INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0006/2021/25–6–1238–1248
DOI: 10.17957/IJAB/15.1785
http://www.fspublishers.org

Full Length Article

A Protective dsRNA is Crucial for Optimum RNAi Gene Silencing in *Chilo partellus*

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Received 02 January 2021; Accepted 25 March 2021; Published 10 May 2021

Abstract

RNAi technology is currently employed as an alternate control measure for agricultural pests. However, the variability of RNAi efficiency in insect pests limits the extensive usage of this technology and demands identifying the best target gene for effective RNAi. Four different bacterially-expressed dsRNA and purified dsRNAs coated on artificial diet were fed to the larvae. The transcripts expression was analyzed at 5 days and 15 days post-exposure to various dsRNAs. In the larvae fed on bacterially-expressed dsRNA, knockdown percentages were 80 and 57% knockdown in Acetylcholinesterase transcript, 40 and 60% gene knockdown in Arginine kinase, 74 and 73% knockdown in Chymotrypsin, and 80 and 20% reduction in V-ATPase transcript expression. Overall, the mRNA knockdown percentages in the targeted genes were more pronounced at 5 days of exposure to bacterially-expressed crude dsRNA than 15 days of exposure. However, most purified dsRNAs rarely induce any significant knockdown except dsARG, which reduced the arginine kinase transcript by 40%. Our findings suggest that for optimum RNAi in *C. partellus*, the dsRNA must be protected from direct access with nucleases. © 2021 Friends Science Publishers

Keywords: Agricultural pest; Bacterially-expressed dsRNA; *C. partellus*; Gene knockdown; RNAi technology

Introduction

Spotted stem borer attacks maize and causes significant yield losses (Mugo *et al.* 2002; Ajala *et al.* 2010). It is a substantial contributor to food insecurity in developing countries because millions of dollars are lost by smallholder farmers each year (Pratt *et al.* 2017). Climate change has a high tendency to accelerate its proliferation and increase the severity of its damages (Adeyinka *et al.* 2018) based on several prediction models (Tamiru *et al.* 2012; Khadioli *et al.* 2014). The rate of damage is still on the increase despite several control techniques currently available. The use of chemical control is mainly favored among several other measures. However, excessive use of pesticides can result in pest resurgence, an outbreak of secondary pests, and poses high risks to the environment and human health (Verger and Boobis 2013). One of the critical strategies to ensure a sustainable food supply is reducing crop losses due to insect pests through ecologically and economically integrated pest management (IPM) practices. Over the past decades, researchers have revealed the potential of RNAi technology to control insect pests.

The exogenous double-stranded RNA (dsRNA) induces degradation of mRNA sequences complementary to guide strand siRNA in organisms (Fire *et al.* 1998; Mello and Conte 2004; Ghosh *et al.* 2017; Worrall *et al.* 2019). RNAi pathway is initiated by RNase-III-like enzymes that cleave various dsRNAs into 20–25 nucleotide (nt) siRNA duplexes (Colmenares *et al.* 2007; Park *et al.* 2008;). The diced siRNAs generate guide strands and passenger strands with different thermodynamic properties based on asymmetry rule (Schwarz *et al.* 2003). The guide strands with the less stable 5' end favorably bind with Argonaute protein (Tim *et al.* 2004) to form a complex known as RNA-induced silencing complex (RISC). On loading to RISC, the targeted complementary regions of mRNA link with the guide strand and cleave the phosphodiester bond at the tenth and eleventh nucleotide from the guide strand 5' end (Elbashir 2001). The RNAi mechanism is currently applied in controlling Agricultural pests.

Recent advances in RNAi technology have drastically increased knowledge on the RNAi mechanism and gene knockdown in several insects (Lü *et al.* 2019; Vogel *et al.* 2019; Adeyinka *et al.* 2020; Husain *et al.* 2021; Jain *et al.* 2021). Scientists are currently improving the dsRNA delivery mode because the internalization of dsRNA is vital

To cite this paper: Adeyinka OS, IA Nasir, S Riaz, I Yousaf, N Toufiq, AP Okiki, B Tabassum (2021). A Protective dsRNA is Crucial for Optimum RNAi Gene Silencing in *Chilo partellus*. *Intl J Agric Biol* 25:1238–1248

for effective RNAi in insects. RNAi silencing signals triggered by dsRNA(s) or siRNA(s) usually transport genetic regulatory information between cells *via* cell-autonomous and non-cell autonomous. In the cell-autonomous RNAi response, the silencing effect is limited to the cell, whereas in non-cell autonomous, the silencing effect is observed in all cells that can take up the dsRNA (environmental RNAi), and the silencing signal is transported from the specific cell to other cells or tissues (Systemic RNAi). The systemic RNA interference-defective-1 (SID-1) protein and endocytosis are currently the best-studied dsRNA uptake mechanisms. Several SID mutants (SID 1, SID2, SID 3, and SID 5) have been studied in *C. elegans* to expand scientific knowledge on systemic RNAi pathways and SID-1 orthologues have been reported in insects. However, the presence of sid-1-like genes does not necessarily result in a robust systemic RNAi response (Miyata *et al.* 2014) in the insect. The endocytosis pathway is the popular dsRNA uptake mechanism in most insects. Clathrin-mediated endocytosis has been investigated as the dsRNA-uptake route in insect species (Li *et al.* 2015; Cappelle *et al.* 2016; Pinheiro *et al.* 2018; Abbasi *et al.* 2020). The efficacy of RNAi in an insect is generally influenced by dsRNA uptake. Several factors, such as physiological pH, targeted genes, and nucleases, influence insects' RNAi efficiency (Dias *et al.* 2020; Zhu and Palli 2020). Identifying the effective targets for specific insect pests is a major challenge in RNAi-mediated biopesticide. This study aims to identify the best target gene and the best dsRNA form that can be used for optimum RNAi silencing of *C. partellus* as potential control measure.

