*Laccase* 1 (LAC1)	CAATCGAAATGTCTATTGATA	21	878-898	28.6	0.4	4c
23.	*Laccase* 1 (LAC1)	CGAAATGTCTATTGATAATCA	21	882-902	28.6	-1.8	4d
24.	*Laccase* 1 (LAC1)	CGACGGCGGTGACATCGAACC	21	924-944	66.6	-0.4	4e

had been reported to be processed by dicer and effective in gene silencing. The optimal length of siRNAs is still to be resolved. The siRNAs gene silencing efficacy is influenced by its GC content. The siRNAs with GC content between 30-60% were effective. In this study, GC content of siRNAs varied from 28.6 to 63.2% (Table 2) and those with GC content less than 30% were discarded. The siRNAs with GC content greater or equal to 33% were finally selected. The low GC content slows down the hybridization of siRNA to target mRNA and high GC content delays unwinding of siRNA duplex mediated by nuclear helicases. The specificity, efficacy and stability of a potent siRNA were determined by MFE (Minimum Free Energy). The siRNA with lower MFE were more stable than those with higher MFE. The nucleotides sequence and their arrangement also affect the MFE and the stability of siRNA. Longer sequences were found to be more stable than those with shorter sequences; therefore, the longer DNA stretches were preferred to design dsRNAs. Moreover, GC rich regions with higher number of hydrogen bonds were more stable than AU rich RNAs.

Target accessibility is another major factor which determines siRNA silencing efficiency. Binding of siRNA to the target mRNA governs the RNAi efficacy which depends on RISC assembly and secondary structures at the target site. Binding of siRNA to the target site in mRNA was accessed using Sirna algorithm of Sfold web-server. The siRNA binds only to single-stranded regions in mRNA free from secondary structures. To access the target sites, energy is needed to open the secondary structures around them. Probability profile that describes the availability of single stranded region in target mRNA for siRNA binding are shown in Fig. 1. The loop specific probability profile reports the folding of a specific nucleotide stretch at target site to form a specific type of loop i.e hairpin (Hplot), bulge (Bplot), internal loop (Iplot) and multi-branched loop (Mplot). The loop specific profiles of target site of Laccase 1 gene are presented in Fig. 2. Gene silencing efficacy of siRNA is also governed by duplex end thermodynamics. Dicer usually binds to the strand which is thermodynamically less

