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RNA INTERFERENCE-MEDIATED KNOCKDOWN OF VACUOLAR-ATPASE GENES IN PINK BOLLWORM (*PECTINOPHORA GOSSYPIELLA*)

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ABSTRACT

Vacuolar H^+-ATPase (V-ATPase) is an ATP-driven proton pump and is essential to the function of eukaryotic cells. In insects, V-ATPaseis located in apical membrane of goblet cells in the midgut. It acts as an energizer of the plasma membrane driving nutrient uptake, fluid secretion and,in some cases, alkalizing the gut lumen. Knocking down V-ATPase gene(s) using RNAimay disturb the food process within pink bollworm, *Pectinophoragossypiella*(*P.gossypiella*) (Lepidoptera:Gelechiidae)midgut and eventually causing the insect death. The full length of V-ATPase subunits A and D transcripts was sequenced. Three dsRNA fragments were designed,two of them (VATPA756-1155 and VATPA347-753) were designed to target subunit A and thethird (VATPD221-703)to knockdown subunit D. Injection of 200 ng of the three dsRNA fragments into the thorax of the third instar caused larval mortality of46.3%,43.9%and 25%,respectively. Furthermore, survived larvae injected with dsRNAs targeting V-ATPase subunit A showed starvation symptoms.

Keywords: dsRNA, Pink bollworm,VATPase Subunit A, VATPase Subunit D

INTRODUCTION

P.gossypiella (Saunders) was firstly described by Saunders in 1842 as *Depressaria gossypiella* (Hennereberry 2007). *P. gossypiella* was recorded

bynearly all the cotton growing countries of the world (CAB Institute of Entomology1990). It damages squares,seeds and bolls of the cotton. The infected seeds by *P.gossypiella* have less weight, vitality and oil content than the healthy seeds (Adkisson*et al*. 1985).The losses are highly increased in humid conditions due to secondary infection of the bolls with other insects and fungi (Ingram 1994). The control strategy of the *P.gossypiella* includes different methods such as mechanical, chemical, biological control…..*etc*. The mechanical method killed about 90% of the larvae attached to the stalks (Noble 1969).In the United States the infestation was reduced 44-82% when this method was applied (Chu and Bariola 1987, 1988). The most effective method is using chemical insecticides (Pimentel2009).Egypt and Mexico were the first countries which applied the chemical control (arsenicals and fluosilicates) but it didn't achieve the expected success(Pearson 1958). The hazards and the side effects of this method limit its applications. Moreover, *P.gossypiella* developed resistance to chemical insecticides such as chlorinated hydrocarbons (Lowry and Berger 1965) and synthetic pyrethroid compounds(Haynes *et al*. 1986, 1987).

RNA interference (RNAi) is a mechanism of post-transcriptional regulation of gene-expression in higher eukaryotes (Berezikov 2011). It was first discovered in *C. elegans* by Fire et al. (1998). The RNAi technology was exploited in many applications as biotechnology, medicine and insect control. RNAi could target the most important gene(s) that play crucial role in insect life. Long list of diverse genes from different insect orders were studied *in vivo* using RNAi as reviewed by Belles (2010)and Terenius *et al*.(2011).Recently, RNAi has been widely used to control a variety of insect orders, including Coleoptera, Hemiptera, Diptera, Hymenoptera and Lepidoptera (Baum *et al*. 2007,Zhao *et al*. 2008, Bautista *et al*. 2009,Walshe*et al*. 2009, Upadhyay*et al*.2011).The dsRNA-transgenic plants showed different levels of resistance towards insect pests(Yu *et al*. 2013). The transgenic tobacco plants *Nicotiana tabacum*, showed higher resistance level compared to non-transgenic for *Helicover paarmigera* (Xiong*et al*. 2013),*Spodopteraexigua* (Zhu *et al*., 2012) and*Bemisiatabaci* (Thakur *et al*. 2014). This success was also achieved with the model plants *Nicotianabenthamiana* and *Arabidopsis thaliana* against the aphids fed on these plants (Pitino*et al*. 2014).But this success wasn't reached in *Spodopteralitura* (Rajagopal *et al*. 2002), *Epiphyaspo stvittana* (Turner *et al*., 2006), *Diatraeas accharalis* (Khajuria *et al*. 2010) and

Trichoplusiani (Terenius *et al*. 2011). Other methods were also suggested such as topical application of dsRNA either by adding these dsRNA to irrigation water or spraying as any other insecticides. However, such methods are not economically applicable due to their high cost and possible degradation of the RNA before reaching its target (Kupferschmidt 2013).

