Full Length Research Paper

RNAi-based silencing of genes encoding the vacuolar-ATPase subunits a and c in pink bollworm (*Pectinophora gossypiella*)

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RNA interference is a post- transcriptional gene regulation mechanism that is predominantly found in eukaryotic organisms. RNAi demonstrated a successful gene silencing in insects that led to the development of novel approaches for insect pest management. In the current study, genes encoding vacuolar ATPase (V-ATPase) subunits a and c from the midgut of pink bollworm, *Pectinophora gossypiella*, were cloned and sequenced. The full length of V-ATPase subunits a and c cDNAs are 2526 and 1140 bp, respectively. The silencing effect of RNAi on these two genes was determined by microinjecting three dsRNA fragments into the thoracic region of pink bollworm larvae. Bioassay results revealed that 200 ng of dsRNAs silenced both genes causing mortality of 18.9 to 26.7%.

Key words: RNAi, dsRNA, Pink bollworm, vacuolar ATPase subunit a, vacuolar ATPase subunit c.

INTRODUCTION

RNA interference (RNAi) is a mechanism of post-transcriptional regulation in higher eukaryotes inhibiting gene expression by RNA transcript degradation (Berezikov, 2011). It was first discovered in the nematode *Caenorhabditis elegans* by Fire et al. (1998) and, later, was exploited as an effective technique for a rapid analysis of gene function by phenotypical changes of the target silenced gene. In this technique, exogenous double stranded RNA (dsRNA) is used to significantly reduce the target gene transcript level (Fire et al., 1998). RNAi technology was harnessed in different applications including insect control, thus, was deployed as a powerful tool in biological control for insect pest management (Gordon and Waterhouse, 2007; Price and Gatehouse, 2008; Zhu, 2013). A variety of methods are used to deliver dsRNA inside the cells such as soaking the animals in dsRNA-containing solution, microinjection and oral feeding (Baum et al., 2007; Chen et al., 2010; Liu et al., 2010; Rosa et al., 2012; Tian et al., 2009; Zhang et al., 2010; Zhao et al., 2011; Zhu et al., 2011).

An advanced progress of RNAi exploitation in the field of insect control was developed in 2007, by expressing insect specific-dsRNAs in transgenic plants. Transgenic corn expressing V-ATPase specific-dsRNAs showed a

significant reduction in feeding damage caused by western corn rootworm, *Diabrotica virgifera virgifera* (Baum et al., 2007). Also, cotton was modified to harbor dsRNA targeting the cytochrome P450 gene (CYP6AE14) of the cotton bollworm, *Helicoverpa armigera* (Mao et al., 2007). Cotton bollworm larvae fed on transgenic cotton showed a reduction in growth rate and lower levels of CYP6AE14 transcript within the insect midgut. Thereafter, other RNAi-mediated transgenic plants were developed such as transgenic tobacco plants *Nicotiana tabacum*, that showed higher resistance level compared to non-transgenic against *H. armigera* (Xiong et al., 2013; Jin et al., 2015), *Spodoptera exigua* (Zhu et al., 2012) and *Bemisia tabaci* (Thakur et al., 2014).

The vacuolar proton pumps, V-ATPases, are ubiquitous holoenzyme among eukaryotes (Dow, 1999). It is a member of ATPases family (A, F and V) which are mainly responsible for ATP hydrolysis (Forgac, 2007). The main function of V-ATPases is acidifying a wide array of intracellular organelles and pump protons across the plasma membranes of numerous cell types (Nelson et al., 2000). V-ATPases utilize the energy derived from ATP hydrolysis to transport protons across intracellular and plasma membranes of eukaryotic cells. In the midgut of lepidopteran larvae, the V-ATPase in the apical cell membranes of the goblet cells plays a role in amino acid absorption, by energizing the plasma membrane through pumping H^+ ions/proton into the goblet lumen (Beyenbach and Wieczorek, 2006). V-ATPase is composed of two complexes; a peripheral, catalytic V_1 complex with subunits $A3B3CDE_XFG_YH$ and a membrane-bound, proton-conducting V_0 complex with subunits ac_6de (Vitavska et al., 2003). The V-ATPase is down regulated during larval moult and starvation periods by the reversible dissociation of the enzyme into its two complexes (Sumner et al., 1995; Gräf et al., 1996) and, in turn, the V_1 complex level increases in the cytoplasm. Thus, biosynthesis of V ATPase subunits is down regulated and transcript levels of these subunits decrease gradually (Wieczorek et al., 2000).

Knock down of genes encoding different subunits belonging to insect V-ATPase have been reported in different insect species. In the current study, the effect of RNAi on V-ATPase genes encoding subunits a and c was determined in *P. gossypiella* by injecting the larval instar with V-ATPase subunits a and c-specific dsRNA fragments.