We examined four dsRNA stability and their ability to initiate the RNAi pathway in *C. partellus*. Bacterially expressed dsRNA and purified dsRNA of the genes (V-ATPases, Acetylcholinesterase, Chymotrypsin, Arginine kinase) that have been explored in Lepidoptera were fed to *C. partellus.* The knockdown efficiency was evaluated at different time intervals. The data presented in this study establishes that oral feeding of bacterially expressed dsRNA induced significant RNAi knockdown effects.

Materials and Methods

Insect management

Maize stem borers (*C. partellus*) larvae were reared at our insectary facility in the Centre of Excellence in Molecular Biology, Pakistan. To adapt to the artificial new rearing environment (26 ± 2°C under a 14:10 h light: dark and 65 ± 5% relative humidity), the larvae were maintained for 2 days on fresh maize stem before given an artificial diet.

Target selection and primer design

Several essential genes necessary for insects' survival, especially in Lepidoptera, were mined from literature. The genes were selected across functions like digestive enzymes (Chymotrypsin), cellular energy metabolism (Arginine kinase), central nervous system (Acetylcholinesterase) and ATP hydrolysis to transport protons across intracellular and plasma membranes (Vacoular-ATPase). *C. partellus* lacks adequate nucleotides information for the selected genes, so primers were designed for consensus sequences from available lepidopteran homologs in NCBI databases. We selected nucleotide information of 7 different insect limited to the Pyraloidea subfamily of Lepidoptera (*Chilo suppressalis, Helicoverpa armigera, Bombyx mori, Spodoptera litura, Plutella xylostella, Scirpophaga incertulas, Amyelois transitella*). The FASTA format sequences were imported into Ugene software and aligned with Clustal omega add-in to obtain the consensus sequence. Gene-specific primers (Table 1) were designed with HindIII and Xbal restriction enzymes using online primer 3 plus software (www.bioinformatics.nl/primer3plus).

RNA Isolation and cDNA preparation

Total RNA was extracted from a pool of five larvae comprised of different developmental stages using TRI Reagent (Sigma-Aldrich, St. Louis, U.S.A.) according to the manufacturer instructions. DNaseI was used to remove DNA impurities and RNA's concentration and purity was determined by A Nanodrop ND-1000 spectrophotometer. The RNA's integrity was verified on 1.5% TAE gel. 1 μg of the RNA was used to synthesize cDNA with Oligo dT primers, using RevertAid First Strand cDNA synthesis kit according to the manual instruction.

Targeted gene sequences identification

To identify the targeted gene sequences, PCR amplification was performed in GeneAmp PCR system 9700 Thermal Cycler (Applied Biosystems, Singapore). The reaction mixture (20 μL) contained 2 μL of 10 X Tag Buffer, 0.4 m*M* of $MgCl_2$, 0.15 m*M* of dNTP 1.25U of Taq, 0.5 μM of both forward and reversed primers, and 1 μg of cDNA. The amplification was performed according to the following profile: initiation at 95°C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58°C for 30 s, and 72°C for 30 s. The amplified products were ligated in pCR2.1 vector and transformed into *E. coli* top 10 competent cells. Positive clones were confirmed through restriction digestion and sequencing.

Bioinformatics analysis

The revealed sequences of four genes used in this study were compared to known the sequences in NCBI database using the NCBI BLAST server (http://www.ncbi.nlm.gov/BLAST). The molecular weights and theoretical pIs were predicted using the Compute pI/Mw tool (https://web.expasy.org/compute_pi/). The

sequences were aligned by muscle algorithm and used to construct phylogenetic tree analysis based on the neighbor-joining method using MEGA X with 1,000 Bootstrap values.

Construction of dsRNA expression vector and induction

Each of the four individual genes was digested with Hind III and Xbal restriction enzymes and ligated to L4440 vector (Fig. 1). We first transformed and confirmed the transformant in *E. coli* top 10 followed by transformation into *E. coli* HT115 strain. Positive *E. coli* HT115 colonies were grown overnight in 5 mL YT media (Yeast extract 10 g/L, Bacto peptone 5 g/L, NaCl 10 g/L) containing 100 µg mL−1 ampicillin at 37°C. The cultures were diluted to 500 mL and incubated until it reaches OD_{595} = 0.4. Then, the dsRNA synthesis was induced by the addition of 0.6 mmol l−1 IPTG at 37ºC for another 3–4 h. Raw bacterially-expressed dsRNAs were harvested and some of the harvested cells were purified as we described in our initial publication (Adeyinka *et al.* 2019).

Fitness of dsRNA at insectary condition and on artificial diet

We investigated if the condition at the insectary would have any effect on dsRNA stability. For this, dsRNA was diluted with nuclease-free water and exposed in the growth chamber for various time intervals: 0 h, 24 h, 48 h, and 72 h. The samples were later resolved on 1% agarose gel electrophoresis to evaluate the effect of environmental conditions. Furthermore, the dsRNA was overlaid on the artificial diet for 36 h and 72 h to assess the time taken for dsRNA degradation. The dsRNA was isolated from the treated diets by Tri-reagent and resolved on 1% gel to estimate the effect of diet on dsRNA stability.

In vitro stability of dsRNA in Insect heamolmyph

To extracted haemolymph, an incision was made at the larvae's prolegs with a surgical blade, and the haemolymph was gradually extracted with a 10 µL pipette. Furthermore, 1 µg of dsRNA was used to incubate 2 μL of the haemolymph at various time intervals to reveal their stability.

Gut content stability test

C. partellus larvae were dissected to extract the entire gut content. Briefly, a surgical blade was used to make minor incisions around the anus and the larvae's neck region; forceps were used to pull out the whole gut into 1X PBS in a 0.2 mL tube. The whole gut was inserted into a 500 μL Eppendorf tube with a slight cut and then centrifuged at centrifugation at 13000 × *g* for 15 min at 4°C into a 1.5 mL Eppendorf tube containing ice-cold 1× phosphate-buffered saline (PBS, pH 7.4) with 1 mg phenylthiourea (PTU). 1 μg of dsRNA was mixed with 2 µL of the haemolymph and incubated for various time intervals to evaluate its stability. The dsRNA integrity was examined on 1% (w/v) agarose gels.