V-ATPaseis a multi-subunitenzyme. It has fourteen subunits in two domains and it is a member of ATPases family (A, F and V) which are mainly responsible for ATP hydrolysis(Forgac 2007).The V-ATPase is essential to the function of eukaryotic cells (Muench*et al*. 2014). The main function of V-ATPases is acidifying a wide array of intracellularorganelles andpump protonsacross theplasma membranesof numerous cell types (Nelson *et al.*2000).V-ATPases couple the energy ofATP hydrolysis to protontransportacross intracellular and plasma membranes of eukaryotic cells. The V-ATPase has a certain function in each eukaryotic cell. In the normal conditionsof theyeast,any subunit of the enzyme could kill the yeast itself if it was mutated(Nelson *et al.*2000). In mammalian cells, the enzyme is as an acidifier of endomembrane–bounded vacuoles and vesicles (Forgac 2000). In insects, it is located in the apical membrane of the goblet cells (Wieczorek *et al.*2000), and actsas an energizer of plasma membrane. The generated voltage across the transmembrane drives nutrient uptake, fluid secretion and in some cases alkalizing the gut lumen (Harvey *et al.* 1998).Due to the importance of the V-ATPase enzyme,it was targeting in many insects to knock it down by diverse ways.

In this study the effect of RNAi on V-ATPase subunits A and D was determined in *P.gossypiella*by injecting the larval instar with dsRNA fragments targeting these subunits and the mortality ratio was recorded within four,six,eight and ten days post-injection.

MATERIALS AND METHODS

Insect Culture

P.gossypiella colony was reared on artificial diet till pupation,at temperature 25±2ºC and light period of 16 light /8 dark hours. The diet wasdistributed in 50 ml glass tubes (5 gm/tube), covered by cotton plugs. The pupae were collected in glass jars covered by filter papers. The jars were supplied with cotton pads wet with 10% sugar solution. The emerged adults laid eggs on the filter papers. The eggs were collected and used for either rearing or experiments.

Total RNA Extraction

The *P.gossypiella*midguts were dissected in insect physiological buffer, 77 mMNaCl, 1.34 mMKCl, 0.9mM $CaCl_2$, 1.05 mM

$MgCl_2$, 1.19 mM $NaHCO_3$, 11.10 mM glucose (Ghanim*et al.*, 2001). The dissection was performed on paraffin wax plates. The dissected midguts were immediately immersed in RNAlater (Qiagen, USA) and stored at -80ºC. The total RNA was isolated from midguts either by Trizole® reagent (Invitrogen, USA) or by RNeasy®Mini kit (Qiagen,USA) according to manufacturer's instructions.

First strand cDNA Synthesis

First strand cDNA was synthesized from total RNA isolated from *P.gossypiella*midguts using M-MulV reverse transcriptase (New England Biolabs, USA) as manufacturer'sinstructions.

Cloning and Sequencing of V-ATPase Subunits A and D

Primers for subunit A

Degenerate primers were designed based on the conserved regions of the V-ATPase subunit Asequences that are published in NCBI database of*Manducasexta* (X64233.1), *Bombyxmori* (NM_001098359), *Ostirinafurnacalis* (HQ434762*), Drosophila melanogaster* (U19745) *Aedesalbopictus* (AY864912) using theVector NTI® program software (Life technologies, USA).