MATERIALS AND METHODS

Insect culture

P. gossypiella colony was reared on an artificial diet until pupation, at temperature 25±2°C and photoperiod of light:dark (16:8) hours (Bell and Joachim, 1976). A pair of neonate larvae was added to approximately 5 g diet in 35 ml glass vial covered by cotton plugs and pupae were collected in glass jars covered by filter paper as oviposition site.

cDNA synthesis and target gene cloning

Total RNA was extracted from *P. gossypiella* midgut tissues using Triazol® (Invitrogen) according to manufacturer's instructions. First strand cDNA was prepared from total RNA using the Superscript II cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Degenerate primers were designed based on the conserved regions of the V-ATPase subunits a and c sequences in NCBI database using the Vector NTI® software (Life technologies). The nucleotide sequences of degenerate, specific and RACE primers are presented in Table 1. One fragment of subunit a was amplified using degenerate primers; VATPavo2264FD / VATPavo2484RD and two more fragments were amplified using degenerate and specific primer sets VATPavo776FD/ VATPavo1559RS and VATPavo1439FD/VATPavo2416RS, respectively. On the other hand, two fragments of subunit c were amplified using degenerate primer set (VATPcvoFD/ VATPcvoRD) and degenerate primer VATPcvo146FD and specific primer VATPcvo901RS. The template cDNA was denatured at 95°C for 5 min followed by 25 cycles of 95°C for 30 s and annealing temperature at 55°C for another 30 s followed by 30 s at 72°C the PCR reaction was extended at 72°C for 7 min. The PCR product was cloned into a pGEM-T Easy vector (Promega, Madison, WI), sequenced using the Big Tri Dye sequencing kit (ABI Applied Biosystems) at Macrogen, Korea.

Both 5' and 3' ends were synthesized using First Choice® RLM-RACE kit (Ambion life technologies) following manufacturer's procedures. RACE adaptors and specific primers (Table 1) were used for two rounds of PCR to amplify both ends of subunits a and c with annealing temperatures of 57 and 65°C, respectively. For subunit a; the outer adaptors were mixed with specific primers VATPavo864RS and VATPavo2265 FS to amplify 5'and 3'-end, respectively. The second PCR round was performed using inner adaptors with specific primers VATPavo795 RS and VATPavo2431FS for 5' and 3'-end, respectively. On the other hand, outer and inner adaptors and specific primers VATPcvo244 RS and VATPcvo185 RS were used to amplify the 5'-end of subunit c. The 3'-end was amplified using specific primers VATPcvo819 FS and VATPcvo847 FS.

Sequence, alignment, and phylogenetic analysis

The obtained sequences of V-ATPase subunits a and c were analyzed using the BLAST algorithm at NCBI for comparative analysis. Multiple alignments of both nucleotide and deduced amino acid sequences were performed by Vector NTI® advance 10 (Life Technologies). The deduced amino acid sequences were also scanned for motifs against the PROSITE database. A phylogenetic tree was constructed using the neighbor-joining method in MAGE version 6 (Tamura et al., 2013).

dsRNA synthesis

The dsRNA fragments were generated using MEGA script® RNAi Kit (Ambion) according to manufacturer's instructions. Two dsRNA fragments; a-1 and a-2 (220 and 466 bp) were prepared to target the *P. gossypiella* V-ATPase subunit a transcript using the following primer sets "VATPavo2264F/VATPavo2484R" and "VATPavo1950F/VATPavo2416R". On the other hand, the gene encoding subunit c was knocked down by only one dsRNA fragment (740 bp) that was synthesized by primer set "VATPcvo161F/ VATPcvo901R" (Table 1).

dsRNA injection

RNAi efficiency of gene silencing in *P. gossypiella* was determined

Table 1. Primers used in PCR, RACE and dsRNA synthesis.

Primer	Sequence
Cloning primers	
Subunit a	
VATPavo2264 FD	5'-GTGGGCDYTKTCRCTKGCBC-3'
VATPavo2484RD	5'-GAAYTTRCTCWKGAACTCCACCC-3'
VATPavo1439FD	5'-TTCTTYGSYGGDCGTTACAT-3'
VATPavo2416 RS	5'-GACGAGGATGGCGAGCGTGAA-3'
VATPavo776 FD	5'-GTVTTYGTGGYBTTCTTCCA-3'
VATP avo1559 RS	5'-CGG AATGCGCCACGACGAGC-3'
Subunit c	
VATPcvoFD	5'-GCYCCYGGCGAYAAGACMTG-3'
VATPcvoRD	5'-GCCSAGRTCBGASAGSCCCAC-3'
VATPcvo146 FD	5'-TGGMACDGCCAAGTCVGGMACBGGT-3'
VATPcvo901 RS	5'-CATCCACTATAAGACAGAAAGCT-3'
RACE primers	
Subunit a	
5'-end	
VATPavo864 RS	5'-GTTGGAAGGCGGACACGGGTA-3'
VATPavo795 RS	5'-CTGCTCACCTTGGAAGAACACC-3'
3'-end	
VATPavo2265 FS	5'-CTTTCGCTGGCTCACGCAGAGTTATC-3'
VATPavo2431 FS	5'-TCTCGGCCTTCCTCCACACCCT-3'
Subunit c	
5'-end	
VATPcvo244 RS	5'-ATAATACCCGCCATGACAACG-3'
VATPcvo185 RS	5'-CGACATAGCGGCGATACCCGT-3'
3'-end	
VATPcvo819 FS	5'-ACTGTGTACCACCATCTTTGC-3'
VATPcvo847 FS	5'-GGTAAAACCTGGCTTAAGTAC-3'
dsRNA primers	
Subunit a	
a-1	
VATPavo2264F	5'-TAATACGACTCACTATAGGGCTTTCGCTGGCTCACGCAGAGT-3'
VATPavo2484R	5'-TAATACGACTCACTATAGGGCAGGGTGTGGAGGAAGGCCGA-3'
a-2	
VATPavo1950F	5'-TAATACGACTCACTATAGGGGAAGATGGCTGCCACGAATA-3'
VATPavo2416R	5'-TAATACGACTCACTATAGGGGACGAGGATGGCGAGCGTG-3'
Subunit c	
VATPcvo161F	5'-TAATACGACTCACTATAGGGCGGCACGGGTATCGCCGCTAT-3'
VATPcvo901R	5'-TAATACGACTCACTATAGGGCATCCACTATAAGACAGAAAGC-3'