Temporal expression of the four targeted genes

To estimate the targeted genes relative transcription levels, samples were collected across developmental stages from three technical replicates and two independent biological replicates. 1st instar larvae were used as reference samples for the temporal expression profiling analyses. RNA isolation and synthesize cDNA methods were the same described above. Beta-tubulin was used as an internal control for normalization of transcript abundance across developmental stages based on our initial validation report (Adeyinka *et al.* 2019). RT-qPCR was conducted using SYBR premix with the primers shown in Table 1. Data were analyzed by the $2^{-\Delta\Delta CT}$ method, ANOVA was used to analyzed expression, and the means were separated using Fisher's protected Least Significant Difference (LSD) test for significance using GraphPad Prism 7 software

Insect bioassay to evaluate the effect of various dsRNAs used in this study

To compare the knockdown efficiency in bacterially expressed dsRNA and purified dsRNA of various targeted genes, 2nd instar larvae were starved for about 1 h before exposure to the dsRNA treatments. For the crude bacteria feeding experiment, 100 μL of dsRNA inducing bacteria was overlaid on artificial diet and fed to 2nd instar larva for 21 days. All diets were replaced after 24 h. In a parallel experiment, 20 μg of purified dsRNA was overlaid on the artificial diet. A total of 20 larvae were used in each treatment in triplicate, and an empty L4440 vector was used as the control treatment. After 5 days and 15 days of exposure to dsRNA, two larvae per replicate were collected and ground in Trizol for RNA isolation and subsequent RT-qPCR.

Transcript expression by RT-qPCR and statistical analysis

Real-time PCR was performed to evaluate the knockdown in mRNA expression of all four targeted genes after dsRNA exposure. The synthesized cDNA was diluted with nuclease-free water in 1:10 ratio and used for RT-qPCR. The RT-qPCR assays were conducted according to Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. RT−qPCR was performed in triplicate in a Piko-real 96 Realtime PCR system (Thermo scientific) to evaluate knockdown brought about by each specific dsRNA. *ELF* gene expression was used as an internal reference to normalized the data based on our validation findings (Adeyinka *et al.* 2019).

Table 1: List of primers used in the study with specific amplicons size

Gene name	PCR Primers (5'-3')
Acetylcholinesterase	F:AAGCTTTGCCTTCTTTCATCATCGTG R:TCTAGATGGGATCAACAGTTGGCTATC
Arginine kinase	F:AAGCTTTTGTACTGGGCCTCTGTGA R:TCTAGACGCAACCCTTGAGAAATTG
Vacuolar ATPase	F:AAGCTTTCACGGAAGTACTCGGATAGAG R:TCTAGAACGGCGAGAAGGAGAAGTA
Chymotrypsin	F:AAGCTTTGTGCAAATGTTGGAGTCCT R:TCTAGAGTCCACCTCGAGGATTCTATTG
	RT-qPCR Primers (5'-3')
Acetylcholinesterase	F:TGCCTTCTTTCATCATCGTG R:GACTGCATGCGTGGAGTAGA
Arginine kinase	F:ATTCCAACACCAGAGTCCAAGT R:AAGTCGCTGCTGAAGAAGTACC
Chymotrypsin	F:CAGCTACCGTGCATAACAACAT R:GTACTGACCGCTGCTCACTG
Vacuolar ATPase	F:CTACAGGCATGTTGGATGTGTT R:CGTGGTAACGAGATGTCTGAAG

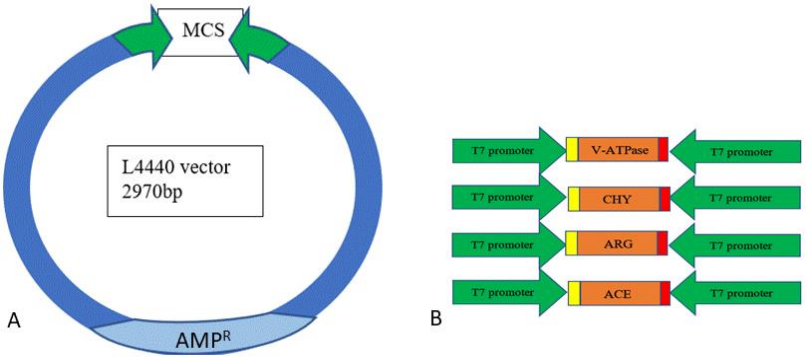

Fig. 1: Schematic representation of L4440 construct transformed in *E. coli* HT115 host. **A**) Demonstrating the L4440 vector containing multiple cloning site (MCS) with double T7 promoters (green colour), **B**) indicating the various gene that was cloned into the HindIII (yellow colour) and Xbal (red colour) restriction enzymes sites

Livak method (Livak and Schmittgen 2001) was used to determine the extent of gene expression. The amplifications cycling profile used comprise of an initial denaturation at 95ºC for 5 min, 35 cycles of denaturation at 95ºC for 30 s, annealing at 58°C for 30 s, and extension at 72ºC for 30 s. The reaction mixture comprises of nuclease free water, cDNA Maxima SYBR Green qPCR 2X Master mix, and 500 n*M* of primers. Analysis of variance was conducted to analyse the mean transcript expression of various dsRNA treatments to nuclease-free water fed larvae. Pairwise comparisons between treatments were performed with the Turkey test at $P < 0.05$.

Results

Amplification of targeted genes

Several crucial genes necessary for the survival of insects have been effectively silenced in the insect. Among these crucial genes were Vacuolar-ATPase, Arginine kinase (Camargo *et al.* 2016), Acetylcholinesterase (Ye *et al.* 2017), and Chymotrypsin previously reported as potential target for RNAi mediated pest control. These four essential insect genes were amplified from *C. partellus* using specific primers (Table 1). The PCR products resolved on 1% agarose gel revealed amplification of a single fragment indicating the high specificity of individual genes (Fig. 2).