One specific and five degenerateprimers were designed for VATPasesubunit A from the conserved regions. The first set was "VATPA220FD,VATPA504RD", the second set was"VATPA874FD,VATPA1008RD" and the third set was "VATPA1340FD,VATPA1508RS". The three amplified fragments were cloned and sequenced. To amplify the gaps between fragments, one forward specific primer (VATPA347FS) was designed with the previous reverse specific primer (VATPA1508RS).The primers sequence and the expected amplified fragments are presented in Table (1).

Primers for Subunit D

A set ofdegenerate primers "VATPD71FD,VATPD266RD" was designed from conserved regions of VATPase subunit D sequences that are published in NCBI for*M. sexta* (AJ251992) and *B. mori*(DQ311428)**.**The primers sequence and the expected amplified fragments are presented in Table (1).

The template cDNA was denatured at 95°C for 5 min followed by 25 cycles of denaturing at 95°C for 30 sec, different annealing temperature was used for each set (shown in Table 1) for 30 sec and extension at 72°C for30 sec and the reaction was ended by 7 min at 72°C. The PCR products were cloned in PGEM-T-easy vector (Promega). The positive clones were analyzed by restriction enzyme digestion using fast digest®*Eco*R1(Thermo

Scientific,USA). The cloned fragments were subjected to sequence analysis using the Big TriDye sequencing kit (ABI Applied Biosystems) by the facility of Macrogen, Korea.

5' and 3' Rapid Amplification of cDNA Ends (RACE)

The full length cDNAs of PgV-ATPase subunits A and D was obtained by identifying both 5' and 3' ends using FirstChoice®RLM-RACE kit (Ambion, USA) according to manufacturer's procedure. All specific primers used to amplify the 5' and 3' ends are presented in Table (1).

Bioassay of dsRNA Fragments

The efficiency of gene silencing using dsRNAs was determined by delivering dsRNA into larval body by direct injection. This technique was adopted by different authors such as Chen*et al*. (2008), Rong *et al*. (2013) and Yao *et al*., (2013).

The third larval instars were collected from the diet for injection. Larvae were injected using Neuros Syringe model 1701RN controlled with dispenser (Hamelton, Höchst, Germany). The dsRNA was diluted with injection buffer (0.1mM $NaPO_4$ pH6.8, 5mMKCl) to final concentration of 1µg/µl and used to inject larvae with 0.2µl between meso and metathoracic segments.

Preparation of dsRNA Fragments

The dsRNA fragments were generated using MEGAscript®RNAi Kit (Ambion, USA) according to manufacturer's instructions.Two dsRNA fragments were prepared targeting the PgV-ATPase subunit A using the following primer sets"VATPA756FDS/ VATPA1155RDS" and "VATPA374FDS/ VATPA753RDS". On the other hand, one dsRNA fragment was prepared to knock out subunit D using "VATPD221FDS/ VATPD703RDS" primer set. All primer sequences contained T7 promoter on the 5'-end as shown in Table (1).

RESULTS

Amplification the Full Length of PgV-ATPase Subunits Aand D

First strand cDNA, prepared from total RNA from the *P.gossypiella*midgut, was used as a template for amplification of V-ATPase subunit A and D. Three sets of primers were designed for subunit A from the conserved regions for the same subunit in other insects and used in the PCR reactions; one of them was specific and the rest were degenerate. The first set "VATPA220FD/ VATPA503RD" amplified aPCR fragment about 280bp in size.

Table 1: The nucleotide sequences of the cloning, RACE and dsRNA primers for VATPase subunits A and D, the annealing temperature and the expected size of the amplified fragments.