by microinjection of dsRNA into larval hameolymph. The third larval instars were injected using Neuros Syringe model 1701RN controlled with dispenser (Hamelton). The dsRNA was diluted with injection buffer (0.1mM NaHPO$_4$ pH 6.8, 5 mM KCl) to final concentration of 1 µg/µl and 0.2 µl were injected into larvae between meso and meta-thoracic segments. The injection was repeated three times for each dsRNA fragment, each replica contained a group of 40 to 45 larvae. Control larvae were injected with injection buffer and treated as the same as experimental individuals. Larvae that died within the first for 24 h were removed and not counted. Larval mortality was recorded five days after injection. Statistics of data was performed with Student's t-test in the Excel program.

RESULTS

Sequence analysis of the V-ATPase subunits a and c

The initial sequences of both transcripts were amplified using degenerate primers designed based on

homologous proteins. A partial sequence (220 bp) of V-ATPase subunit a was obtained from *P. gossypiella* by RT-PCR using degenerate primer set (VATPavo2264FD / VATPavo2484RD), and two longer fragments of 977 and 783 bp were isolated using two sets of combined degenerate and specific primers (VATPavo776FD/ VATPavo1559RS and VATPavo1439FD / VATPavo2416RS) (Supplementary Figure S-1). A 150 bp fragment of V-ATPase subunit c was also isolated based on degenerate primer set (VATPcvoFD/ VATPcvoRD) while a longer 755 bp sequence was amplified by degenerate and specific primers (VATPcvo146FD/VATPcvo901RS). However, the full lengths of the two V-ATPase transcripts were identified by cloning and sequencing the 5' and 3' ends (Supplementary Figure S-2). The RACE strategy demands two PCR reactions, the outer and the inner rounds using RACE adaptors with specific primers. Therefore, two specific primers were designed for each end (Table 1). Specific primers VATPavo864RS and VATPavo795 RS were used to amplify the 5'-end of subunit a, while the 3'-end was synthesized using VATPavo2265 FS and VATPavo2431FS primers. Likewise, the 5'-end of subunit c was identified using specific primers VATPcvo244 RS and VATPcvo185 RS and the 3'-end was amplified using specific primers VATPcvo819 FS and VATPcvo847 FS. The products of PCR and 5' and 3' RACE reactions were aligned to form a contig and finally the full lengths were assembled.

Full length sequence of subunit a (accession no. KU550964) consists of 2760 bp including open reading frame of 2526 bp encoding for 842 amino acids (aa) with 88% identical and 92% similarity to subunit a of *B. mori* and *Amyelois transitella*. The calculated molecular mass of the protein is 96.4 kDa with isoelectric point (pI) of 5.67. On the other hand, subunit c (accession no. KU550965) is 1140 bp containing an ORF of 480 bp and encoding a protein of 160aa with molecular weight of 16.2 kDa and pI of 8.96.

Searching the PROSITE database revealed no common motifs for both subunits sequences. The phylogenetic tree analysis shows that the V-ATPase subunit a of both *P. gossypiella* and *Tribolium castaneum* are located on the same branch as *Bactrocera dorsalis* with 94% bootstrap support (Figure 1a). On the other hand, the *P. gossypiella* V-ATPase subunit c is in close proximity with other lepidopteran species; *H. virescens*, *M. sexta* and *Plutella xylostella* by 50% bootstrap with distal scale length of 0.02 (Figure 1b). The similarity of PgVATPase subunit a and other cognate of other insect orders is presented in Figure 2 whereas subunit c belongs to highly conserved proteolipid protein family known as ductin (Pietrantonio and Gill, 1997). The amino acid sequence of PgVATPase subunit c is highly identical with other lepidopteran insects (Figure 3). The amino acid is 95% identical and 97% similar to that of *Heliothis virescens* and *Manduca sexta*.

dsRNA injection

Third larval instars were injected with 200 ng dsRNA in between thoracic segments. Larval mortality within 24 h after injecting the dsRNA was neglected to make sure that mortality was not caused by injury from injections. Larval mortality was recorded between 24 and 96 h post injection. Although two dsRNAs fragments, a-1 and a-2, were designed to target the same site on the V-ATPase subunit a transcript, each caused different mortality to the injected larvae. The first fragment (a-1) is 220 bp in length (nucleotides 2264-2484), whereas the length of the second dsRNA (a-2) is 466 bp from 1950 to 2416 (Supplementary Figure S-3). Injection of a-1 fragment into the haemolymph causes larval mortality of 18.9%, with P value <0.05 compared to control larvae (5.7%). On the other hand, lethal effect of a-2 fragment results in 26.7% and shows significant difference with the control (P <0.05). Likewise, V-ATPase subunit c specific-dsRNA reveals similar effect on pink bollworm larvae with morbidity of 23.5% (P <0.05) (Figure 4a). Statistical analysis revealed no significance difference among the three dsRNAs treatments (P >0.05). Survived larvae from injection bioassays were kept on diet to observe larval development and pupation. Bodies' shrinkage and retardation of larval development are clearly shown of dsRNA-treated larvae due to starvation effect (Figure 4b). Some of these larvae failed to pupate due to deficiency in food absorption.