Table 3. The details of siRNAs designed through i-score designer

S. No.	Gene	Sense	Antisense	Location of siRNA within gene	5'end	3'end	Whole dG	GC stretch	i-score	DSIR	s-Biopr-edsi	MFE (kcal/mol)
1.	*Vitellogenin* receptor	GGCACUGUAGA AGUGUUCAGU	ACUGAACACUU CUACAGUGCC	2101-2121	-3.3	-2.1	-38.1	3	66.2	94.1	0.8	-33.00
2.	*Vitellogenin* receptor	CUGUAGAAGUG UUCAGUUUCU	AGAAACUGAAC ACUUCUACAG	2105-2125	-2.1	-0.9	-33.2	1	68.0	97.0	0.83	-27.80
3.	*Vitellogenin* receptor	GGAAGUUGUU GCUCCGGGAAU	AUUCCCGGAGC AACAACUUCC	2138-2158	-3.3	-2.4	-41.7	5	70.0	94.4	0.84	-36.60
4.	*Vitellogenin* receptor	CCGGGAAUUUG ACGAUGAAAA	UUUUCAUCGUC AAAUUCCCGG	2151-2171	-3.3	-0.9	-36.5	5	78.3	95.1	0.87	-31.40
5.	*Vitellogenin* receptor	CGGGAAUUUG ACGAUGAAAAA	UUUUUCAUCGU CAAAUUCCCG	2152-2172	-2.4	-0.9	-34.1	4	74.6	91.7	0.86	-29.00
6.	*Vitellogenin* receptor	GGGAAUUUGAC GAUGAAAAAC	GUUUUUCAUCG UCAAAUUCCC	2153-2173	-3.3	-0.9	-32.6	3	77.0	94.9	0.86	-27.50
7.	*Vitellogenin* receptor	GGAAUUUGAC GAUGAAAAACC	GGUUUUUCAUC GUCAAAUUCC	2154-2174	-3.3	-0.9	-30.2	2	85.7	98.9	0.88	-24.70
8.	*Ryanodine* receptor	CGAACUUUACU GUAUGGAAAU	AUUUCCAUACA GUAAAGUUCG	283-303	-2.4	-0.9	-32.8	2	79.0	96.5	0.86	-27.70
9.	*Ryanodine* receptor	GAACUUUACU GUAUGGAAAUG	CAUUUCCAUAC AGUAAAGUUC	284-304	-2.4	-0.9	-31.3	2	77.6	94.4	0.86	-25.80
10.	*Ryanodine* receptor	CUGCUUCGACA UCAAAAUAGU	ACUAUUUUGAU GUCGAAGCAG	310-330	-2.1	-1.3	-32.8	2	70.8	91.1	0.83	-27.70
11.	*Ryanodine* receptor	GCUUCGACAU CAAAAUAGUGA	UCACUAUUUUG AUGUCGAAGC	312-332	-3.4	-2.2	-32.9	2	71.2	92.9	0.83	-27.60
12.	*Ryanodine* receptor	CGACAUCAAAA UAGUGAUAUG	CAUAUCACUAU UUUGAUGUCG	316-336	-2.4	-1.3	-31.0	2	76.3	98.6	0.85	-25.50
13.	*Ryanodine* receptor	AUUUGAUGUU GGUUUGCAAGA	UCUUGCAAACC AACAUCAAAU	381-401	-1.1	-0.9	-31.6	2	68.2	91.3	0.84	-26.00
14.	*Ryanodine* receptor	AGAACAUUCACA AGGUGAAGC	GCUUCACCUUG UGAAUGUUCU	399-419	-2.1	-0.9	-34.4	2	68.1	94.1	0.85	-28.80
15.	*Nicotinic acetylcholine* receptor beta1 subunit	GGCUUUCGU UCAACUUAUUAA	UUAAUAAGUUG AACGAAAGCC	202-222	-3.3	-0.9	-32.4	3	75.4	91.9	0.86	-27.10
16.	*Nicotinic acetylcholine* receptor beta1 subunit	GAAGAAUCAA AUUAUGAAAUC	GAUUUCAUAAU UUGAUUCUUC	232-252	-2.4	-0.9	-26.8	1	80.4	92.0	0.86	-21.30
17.	*Nicotinic acetylcholine* receptor beta1 subunit	GGUUGCGACU GGUAUGGAAUG	CAUUCCAUACC AGUCGCAACC	261-281	-3.3	-0.9	-39.7	3	75.8	101.2	0.86	-34.20
18.	*Nicotinic acetylcholine* receptor beta1 subunit	GGUAUGGAAU GAUUACCAACU	AGUUGGUAAUC AUUCCAUACC	271-291	-3.3	-0.9	-34.0	2	81.7	98.8	0.87	-28.50
19.	*Nicotinic acetylcholine* receptor beta1 subunit	GAUGGGAUGA AACAGAUUACG	CGUAAUCUGUU UCAUCCCAUC	294-314	-2.4	-1.3	-34.1	3	75.2	94.0	0.85	-28.60
20.	*Laccase* 1 (LAC1)	CGAUUUUCAAC GUGAAAAAAG	CUUUUUUCACG UUGAAAAUCG	809-829	-2.4	-0.9	-28.9	2	80.8	93.5	0.87	-23.80
21.	*Laccase* 1 (LAC1)	CGUGAAAAAAG GUGAAAAAUA	UAUUUUUCACC UUUUUUCACG	819-839	-2.4	-0.9	-29.3	2	81.8	93.1	0.88	-23.80
22.	*Laccase* 1 (LAC1)	CAAUCGAAAUG UCUAUUGAUA	UAUCAAUAGAC AUUUCGAUUG	878-898	-2.1	-2.4	-30.8	2	74.0	96.0	0.85	-25.30
23.	*Laccase* 1 (LAC1)	CGAAAUGUCUA UUGAUAAUCA	UGAUUAUCAAU AGACAUUUCG	882-902	-2.4	-1.1	-28.7	2	76.7	96.7	0.85	-23.30
24.	*Laccase* 1 (LAC1)	CGACGGCGGUG ACAUCGAACC	GGUUCGAUGU CACCGCCGUCG	924-944	-2.4	-0.9	-42.0	6	73.8	96.3	0.85	-36.50