Primer Name	Sequence and Direction	Ann. Temp. (°C)	Exp. Size (bp)
Cloning primers:			
Subunit A			
VATPA220FD	**5'-CCGTCTKGARGGYGACATGGCCACC-3'**	**52°C**	**284**
VATPA504RD	**5'-TCTCCDCCRGTGATGTGKGA-3'**		
VATPA874FD	**5'-CGTCGGBTGCGGMGARCGYGGT-3'**	**55°C**	**134**
VATPA1008RD	**5'-GGCATGTTGGAKGTGTTGGC-3'**		
VATPA1340FD	**5'-CAGGTGTTCTGGGGKYTSGACAAG-3'**	**58°C**	**168**
VATPA1508RS	**5'-CCTCCTGCAGGATCTCCTTGACC-3'**		
VATPA347FS	**5'-ATCTCCGACGGCATCCAGCG-3**	**58°C**	**1161**
Subunit D			
VATPD71FD	**5'-GCBATWTTYCCTTCTCGKGGTGC-3'**	**50°C**	**195**
VATPD266RD	**5'-CTTGGTTGAARTCWCCRGTWG-3'**		
RACE primers:			
SubunitA			
VATPA1385FS	**5'-TTC CCC TCC ATC AAC TGG CTC-3'**		
VATPA1484FS	**5'-AAG GTC AAG GAG ATC CTG CAG GAG G-3'**	**55°C**	**3'**
VATPA357RS	**5'-CCG TCG AAG ATG GAG CCG A-3'**		
VATPA503RS	**5'-CC TTG GGG ATG TAG ATG GAC T-3'**	**56°C**	**5'**
Subunit D			
VATPD177FS	**5'-GGT TCC GTA TGA TCC TGG GTA A-3'**		
VATPD221FS	**5'-ATG GGA GAA GTG ATG AAA GAG GC-3'**	**55°C**	**3'**
VATPD198RS	**5'-T TAC CCA GGA TCA TAC GGA ACC-3'**		
VATPD273RS	**5'-GTG AAC TTA GCT TCA GCC AGT-3'**	**52°C**	**5'**
dsRNA primers:			
SubunitA			
VATPA756FDS	**5'-TAATACGACTCACTATAGGGACTCGCTGTTCCCTTGCGTCC-3'**	**60°C**	**399**
VATPA1155RDS	**5'-TAATACGACTCACTATAGGGATCTCCGCCAGACGACCCGA-3'**		
VATPA374FDS	**5'-TAATACGACTCACTATAGGGAAGGACATCAACGAGCTCAC-3'**	**60°C**	**379**
VATPA753RDS	**5'-TAATACGACTCACTATAGGGAGCAGCGGGTGGTTGGCGGG-3'**		
Subunit D			
VATPD221FDS	**5'-TAATACGACTCACTATAGGGATGGGAGAAGTGATGAAAGAGGC-3'**	**60°C**	**482**
VATPD703RDS	**5'-TAATACGACTCACTATAGGGCGCTTCCGCCTTGTCTTTGA-3'**		

T7 promoter sequence at the 5'-end is underlined

Two fragments ranging in size between 150 - 200bp were obtained by primer sets "VATPA874FD/ VATPA1008RD" and "VATPA1340FD/ VATPA1508RS".The three fragments represent different parts of the gene, first, middle and end. The sequence gap was filled by a specific primer set that was designed based on the sequence resulted from the previous three clones. The specific primer set

"VATPA374FS/ VATPA1508RS" amplified a fragment size of approximately 1161bp.The sequence between 220 and 1504 was revealed. However, to identify the full length of PgVATPase subunitA both 5' and 3' ends were sequenced. The RACE strategy demands two rounds of PCR reaction, the outer and the inner. Therefore, two primers were designed for each end, "VATPA503RS, VATPA357RS" primers for 5' end and "VATPA1385FS, and VATPA1484FS" primers for 3' end **(Figure 1 and Table1)**.

Similarlya set of degenerate primers "VATPD71FD/VATPD266RD"was designed to clone the subunit D ofPgV-ATPase. The same PCR rounds strategy was also performed to amplify the 3' and the 5' end of VATPase subunit D. Four specific primers were designed according to the sequencing results of the previous fragment. Two sets of specific primers "VATPD273RS, VATPD198RS" and "VATPD177FS,VATPD221FS" were employed to amplify the 5' and 3' ends respectively**(Figure 2 and Table1)**.