DISCUSSION

V-ATPase plays a crucial rule in the lepidopteran midgut keeping an alkaline environment in the midgut lumen and energizing secondary amino acid absorption. It is distributed with high density across the plasma membrane of the goblet cell with approximate ratio of 5000 μm^{-2} (Vitavska et al., 2005). The dynamic function of V-ATPase was summarized by Osteresch et al. (2012). The headpiece A3B3 hexamer catalyses ATP binding and hydrolysis and the generated energy rotates the stalk that functions as a structural and functional connection between the V_1 and V_0. The stalk is made of the central shaft "D and F subunits" that fills the central cavity of the headpiece (Ma et al., 2011) and the V_0 subunits d with the proteolipid ring of c isoforms. H^+ proton is translocated across the membrane through entering the proteolipid ring via the cytosolic half channel of subunit a. The proton binds to conserved glutamate of subunit c, upon rotation, it dissociates and leave into the lumen through the outer half channel of subunit a. The role played by V-ATPase in the insect midgut makes it a good candidate for gene silencing by RNAi.

In this study, we cloned genes encoding the membrane-bound V_0 subunits a and c from the *P. gossypiella* midgut and dsRNA was *in vitro* synthesized and injected into the
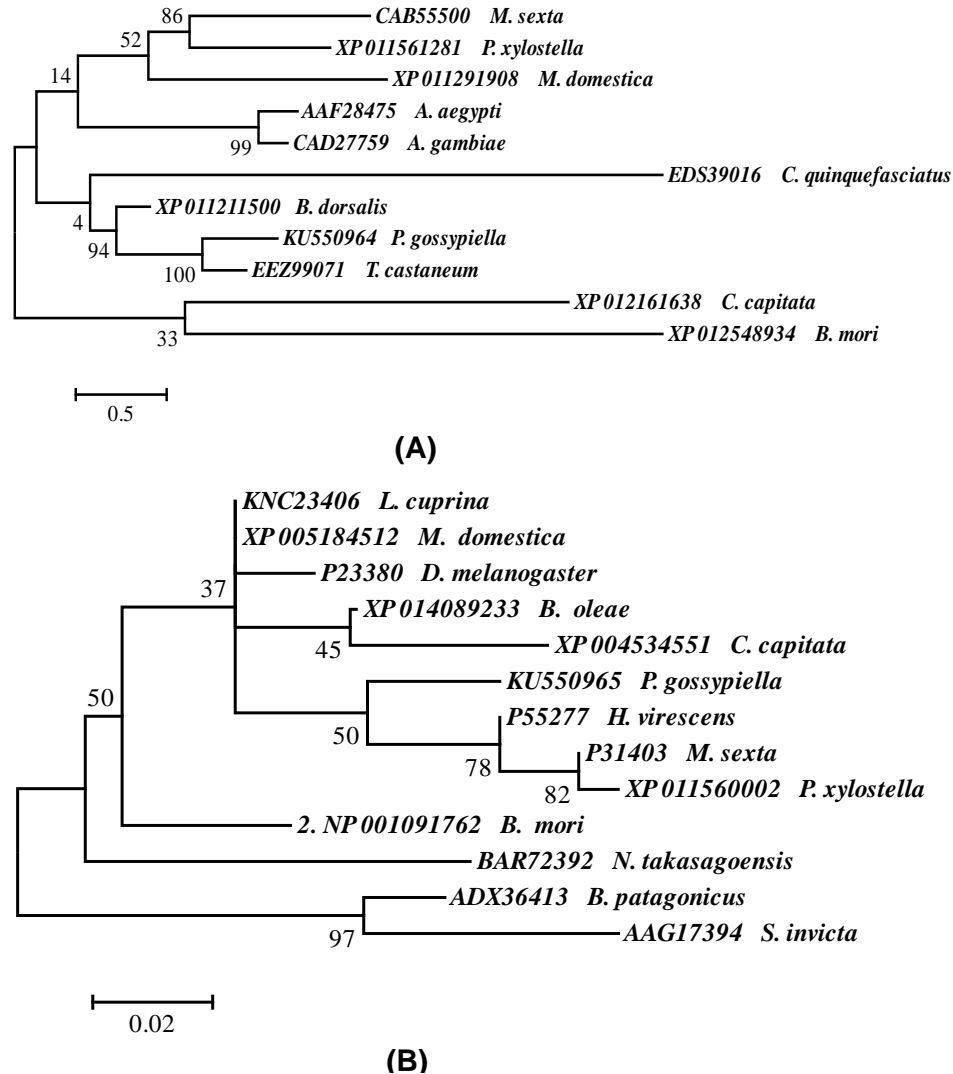
Figure 1. Phylogenetic relationship of V-ATPase subunits. **(A)** V-ATPase subunit a. **(B)** V-ATPase subunit c. This un-rooted phylogenetic tree was constructed by the neighbor-joining method. Nodes indicate bootstrap calculated with 1000 replications support.

third larval instar. RNAi effectiveness depends on multiple factors including the amount of injected dsRNA (Chen et al., 2008). A concentration of 20 ng dsRNA/mg tissue was chosen for the current experiments comparable to previous assessments of dsRNAs on pink bollwormV_1 subunits (Mohammed et al., 2015). Furthermore, high level of silencing is achieved at high doses of dsRNA (0.1 to 1 µg/mg) in some lepidopteran species (Terenius et al., 2011). Direct microinjection is one of the commonly used procedures for delivery of dsRNA into organisms (Bucher et al., 2002; Tomoyasu and Denell, 2004; Chen et al., 2008; Rong et al., 2013; Yao et al., 2013). Nevertheless, microinjection causes pressure on the insect body during injection and may result in wound that reduces insect survival. Therefore, deceased larvae within the first 24 h post injection were not counted. Although the V_0 complex

plays a role in translocating the proton, only few reports on targeting V_0 complex subunits were published. We therefore, cloned and targeted V_0 subunits a and c genes and attempted to knockdown these genes by three dsRNA fragments.