Sequence comparison and phylogenetic analysis

To examine the relatedness of the sequenced genes, the individual sequence was analyzed through BLASTn. The outcome indicated high sequence homology with other lepidopteran insects available in NCBI database. Specifically, the Chymotrypsin gene sequence showed 98% similarity with Chymotrypsin gene of *Helicoverpa armigera*; Vacuolar ATPase was 87% similar to *M. sexta* vacuolar ATPase*;* Arginine kinase gives 87% similarity with *Spodoptera litura* Arginine kinase and Acetylcholinesterase showed 91% identity to *Chilo auricilius* Acetylcholinesterase. The Table 2, highlights some of the chemical properties of the genes used in this study, their molecular weight, isoelectric point and number of amino acids. The BLASTn result were aligned by ClustalW in MEGA-X software while neighbour-joining method was used for the construction of phylogeny tree for individual gene to demonstrate their relatedness among the lepidopteran (Fig. 3). The phylogeny tree indicated that these genetic sequences are highly conserved among Lepidoptera.

Table 2: Chemical properties of the targeted genes revealed through in-silico studies

Gene name	Accession no.	Isoelectric point	Molecular weight	Amino acids
Acetylcholinesterase	MK560447	4.70	15652.83	144
Arginine kinase	MK560449	5.02	15491.43	140
Vacuolar ATPase	MK560450	6.24	26496.39	242
Chymotrypsin	MK560452	8.09	7793.83	74

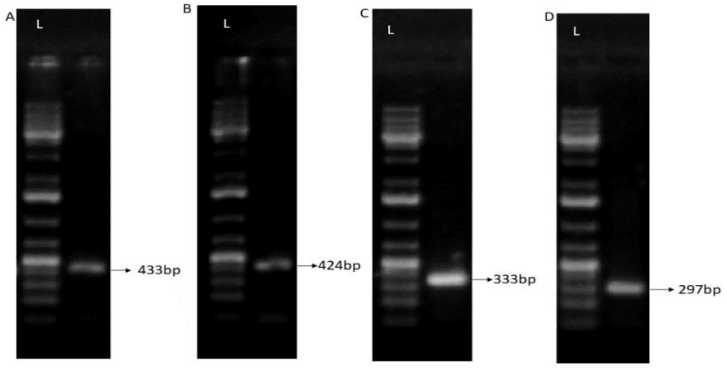

Fig. 2: PCR amplification of genes used in this study from *C. partellus.* **A**) Acetylcholinesterase gene fragment, **B**) Arginine kinase gene fragment, **C**) Chymotrypsin gene fragment, and **D**) *Vacuolar ATPase* gene fragment. L refers to 1kb plus DNA ladder. The gene sequences were submitted in NCBI GenBank and assigned with the following accession numbers; Acetylcholinesterase - MK560447, Arginine kinase - MK560449, *Vacuolar ATPase* - MK560450, and Chymotrypsin - MK560452

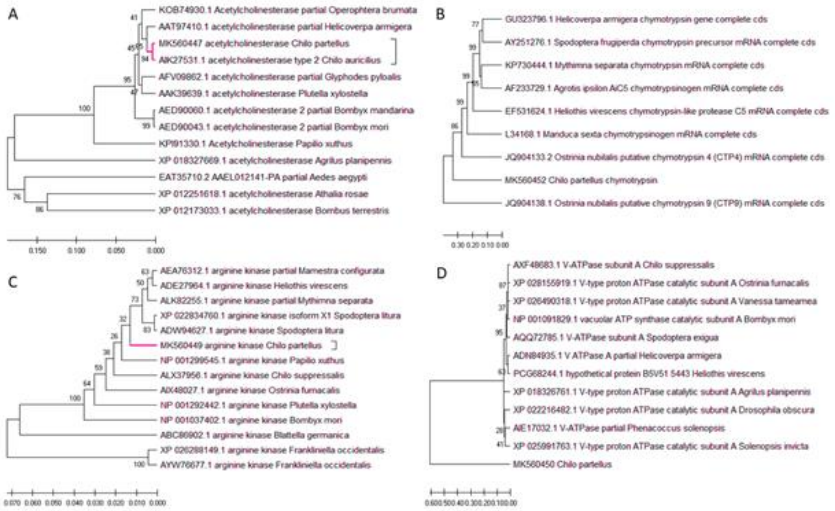

Fig. 3: Phylogenetic tree analysis showing the relationship among *C. partellus* amplified genes and related sequences available in NCBI repository. **A**) Acetylcholinesterase, **B**) Chymotrypsin, **C**) Arginine kinase and **D**) *V-ATPase* genes. The analysis was based on the neighbour-joining method according to amino-acid sequences using MEGA-X. with 1,000 Bootstrap values

E. coli HT115 induces dsRNA synthesis upon IPTG induction

The individual constructs harboring each of the four amplified genes cloned in the L4440 vector were transformed into *E. coli* HT115 strain. The clones were confirmed through restriction digestion, as depicted in Fig. 4. Confirmed clones with two distinctly restricted fragments, one of ~2.7 kb corresponding to the L4440 vector, while the second ~500bp corresponding to transgene (Fig. 4) were selected for subsequent induction. The recombinant HT115 strain transcribes high quantity dsRNA when induced with 0.6 m*M* IPTG (Fig. 5). A clear difference was observed between un-induced and induced cultures in terms of dsRNA production. The sizes of the transcribed dsRNAs correspond to individually targeted dsRNA in *C. partellus*.

Stability test for purified dsRNAs

The stability of the purified dsRNA was evaluated under various conditions before the bioassay analysis.