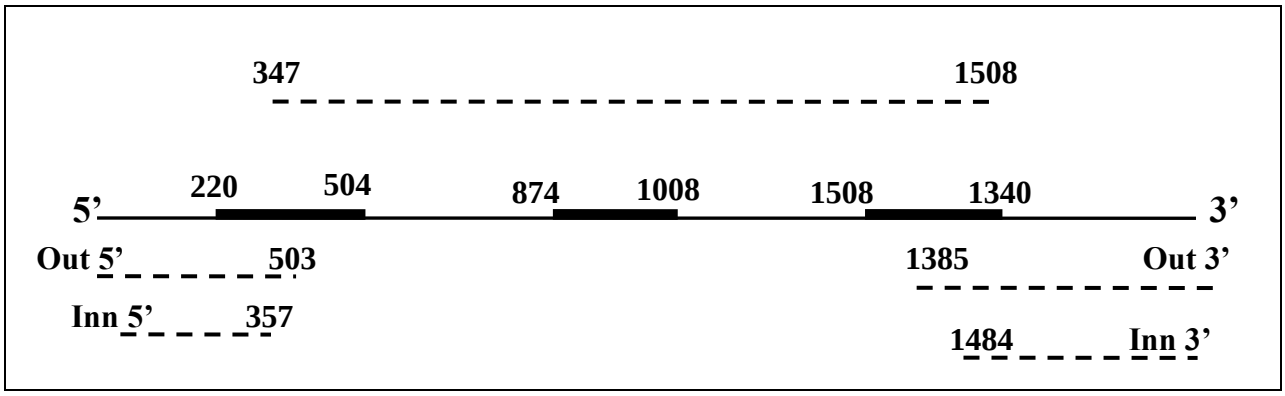

Figure 1: The cloning and RACE primers for pgVATPase subunit A, three degenerate primer sets are shown in solid lines and the specific primer sets are shown in dashed lines.

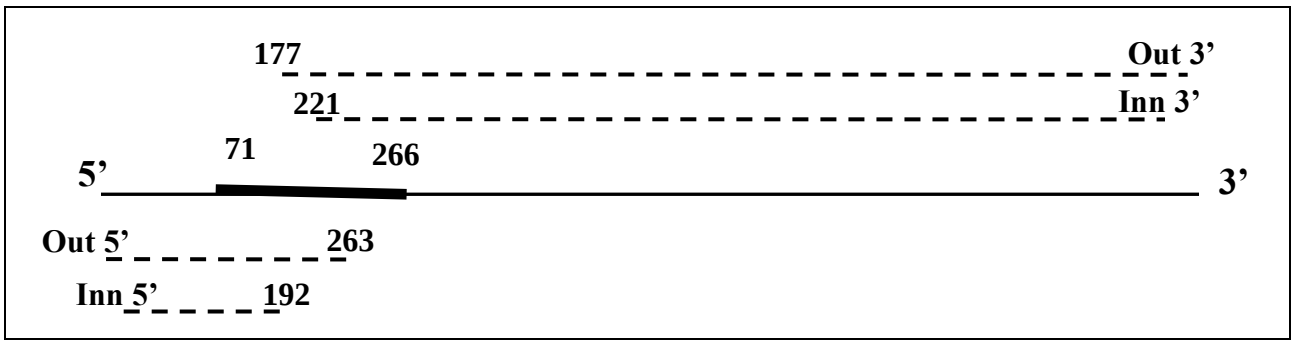

Figure 2: The cloning and RACE primers for pgVATPase subunit D,one degenerate primer set is shown in solid line and the specific primer sets are shown in dashed lines.

Sequence Analysis of the pgV-ATPase cDNAs A and D

The sequences of the cDNA fragments obtained from the amplification by degenerate,specific and RACE primers were assembled together and aligned in one long sequence. The obtained full length sequence of PgV-ATPase subunit A (accession no. KP341655) consists of 2602 nucleotides encoding 618 amino acids. These amino acids showed high similarity with V-ATPases subunit A from other insects **(Figure3)**.

The similarity with lepidopteran insects ranged from 94% to 95% for *B.mori*, and *Ostirinafurnacalis*, respectively. While non-lepidopteran insect as *Drosophila melanogaster* revealed a lower percentage of similarity (91%). The full length sequence of PgV-ATPase subunit D (accession no.KP341656) was comprised of 1467 nucleotides encoding 249 amino acids. As shown in **(Figure4)**, the alignment of these amino acids showed high similarity with V-ATPasessubunit D from*M.sexta* and *B.mori*(94% and 93%, respectively).