Significant mortality was observed in dsRNA treatments compared to control, whereas there were no significant differences in the toxicity of the three dsRNAs. The dsRNA "a-2" was found to be most effective, followed by dsRNA "c" (P <0.05). The dsRNA "a-1" was the least toxic and caused mortality up to 19% only. The observedmortality of pink bollworm larvae by dsRNAs silencing V_0 subunits is consistent with earlier reports with V_1 subunits. Four V_1 subunits (A, B, C and D) of the pink bollworm V-ATPase were knock downed by RNAi with mortality range of 23.5 to 40.5% (Mohammed et al.,

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TcVATPase a  KAPNPREIIDLEAHLEKTEGDIKELSESAVNLKSNYLELIELKQVLEKTQAFFNEQDE
CqVATPase a  RAPNPREIIDLEAHLEKTESEIMELSQNAVNLKSNYLELTELKHVLERTQGFFFEQEG
BdVATPase a  RAPNPREIIDLEAHLEKTENEILELAQNEVNLKSNYLELTELRKVLENTQGFFSDQEV
PgVATPase a  RAPNPREIIDLEAHLEKTENEILELSHNAVNLKQNYLELTELKHVLEKTEAFFVAQEE

TcVATPase a  ANGLDSAHKALINDES-HNVSIRGRLGFVAGVINRERVPGFERMLWRISRGNVFLRQV
CqVATPase a  S---GDTFRNNIIDDP-SNIQTRGRLGFVAGVIQREKVPGFERMLWRISRGNVFLRQA
BdVATPase a  LN-LDSSNRGPNADVD-VGPQNRGRLGFVAGVINRERVFAFERMLWRISRGNVFLKRS
PgVATPase a  FG-MDPLTKSLISDETGQQAATRGRLGFVAGVVQRERVPAFERMLWRISRGNVFLRRA

TcVATPase a  EIEKPLEDPATGNQLYKTVFVAFFQGEQLKTRIKKVCAGYHASLYACPSSLQERNEML
CqVATPase a  ELEKPLEDPSTGNQIYKTVFAAFFQGEQLKTRIKKVCTGYHASLYPCPSAADEREEML
BdVATPase a  DLDDPLKDPSTGHPIYKTVFVAFFQGEQLKNRIKKVCTGFHASMYPCPSSHTEREEMV
PgVATPase a  ELDKPLEDPSTGNEIYKTVFVVFFQGEQLKSRIKKVCTGFHASLYPCPPSNVERQDMV

TcVATPase a  KGVCTRLEDLNLVLNQTQDHRQRVLVSVAKELQNWSVMVSKMKAIYHTLNFFNMDVTK
CqVATPase a  KGVKTRLEDLSMVLNQTQDHRSRVLSTVAKELPRWRIMVKKMKAIYHTLNFFNMDVTK
BdVATPase a  KGVRTRLEDLKLVLSQTEDHRSRVLATVSKNLPSWSIMVKKMKAIYHTLNLFNMDVTK
PgVATPase a  KGVRTRLEDLNMVLNQTRDHRQRVLVSVAKELPSWSIMVRKMKAIYHTLNLFNMDVTK

TcVATPase a  KCLIGECWVSSKDIPIVQKALSDGSSACGSSIPSFLNVINTNEDPPTFNRTNKFTRGF
CqVATPase a  KCLIGECWVPVLDLPLIQKALSDGSAAVGSTIPSFLNVIETSEAPPTFNRTNKFTRGF
BdVATPase a  KCLIGECWVPTKDLPVVQKALSDGSAAVGSTIPSFLNVIDTNEQPPTFNRTNKFTRGF
PgVATPase a  KCLIGECWVPVADLPNVQKALADGSNACGSSIPSFLNCIETDEEPPTFNRTNRFTRGF

TcVATPase a  QNLIDSYGVASYREANPALYTIITFPFLFAVMFGDVGHAMIMALFGGYLVISEKKIMA
CqVATPase a  QNLIDAYGIASYREANPALYTIITFPFLFGIMFGDLGHGMIMAAFGLWMVTNERKLSA
BdVATPase a  QNLIDAYGVASYREANPALYTCITFPFLFAVMFGDLGHGIILALFGGWMVMYEKSLQR
PgVATPase a  QNLIDAYGVASYRECNPALYTIITFPFLFAVMFGDFGHGAIMMLFGAWMVCKEVSLSA
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Figure 2. Deduced amino acid sequence between 117-406 aa of PgV-ATPase subunit a and aligned with *Tribolium castaneum* (Gene Bank™ accession number EEZ99071) (TcVATPase a) *Culex quinquefasciatus* EDS39016 (CqVATPase a) and *Bactrocera dorsalis* XP_011211500 (BdVATPase a). The identical amino acids are shaded in black boxes.