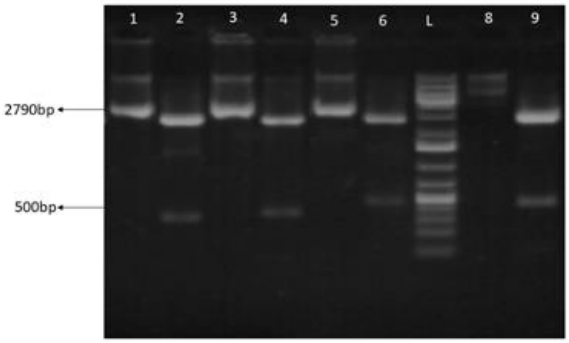

Fig. 4: Restriction digestion of positive clones confirming the specific gene insertion in the L4440 vector. Lane 1; undigested *Vacuolar ATPase*, Lane 2; digested *Vacuolar ATPase*, Lane 3; undigested Chymotrypsin, Lane 4; digested Chymotrypsin, Lane 5; undigested plasmid DNA with Acetylcholinesterase *gene cloned*, Lane 6; digested plasmid DNA harboring Acetylcholinesterase *gene*, L is the 1kp plus Ladder, Lane 8; undigested *Arginine kinase*, Lane 9; digested *Arginine kinase*

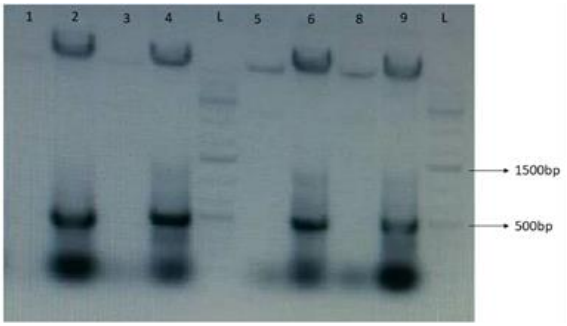

Fig. 5: Synthesis of double-stranded RNAs from IPTG-induced HT115 bacterial culture. Lane 1; un-induced Acetylcholinesterase, Lane 2; induced Acetylcholinesterase, Lane 3; un-induced *Arginine kinase*, Lane 4; induced *Arginine kinase*, Lane 5; un-induced Vacuolar ATPase, Lane 6; induced *Vacuolar ATPase*, Lane 6; induced *Vacuolar ATPase*, Lane 7; un-induced Chymotrypsin, Lane 8; induced Chymotrypsin. L is the 1 kp plus Ladder

There was no significant reduction in the dsRNA quantity after 24 h, 48 h, and 72 h after dsRNAs exposure to general insectary lab conditions. The same level of dsRNA intensity was observed within 24 h to 72 h of exposure (Fig. 6). These results indicated that the insectary lab conditions do not have any effect on dsRNA quality. Furthermore, we evaluated the stability of dsRNA overlaid on an artificial diet. A significantly high intact dsRNA was recovered after 36 h of exposure to an artificial diet (Fig. 7A). However, after 72 h of exposure on diet, almost the whole intact dsRNA was degraded (Fig. 7B). Based on these findings, artificial diet was replaced with a new diet overlaid with fresh dsRNA within every 24–30 h in subsequent feeding assays.

To determine dsRNA's fate inside *C. partellus*, the haemolymph was collected and incubated with dsRNA for various time intervals. The dsRNA was stable for 1 min and the stability prolonged for 5 min; however, it gradually

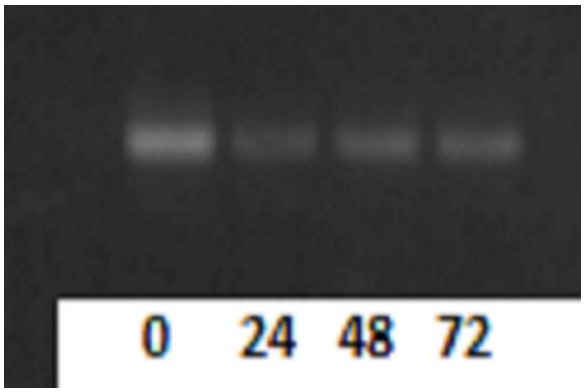

Fig. 6: dsRNA stability test in lab environment. dsRNAs were exposed to lab environment for variable periods (0 h, 24 h, 48 h, 72 h), and their integrity was evaluated through agarose gel electrophoresis

degraded as the time interval increased (Fig. 8A). Surprisingly, the dsRNA degraded very fast in about 30 seconds when it was incubated with whole gut content for the time intervals; 30sec, 1 min, 2 min, 3 min, 4 min and 5 min (Fig. 8B). These results depict that gut contents have a high concentration of dsRNase (s). Based on these findings, we opted for a high dsRNA concentration in subsequent feeding assay to initiate effective RNAi.

Temporal expression of Acetylcholinesterase, arginine kinase, *V-ATPase,* and Chymotrypsin

The expression of the four genes was determined across all the developmental stages. Our data indicated that the analysis of the temporal expression of the targeted four genes: Acetylcholinesterase, arginine kinase *V-ATPase*, and Chymotrypsin were expressed in all the developmental stages with minimal expression in 3rd and 4th instar (Fig. 9).

Bacterially expressed crude dsRNAs exhibit more effective knockdown compared to naked dsRNA

To compare the knockdown sensitivity in larvae fed on purified dsRNA and crude bacterially-expressed dsRNA treatment at 5 and 15 days of exposure. The RT-qPCR analysis indicated Acetylcholinesterase was downregulated by up to 80 and 57% in the larvae fed on bacterially-expressed dsACE at 5 and 15 days exposure, respectively. Whereas larvae fed on purified dsACE did not show any significant knockdown at the same time of exposure (Fig. 10A and 11A). The larvae fed with bacterially expressed dsARG exhibited 40 and 60% knockdown in the Arginine kinase transcript expression after 5 and 15 days of exposure, respectively (Fig. 10B). A similar knockdown level (~40%) in Arginine kinase was observed in larvae fed on purified dsARG (Fig. 11B). Fig. 11C showed that mRNA abundance of Chymotrypsin decreased by 74 and 73% at 5 and 15 days post-exposure to bacterially-expressed dsRNA, respectively.