stable at 5' end especially in antisense strand. The internal stability of siRNA was evaluated for its target site dinucleotide ΔG values at the 5' and 3' ends by i-Score Designer (Table 3). Secondary structure and Minimum Free Energy (MFE) of siRNAs were calculated by using "RNAfold" web-server (*http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi*). Minimum free energy calculated for siRNAs is presented in Table 3 and secondary structures of single stranded siRNAs are shown in Fig. 3 (1a-4e). The asymmetry of siRNA duplex ends is essential for the assembly of RISC. siRNA binding to the target disrupts its secondary structure. The free energy required to open the secondary structure around the target site is lower than the free energy gain during the hybridization of the target site and the guide siRNA strand. Therefore, simple secondary structure of target site leads to more silencing effect than highly structured target site. In conclusion, highly specific, efficient and off-target free dsRNAs and 24 promising siRNAs against four vital genes of whitefly have been identified in the present study to develop a novel long-term RNAi based approach for whitefly control.

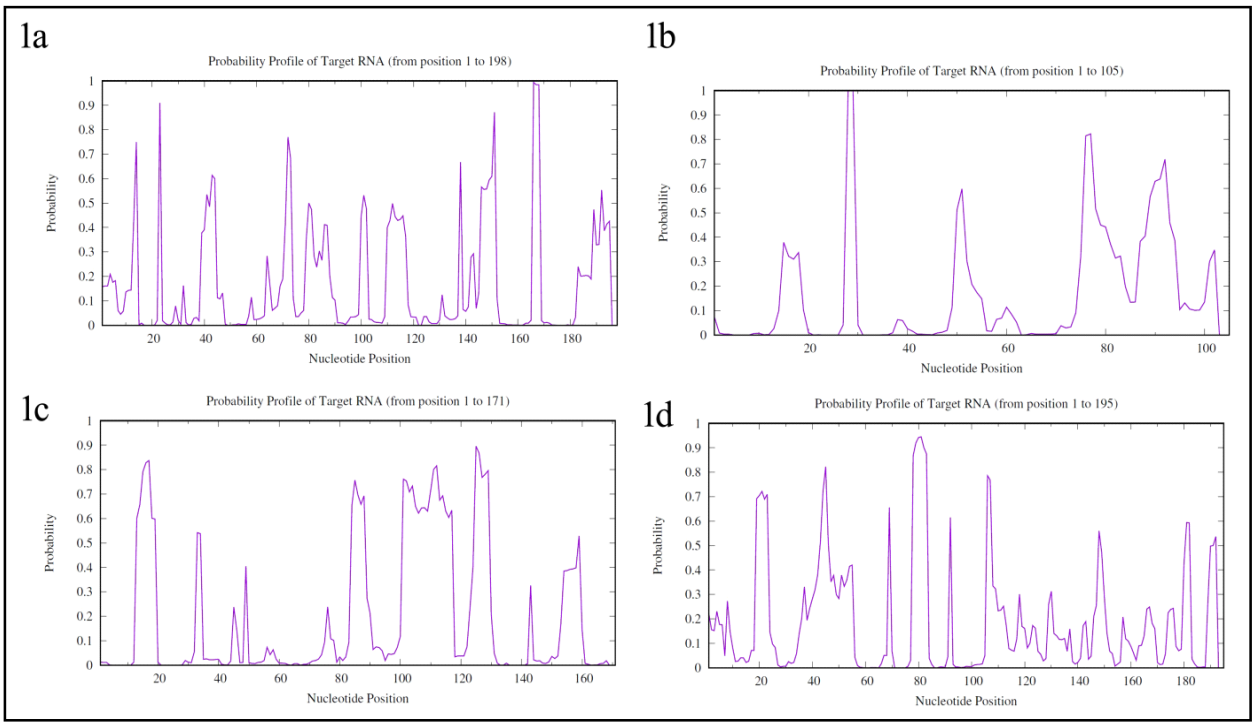

Fig. 1. Probability profiles of target mRNA to remain single stranded. (a) target site of *Laccase*, (b) target site of *Vitellogenin,* (c) target site of *Ryanodine receptor beta* gene and (d) target site of *Nicotinic acetylcholine* receptor β-subunit.