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PgVATPase c  MSSPAADNPAYGPFFGVMGAASAIIFSALGAAYGTAKSGTGIAAMSVMRPELIMKSII
HvVATPase c  ----MAENPIYGPFFGVMGAASAIIFSALGAAYGTAKSGTGIAAMSVMRPELIMKSII
MsVATPase c  ----MAENPIYGPFFGVMGAASAIIFSALGAAYGTAKSGTGIAAMSVMRPELIMKSII
PxVATPase c  ---MSAENPIYGPFFGVMGAASAIIFSALGAAYGTAKSGTGIAAMSVMRPELIMKSII

PgVATPase c  PVVMAGIIAIYGLVVAVLIAGAMDQPSAGYTLYKGFIHLGAGLAVGFSGLAAGFAIGI
HvVATPase c  PVVMAGIIAIYGLVVAVLIAGSLDAPSNNYTLYKGFIHLGAGLAVGFSGLAAGFAIGI
MsVATPase c  PVVMAGIIAIYGLVVAVLIAGSLDSPSNNYTLYRGFIHLGAGLAVGFSGLAAGFAIGI
PxVATPase c  PVVMAGIIAIYGLVVAVLIAGSLDSPAN-YTLYRGFIHLGAGLAVGFSGLAAGFAIGI

PgVATPase c  VGDAGVRGTAQQPRLFVGMILILIFAEVLGLYGLIVAIYLYTKQ
HvVATPase c  VGDAGVRGTAQQPRLFVGMILILIFAEVLGLYGLIVAIYLYTKQ
MsVATPase c  VGDAGVRGTAQQPRLFVGMILILIFAEVLGLYGLIVAIYLYTKQ
PxVATPase c  VGDAGVRGTAQQPRLFVGMILILIFAEVLGLYGLIVAIYLYTKQ
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Figure 3. Deduced amino acid sequence of PgV-ATPase subunit c and aligned with Heliothesis virescnce (HvVATPase c) and Maduca *sexta* CAB55500 (MsV-ATPase c) and *Plutella xylostella* XP_011561281 (PxVATPase c) (PxV-ATPase c). The identical amino acids are shaded in black boxes.

2015). Current results suggest that silencing either V_1 or V_0 subunits have similar toxicity effects in pink bollworm.

However, other reports on other insect species focused on V-ATPase subunit A as a target for RNAi more than
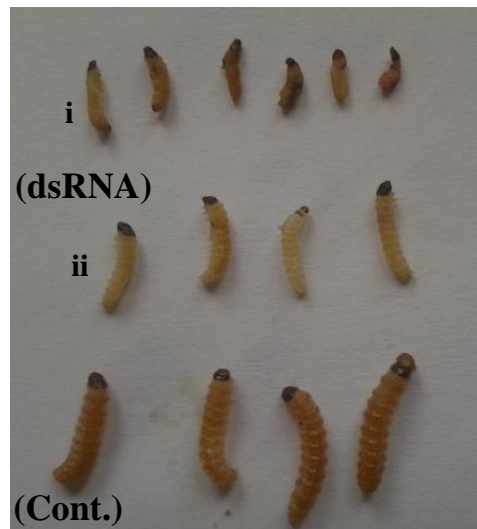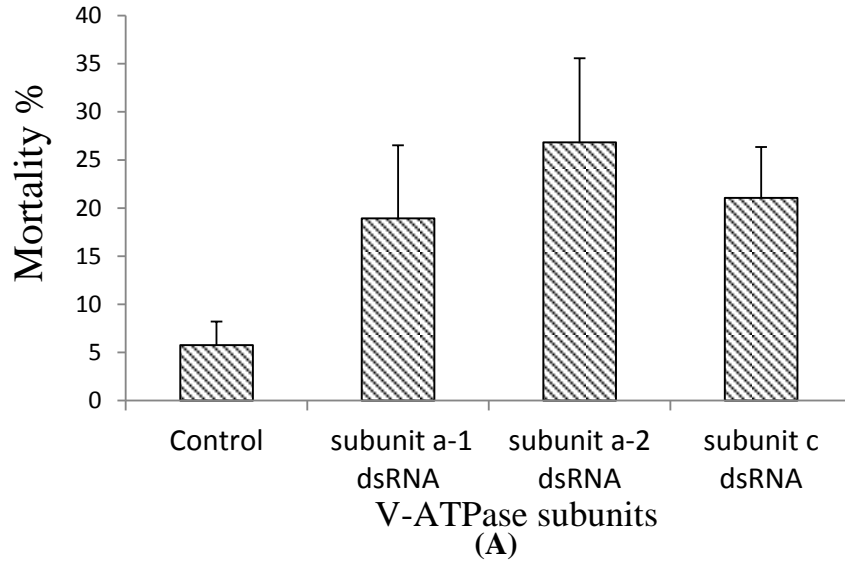
Figure 4. The lethal effect of injecting dsRNA targeting V-ATPase subunits a and c transcripts into the haemolymph of pink bollworm larvae. The larval mortality was counted 120 h after injection. **(A)** Death bar demonstrate the mortality percentage of the injected larvae against either subunit a or c-specific dsRNAs as well as control larvae. The results are shown as the mean ± SE. **(B)** Image of pink bollworm larvae were injected with V-ATPase specific-dsRNA (dsRNA); "i" dead larvae and "ii" retardation of larval development. Control larvae were injected with buffer showing normal development of control larvae (Cont.).

other subunits. As far as we know, transgenic plant knocking down V-ATPase was exclusively used to silence subunit A gene. Transgenic corn showed significant reduction in root damage by western corn rootworm (Baum et al., 2007). Thakur et al. (2014) developed transgenic tobacco expressing VATpase-A specific dsRNA, resulted in 62% reduction of the V-ATPase A transcripts level within whiteflies midguts. Transgenic plants showed high resistance to heavy infestation of whiteflies compared to control plants. Recently, three genes encoding P450, chitin synthase B and V-ATPase A from the midgut of *H. armigera* were targeted by dsRNAs that are expressed in the chloroplast genome of transplastomic tobacco plants (Jin et al. 2015). The transcripts of these three genes were not detected in the midgut of larvae fed on tobacco leaves expressing dsRNA. As a result, the net weight and growth of fed larvae were retard and pupation rate was significantly

reduced.