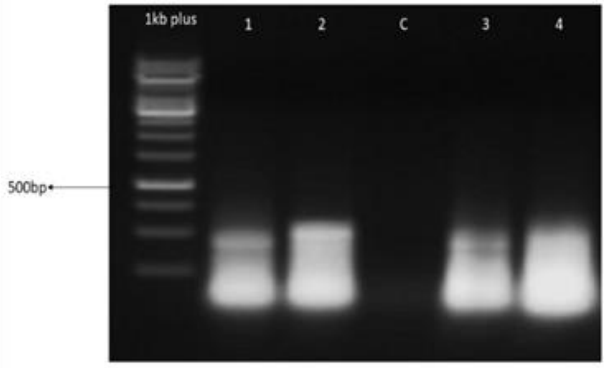

Fig. 7A: dsRNA stability test recovered from artificial diet after 36 hours of exposure. Lane 1: dsV-ATPase, Lane 2: dsACE, Lane 3: dsCHY, Lane 4: dsARG, while C refers to control diet overlaid with nuclease-free water

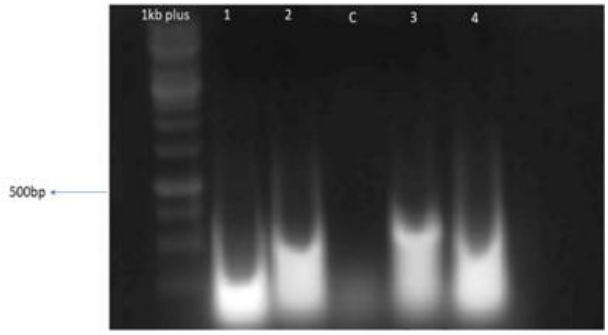

Fig. 7B: dsRNA stability test recovered from artificial diet after 72 hours of exposure. Lane 1: dsV-ATPase, Lane 2: dsACE, Lane 3: dsCHY, Lane 4: dsARG, while C refers to control diet overlaid with nuclease-free water

In contrast, purified dsCHY failed to exhibit any knockdown in the mRNA expression of the chymotrypsin gene in larvae fed with purified dsCHY (Fig. 11C). V-ATPase mRNA expression was reduced to about 80% when fed with bacterially-expressed V-ATPase dsRNA for 5 days, while extended exposure to 15 days resulted in 20% reduction in the transcript expression (Fig. 10D). Overall, the knockdown percentages for all targeted genes were more pronounced and significant at 5 days of exposure to bacterially-expressed dsRNA as compared to 15 days of exposure. On the other hand, purified dsRNAs rarely induced any significant knockdown percentage for all targeted genes except dsARG (Fig. 11B).

Discussion

The knockdown of essential genes in agricultural pests has been investigated as an alternative technique to control insect pests. However, some lepidopterans are refractory to RNA interference, and silencing has not been effective. Identifying relevant target genes for RNAi in insects is one of the hurdles that must be solved for effective RNAi-

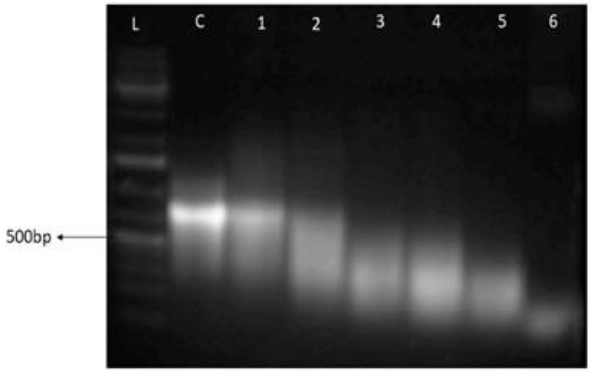

Fig. 8A: Degradation of dsARG incubated with haemolymph derived from *C. partellus* for different time intervals. L refers to the 1kb plus Ladder, and C refers to control (dsRNA without haemolymph). dsRNA incubated with haemolymph for; 1 min (lane 1), 5 min (lane 2), 10 min (lane 3), 15 min ((lane 4)), 30 min (lane 5), 60 min (lane 6) respectively

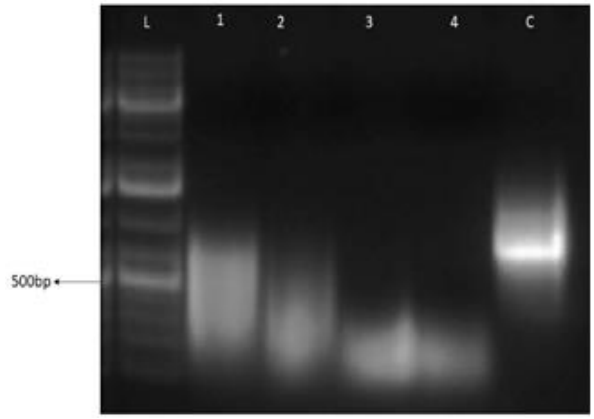

Fig. 8B: Degradation of dsARG incubated with gut contents derived from *C. partellus* for different time intervals. L refers to the 1kb plus Ladder, and C is control dsRNA without gut content, dsRNA incubated with gut contents for; 30 seconds (lane 1), 1 min (lane 2), 2 min (lane 3), 5 min (lane 4) respectively

mediated biopesticides. This study investigated the RNAi effectiveness in *C. partellus* by targeting four candidate genes (specifically; *V-ATPases*, Acetylcholinesterase, Chymotrypsin and Arginine kinase) involved in the various biological functions of *C. partellus*. *V-ATPase* is multi-subunit proton pumps that energize transport across plasma membranes in insect cells and epithelia. Acetylcholinesterase is an essential enzyme in the insect central nervous system, which terminates nerve impulse transmission at synaptic junctions of cholinergic neurons through neurotransmitter acetylcholine hydrolyzation. Insects usually exhibit high Acetylcholinesterase expression for accurate nerve impulse termination, a minor decrease in the enzyme activity disrupt nerve impulse transmission and affect insect survival. Chymotrypsin belongs to the serine proteases family involved in various biological functions,