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Pg-VATPA    MTTTRPLKTIAMSDDSEEKFGYVFAVSGPVVTAERMSGSA
Ms-VATPA    MASKGGLKTIANEEN-EERFGYVFAVSGPVVTAEKMSGSA
Of-VATPA    MASKGGLKTIANEEN-EEKFGYVFAVSGPVVTAEKMSGSA
Bm-VATPA    MASKGGLRTIANEEN-EERFGYVFAVSGPVVTAEKMSGSA
Aa-VATPA    ---MSTLKKISDEDR-ESKFGYVFAVSGPVVTAERMSGSA
Dm-VATPA    ---MSNLKRFDDEER-ESKYGRVFAVSGPVVTAEAMSGSA

Pg-VATPA    MYELVRVGYNELVGEIIRLEGDMATIQVYEETSGVTVGDP
Ms-VATPA    MYELVRVGYNELVGEIIRLEGDMATIQVYEETSGVTVGDP
Of-VATPA    MYELVRVGYNELVGEIIRLEGDMATIQVYEETSGVTVGDP
Bm-VATPA    MYELVRVGYNELVGEIIRLEGDMATIQVYEETSGVTVGDP
Aa-VATPA    MYELVRVGYYELVGEIIRLEGDMATIQVYEETSGVTVGDP
Dm-VATPA    MYELVRVGYYELVGEIIRLEGDMATIQVYEETSGVTVGDP

Pg-VATPA    VLRTGKPLSVELGPGILGSISDGIQRPLKDINELTQSIYI
Ms-VATPA    VLRTGKPLSVELGPGILGSIFDGIQRPLKDINELTQSIYI
Of-VATPA    VLRTGKPLSVELGPGILGSIFDGIQRPLKDINELTQSIYI
Bm-VATPA    VLRTGKPLSVELGPGILGSIFDGIQRPLKDINELTQSIYI
Aa-VATPA    VLRTGKPLSVELGPGIMGSIFDGIQRPLKDINELTSSIYI
Dm-VATPA    VLRTGKPLSVELGPGIMGSIFDGIQRPLKDINELTESIYI


Pg-VATPA    PKGVNVPCLARETSWEFNPLNVKVGSHITGGDLYGIVHEN
Ms-VATPA    PKGVNVPSLAREVDWEFNPLNVKVGSHITGGDLYGIVHEN
Of-VATPA    PKGVNVPCLARNHDWEFNPLNVKVGSHITGGDLYGIVHEN
Bm-VATPA    PKGINVPSLAREVDWEFNPLNVKVGSHITGGDLYGIVHEN
Aa-VATPA    PKGVNIPCLSRTQSWGFNPLNVKVGSHITGGDLYGLVHEN
Dm-VATPA    PKGVNVPSLSRVASWEFNPLNVKVGSHITGGDLYGLVHEN
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Pg-VATPA    TLVKHKMLMPPRAKGTVTYIAPNGNYKVTDVVLETEFDGE
Ms-VATPA    TLVKHKMLMPPRAKGTVTYIAPAGNYKVTDVVLETEFDGE
Of-VATPA    TLVKHKMLMPPKAKGTITYIAPAGNYNVTDVVLETEFDGE
Bm-VATPA    TLVKHRMLVPPKAKGTVTYIAPAGNYKVTDVVLETEFDGE
Aa-VATPA    TLVKHKLLVPPRAKGTVRYIAPPGNYTVDDIILETEFDGE
Dm-VATPA    TLVKHKMIVNPRAKGTVRYIAPSGNYKVDDVVLETEFDGE