Suppression of endogenous V-ATPase subunits genes showed variable results according to targeted subunit and insect species. The V_1 subunits A, D and E genes were targeted by oral feeding of specific dsRNA causing mortality against WCR (Baum et al., 2007). There was no apparent significant difference in the assessed dsRNAs. The V-ATPase A-dsRNA caused variable mortality; 97.5% in *B. tabaci* (Upadhyay et al., 2011), 27.3 to 54.5% in *Tetranychus urticae* (Kwon et al., 2013) and 35% in *B. dorsalis* (Li et al., 2011a). Likewise, the V-ATPase E transcript levels was reduced between 55 and 85% in *Leptinotarsa decemlineata* larvae after ingesting two dsRNA fragments (Ky et al., 2014), low silencing level detected in *M. sexta* (Whyard et al., 2009), and no observed response in *Nilaparvata lugens* (Li et al., 2011b). The V- ATPase B and D transcripts were targeted by both feeding and microinjection of dsRNAs in the corn plant hoper, *P. maidis* (Yao et al., 2013). Quantitative PCR analysis indicated a reduction of 27-fold of V-ATPase transcripts two days post injection, while ingestion of dsRNA resulted in only two fold reduction after six days of feeding. Higher mortality and lower fecundity as well as phenotypic deformation were observed in nymphs injected with 200 ng of either V-ATPase B or D dsRNA.

Different factors influence RNAi efficiency in insects such as; effective dose of dsRNA that efficiently knockdowns target transcripts (Kumar and Sarin, 2013), ability of insect cells to uptake dsRNA molecules and to involve them within the RNAi pathway (Terenius et al., 2011) and RNAi processing machinery within the cells and signal propagation across neighboring cells (Roignant et al., 2003; Miller et al., 2008). Also, length of the dsRNA, life-stage of the insect, and persistence of gene silencing, are important factors for successful RNAi application (Thakur et al., 2014).

Despite the low mortality achieved in the current study, deleterious effects on pink bollworms were noticed. V-ATPase is still a potential target for RNAi and could be deployed in the control of pink bollworm, if multiple genes could be targeted simultaneously by dsRNA, or by targeting different sites on the same target gene(s) causing an increase of larval mortality. RNAi is a promising strategy in insect pest management and further investigation is required to enhance its effect.