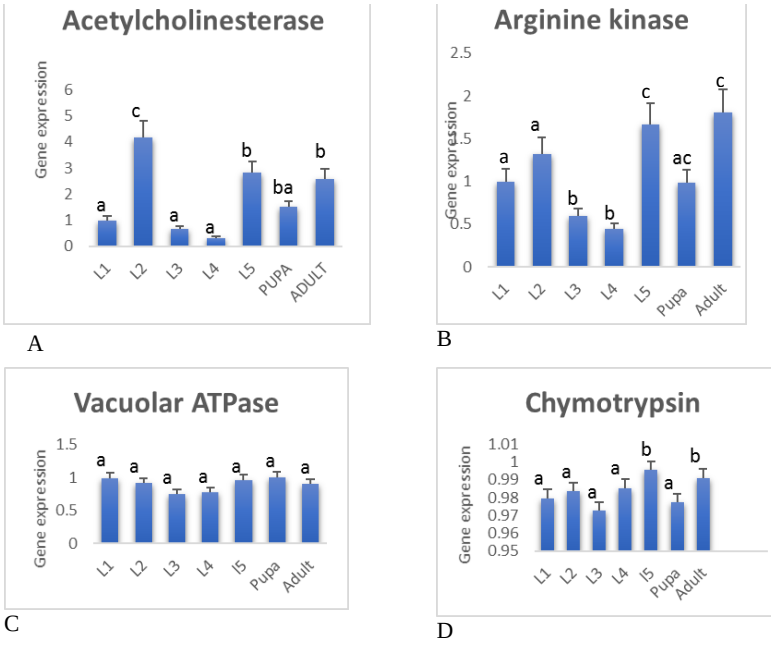

Fig. 9: Temporal expression profiles of the four genes being studied in *Chilo partellus*. **A**) acetylcholinesterase expression across all developmental stages, **B**) arginine kinase expression across all developmental stages, **C**) vacuolar ATPase expression across all developmental stages, and **D**) chymotrypsin expression across all developmental stages. Values are expression mean ± standard error, and different letter indicate significant different ($P < 0.05$)

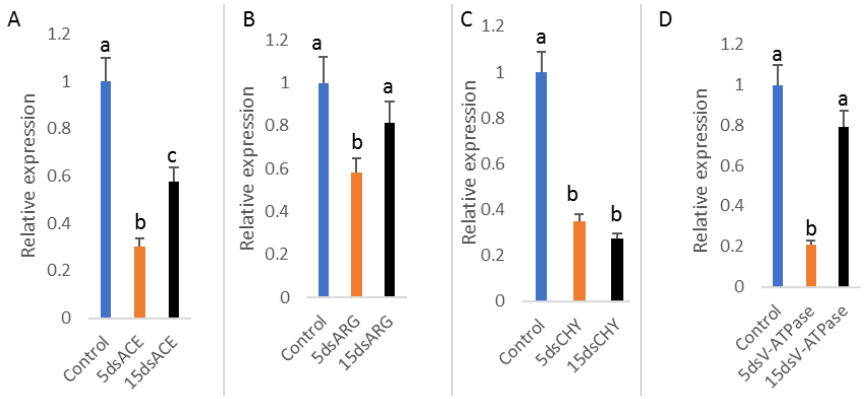

Fig. 10: Relative knockdown in transcript levels of targeted genes in *in-vitro* feeding assay with bacterially expressed dsRNA for a period of 5 and 15 days. **A**) Acetylcholinesterase, **B**) Arginine kinase, **C**) Chymotrypsin, **D**) V-ATPase. Orange coloured bar refers to exposure for 5days while the Black coloured bar represents 15 days exposure. Values are expression mean ±standard error and different letter indicate significant differences ($P < 0.05$) between the two genes and control (without dsRNA treatment

including food digestion, immune defense, and zymogen activation. Arginine kinase (AK) is a phosphotransferase involved in cellular energy metabolism; it catalyzes the transfer of a high-energy phosphate group from ATP to L-arginine to produce phospho arginine (Bragg *et al.* 2012). Different dsRNA delivery methods such as oral feeding, microinjection, soaking, transfection and host plant delivery have evolved over the years. Oral delivery of dsRNA is a proven delivery method of inducing RNAi in Lepidoptera (Choi and Meer 2019). We used an oral route to deliver bacterially-expressed dsRNA and purified dsRNA into the insect gut microenvironment to examine the knockdown efficiency of the targeted genes in *C. partellus.*

Another critical element in determining the RNAi efficiency is the rate of degradation of dsRNA by haemolymph and gut extracellular ribonucleases (Wang *et al.* 2016; Song *et al.* 2017; Spit *et al.* 2017). It has been well established that for fruitful RNAi induction, the dsRNA must persist in being absorbed into the insect cell without degradation. We investigated our purified dsRNA under various conditions; to check if the insectary condition would influence dsRNA, we found out that the dsRNA remained