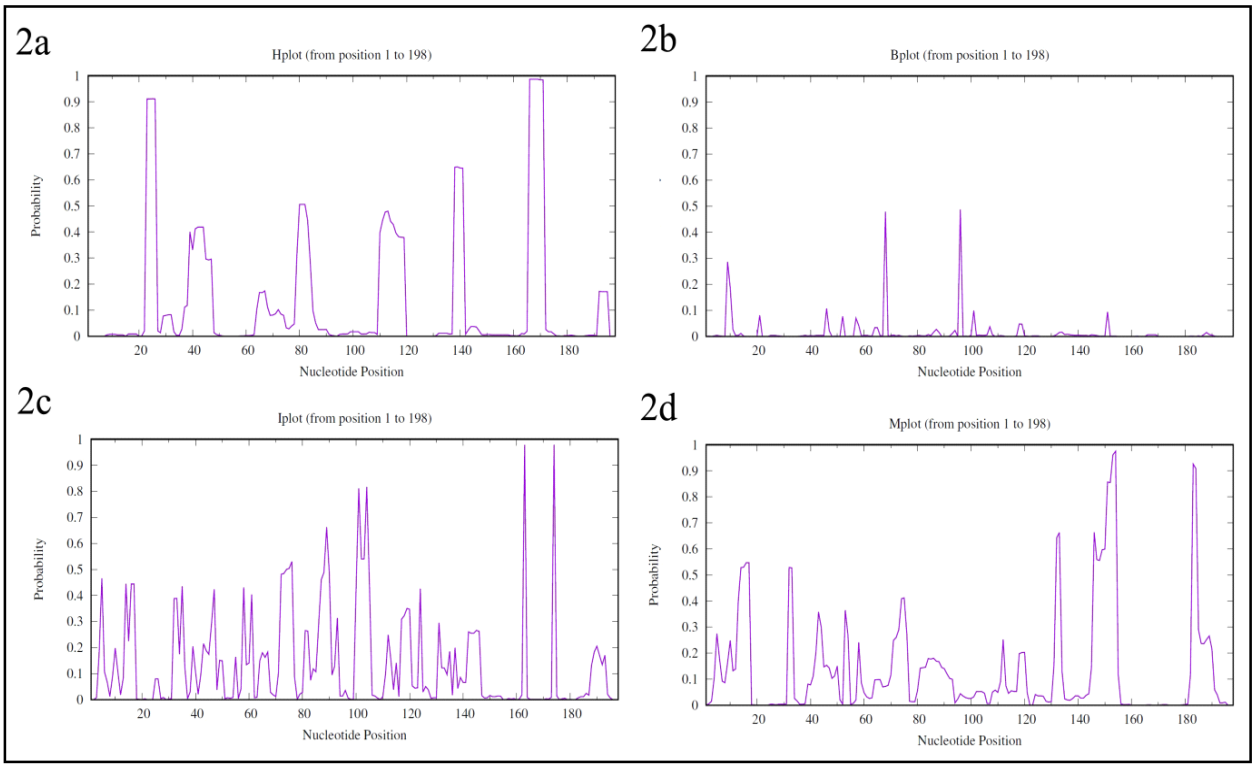

Fig. 2. Loop specific profiles of target site of *Laccase 1* gene. (a) displays the probability that a base is involved in hairpin loop (H plot), (b) the probability that a base is involved in a bulge (b plot), (c) the probability that a base is involved in an internal loop (Iplot) and (d) the probability that a base is involved in a multibranched loop (M plot).

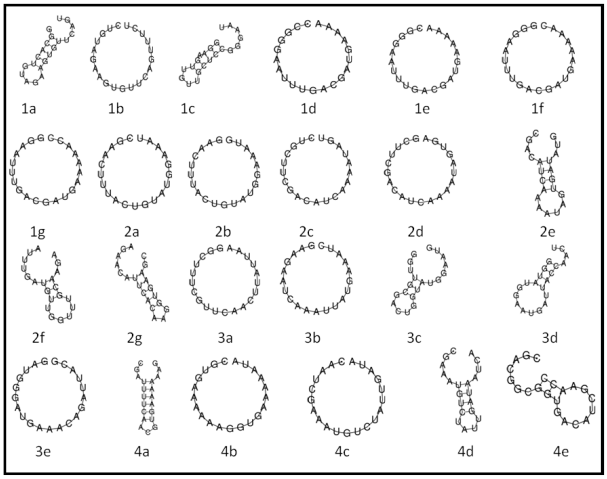

Fig. 3. Most stable secondary structures of single stranded siRNAs of four target genes. 1a-1g: *Vitellogenin receptor* gene (*BtVgR*), 2a-2g: *Ryanodine receptor* gene (*BtRyR*), 3a-3e: *Nicotinic acetylcholine* receptor beta 1 gene (*BtnAChRB1*) and 4a-4e: *Laccase* gene (*BtLAC1*).

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