Conflicts of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

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SUPPLEMENTARY FIGURES
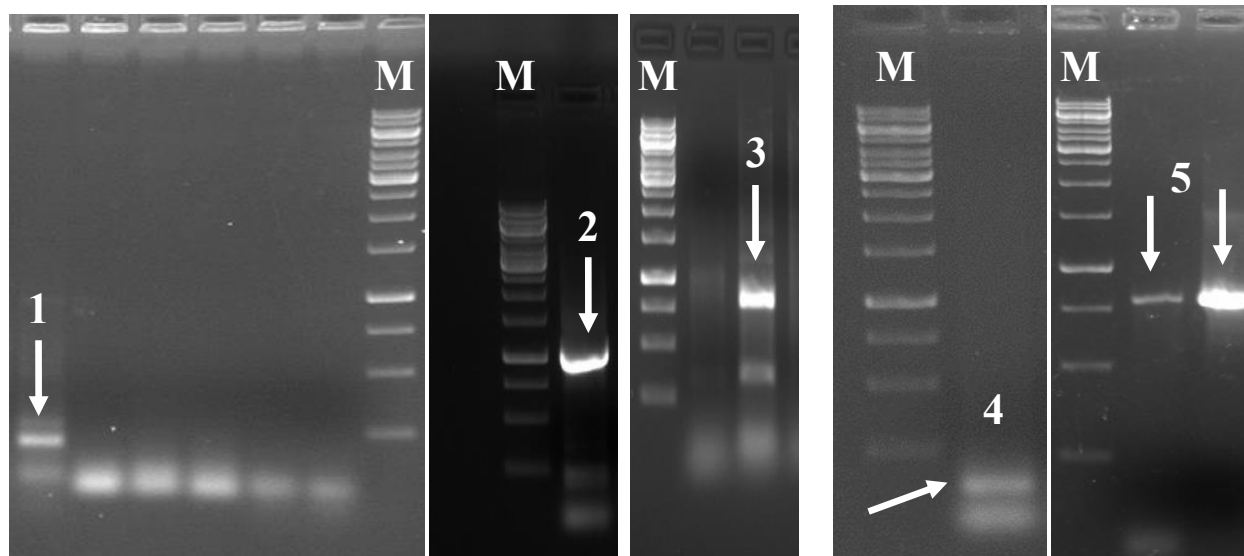
Figure S-1. Cloning of VATPase subunits a and c. PCR products of subunit a; (1) 220 bp using degenerate primer set (VATPavo2264FD/VATPavo2484RD), (2) 977 bp using degenerate/specific primers (VATPavo1439FD/VATPavo2416RS), (3) 783 bp using (VATPavo776FD/ VATPavo1559RS) and subunit c (4) 150 bp using degenerate primer set (VATPcvoFD/ VATPcvoRD), (5) 755 bp using degenerate/specific primers (VATPcvo146FD/ VATPcvo901RS). (M) 1 Kb DNA marker.
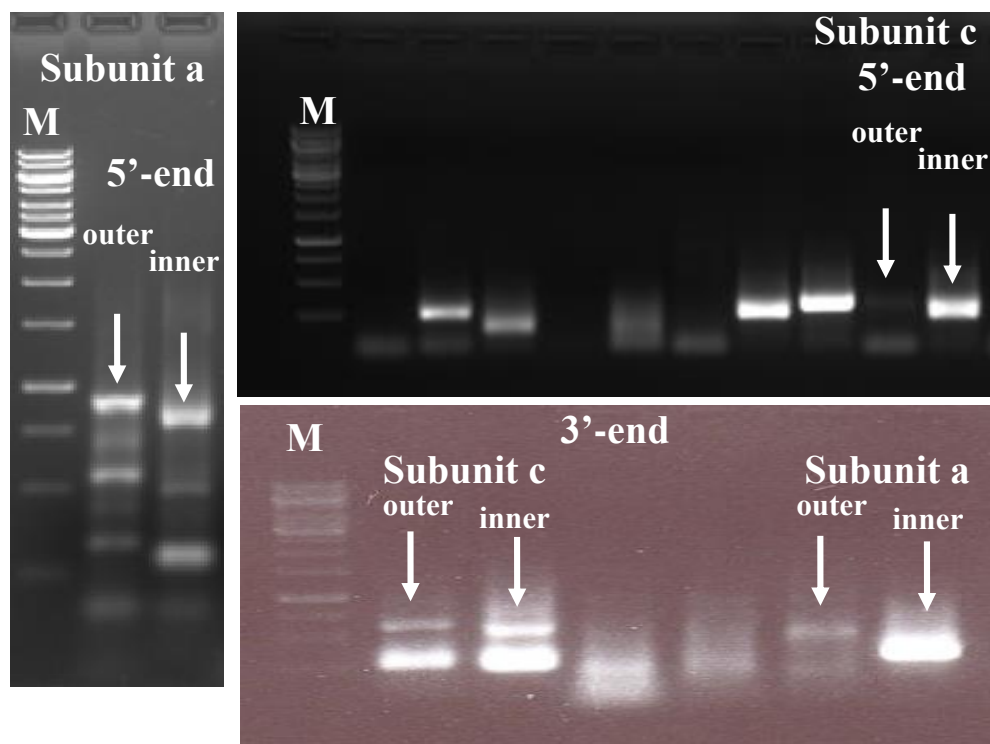
Figure S-2. RACE reactions; (1) 5'-end of subunit a was amplified using outer 5 prime supplied with the kit with VATPavo864RS specific primer followed by PCR reaction using inner 5 prime with VATPavo795RS specific primer. (2) 5'-end of subunit c was amplified using outer and inner prime with specific primers VATPcvo244RS and VATPcvo185RS, respectively. (3) 3'-end of both subunits a and c were synthesized using outer and inner 3 prime supplied with the kit with specific primers VATPcvo819FS and VATPcvo847FS for subunit c, and with specific primers VATPavo2265 FS and VATPavo2431FS for subunit a.
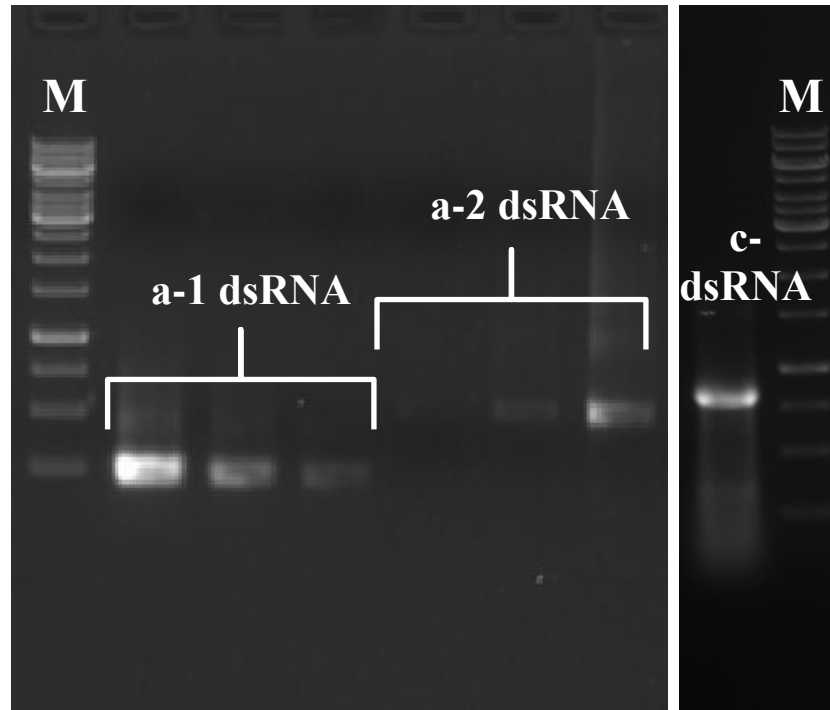
Figure S-3. Synthesis of dsRNA; a-1 dsRNA fragment of 220 bp using VATPavo2264F/ VATPavo2484R primer set; a-2 dsRNA fragment of 466 bp using VATPavo1950F/VATPavo2416R and c-dsRNA fragment of 740 bp using VATPcvo161F/ VATPcvo901R. (M) 1 Kb DNA marker.