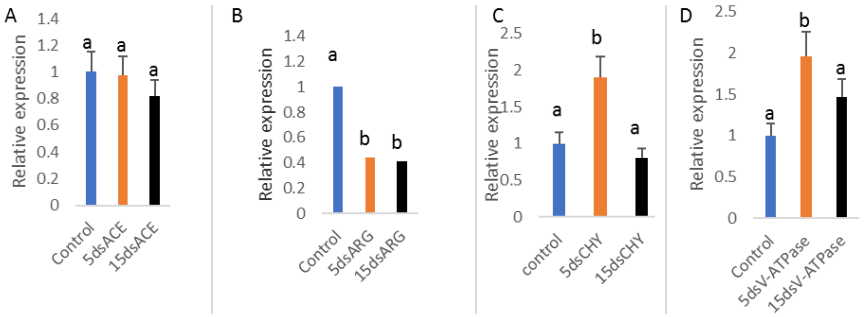

Fig. 11: Relative knockdown in transcript levels of different targeted genes after feeding larvae with purified dsRNA for 5 days and 15 days. **A**) Acetylcholinesterase **B**) Arginine kinase, **C**), Chymotrypsin, **D**) V-ATPase. Orange coloured bar refers to exposure for 5 days, while the black coloured bar represents 15 days of exposure. Values are expression mean ±standard error, and the different letter indicates a significant difference ($P < 0.05$) between the two genes and control (without dsRNA treatment)

stable for approximately 72 h. However, the dsRNA was found intact on an artificial diet for about 36 h and start degrading after 48 h of exposure. These findings were similar to initial reports documenting that it takes 48 h to 84h for nucleases present in an artificial diet to degrade dsRNA (Christiaens *et al.* 2014; Cao *et al.* 2018). Additionally, we found that the *C. partellus* haemolymph and gut content degrade dsRNA at a very rapid rate. The *C. partellus* haemolymph starts dsRNA degradation at about 1 min with complete degradation at 30 min. The instability of dsRNA in gut content and haemolymph limits the RNAi efficiency in *C. partellus.* This finding is similar to Cooper *et al.* (2020), who measured dsRNA stability with RT-qPCR and reported high dsRNA degradation in *Ostrinia nubilalis's* gut content and heamolymph. The rapid gut content degradation of dsRNA in this study is in accordance with previous studies that reported a correlation between dsRNA degradation and low RNAi efficiency in insects (Cooper *et al*. 2021; Peng *et al.* 2018).

Several earlier studies have established that feeding-based RNAi can precisely induce an RNAi response in agricultural insects (Miller *et al*. 2012; Upadhyay *et al.* 2013; Abdel-latief and Hoffmann 2014; Xiao *et al.* 2015; Cappelle *et al.* 2016). RNAi effectiveness generally depends on the sufficient concentration of ds/siRNA able to initiate the RNAi pathway. However higher concentration is most often used while executing oral feeding assays. We found out that prolonged exposure of *C. partellus* larvae to dsRNA does not lead to enhanced silencing in most targeted genes but rather attempt to nullify the suppression effects. We hypothesize that an alternative pathway might be activated since the mechanisms for this is not clear; further experiments are needed to understand the multiple mechanisms that might contribute to low RNAi efficiency in *C. partellus*. Our suggestion is that siRNA and shRNA targeting a specific gene may induce nonspecific effects in the stress response pathways (Olejniczak *et al.* 2010), which may rescue the targeted gene transcript reduction.

The comparison between the transcript knockdowns in larvae fed on bacterially-expressed dsRNA and purified dsRNAs indicated a significant knockdown of all the four genes evaluated in this study. However, larvae fed on purified dsRNAs failed to induce significant knockdown in most of the targeted genes. The low sensitivity of purified dsRNA can be ascribed to its degradation in the gut due to the presence of nuclease. The knockdown observed in this study indicated that RNAi efficiency varied between target genes in *C. partellus*. Recently, studies have shown that REase competes with Dicer-2 for targeted dsRNA, influences the unique total reads of target gene siRNAs, and consequently affects RNAi efficiency (Guan *et al.* 2018). Although different physiological conditions in other tissues modulate enzyme activity and various insects produce a variety of dsRNA degrading enzymes in different quantities (Peng *et al.* 2018; Cooper *et al.* 2021).

These results further strengthen the notion that the rapid degradation of dsRNAs affects the ability to induce RNAi mechanism and influence its stability. Since most bacterially-expressed double-stranded dsRNAs exhibited significant knockdown compared to purified dsRNAs. The challenges of the instability of dsRNA and rapid degradation of dsRNA must be overcome to enhance RNAi efficiency in *C. partellus.* The protection of dsRNA from nuclease will be a better way of achieving optimum knockdown in *C. partellus* and facilitating RNAi-mediated control strategy. Efforts to protect dsRNA from nucleases degradation by silencing nuclease have been demonstrated to enhance dsRNA uptake in agricultural pests and subsequently improve RNAi efficiency (Giesbrecht *et al.* 2020; Wang *et al.* 2020; Sharma *et al.* 2021). Research evidence has shown that nanoparticle-mediated delivery overcomes poor cellular internalization and nucleolytic degradation of dsRNAs for effective RNAi response in insect cells (Christiaens *et al.* 2018; Dhandapani *et al.* 2019; Wang *et al.* 2019; Baddar *et al*. 2020; Yan *et al.* 2021).

Conclusion

The data presented in this study establish that oral feeding of bacterially expressed dsRNA through an artificial diet

effectively induces RNAi-mediated knockdown of the targeted genes. In contrast, purified dsRNA rarely initiates the RNAi mechanism in *C. partellus*. We suggest that the dsRNA should be protected from direct access with nucleases for optimum RNAi in *C. partellus*. Nanoparticles are currently being employed as novel delivery vehicles that enhance dsRNA uptake into insect cells and protect dsRNA against RNases. Future nanoparticle-mediated delivery of the dsRNAs examined in this study is worth investigation to evaluate the best dsRNA delivery approaches in *C. partellus.*

Acknowledgements

We acknowledge The World Academy of Sciences for awarding the TWAS-CEMB Postgraduate Fellowship and International Foundation for Science for providing the grant for this research.

Author Contributions

Olawale Samuel Adeyinka performed the experiment and write the manuscript, Bushra Tabassum and Idrees Ahmad Nasir designed and supervised the experiment and edit the manuscript. Nida Toufiq, Iqra Yousaf and Abimbola Pius Okiki reviewed the manuscript and effect corrections and Samam Riaz executed the insect bioassay and real time assays.

Conflict of Interest

All the authors declare no conflicts of interest

Data Availability

All the data used to support the findings of this study are included in the article.

Ethics Approval

Not applicable.

Funding Source

This study was supported by International Foundation for Science grant (No. C/6194-1).

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