Pg-VATPA    KSSYSMLQVWPVRRPRPVTEKLPANHPLLTGQRVLDSLFP
Ms-VATPA    KAQYTMLQVWPVRQPRPVTEKLPANHPLLTGQRVLDSLFP
Of-VATPA    KNSYTMLQVWPVRQPRPCTEKLPANHPLLTGQRVLDSLFP
Bm-VATPA    RQKYSMLQVWPVRQPRPVTEKLPANHPLLTGQRVLDSLFP
Aa-VATPA    INKWSMLQVWPVRQPRPVTEKLPANHPLLTGQRVLDSLFP
Dm-VATPA    ITKHTMLQVWPVRQPRPVTEKLPANHPLLTGQRVLDSLFP

Pg-VATPA    CVQGGTTAIPGAFGCGKTVISQALSKYSNSDVIVYVGCGE
Ms-VATPA    CVQGGTTAIPGAFGCGKTVISQALSKYSNSDVIIYVGCGE
Of-VATPA    CVQGGTTAIPGAFGCGKTVISQALSKYSNSDVIVYVGCGE
Bm-VATPA    CVQGGTTAIPGAFGCGKTVISQALSKYSNSDVIIYVGCGE
Aa-VATPA    CVQGGTTAIPGAFGCGKTVISQALSKYSNSDVIIYVGCGE
Dm-VATPA    CVQGGTTAIPGAFGCGKTVISQALSKYSNSDVIIYVGCGE

Pg-VATPA    RGNEMSEVLRDFPELTVEIEGVTESIMKRTALVANTSNMP
Ms-VATPA    RGNEMSEVLRDFPELTVEIEGVTESIMKRTALVANTSNMP
Of-VATPA    RGNEMSEVLRDFPELSVEIDGVTESIMKRTALVANTSNMP
Bm-VATPA    RGNEMSEVLRDFPELTVEIEGVTESIMKRTALVANTSNMP
Aa-VATPA    RGNEMSEVLRDFPELSVEIDGVTESIMKRTALVANTSNMP
Dm-VATPA    RGNEMSEVLRDFPELSVEIDGVTESIMKRTALVANTSNMP

Pg-VATPA    VAAREASIYTGITLSEYFRDMGYNVSMMADSTSRWAEALR
Ms-VATPA    VAAREASIYTGITLSEYFRDMGYNVSMMADSTSRWAEALR
Of-VATPA    VAAREASIYTGITLSEYFRDMGYNVSMMADSTSRWAEALR
Bm-VATPA    VAAREASIYTGITLSEYFRDMGYNVSMMADSTSRWAEALR
Aa-VATPA    VAAREASIYTGITLSEYFRDMGYNVSMMADSTSRWAEALR
Dm-VATPA    VAAREASIYTGITLSEYFRDMGYNVSMMADSTSRWAEALR

Pg-VATPA    EISGRLAEMPADSGYPAYLGARLASSYERAGRVKCLGNPD
Ms-VATPA    EISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPD
Of-VATPA    EISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPD
Bm-VATPA    EISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPD
Aa-VATPA    EISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPE
Dm-VATPA    EISGRLAEMPADSGYPAYLGARLASFYERAGRVKCLGNPE

Pg-VATPA    REGSVSIVGAVSPPGGDFSDPVTAATLGIVQVFWGLDMKL
Ms-VATPA    REGSVSIVGAVSPPGGDFSDPVTAATLGIVQVFWGLDKKL
Of-VATPA    REGSVSIVGAVSPPGGDFSDPVTAATLGIVQVFWGLDKKL
Bm-VATPA    REGSVSIVGAVSPPGGDFSDPVTAATLGIVQVFWGLDKKL
Aa-VATPA    REGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL
Dm-VATPA    REGSVSIVGAVSPPGGDFSDPVTSATLGIVQVFWGLDKKL

Pg-VATPA    AQRKHFPSINWLISYSKYMRALDDFYDKNYPEFVPLRTKV
Ms-VATPA    AQRKHFPSINWLISYSKYMRALDDFYEKNYPEFVPLRTKV
Of-VATPA    AQRKHFPSINWLISYSKYMRALDDFYEKNYPDFVPLRTKV
Bm-VATPA    AQRKHFPSINWLISYSKYMRALDDFYEKNYPEFVPLRTKV
Aa-VATPA    AQRKHFPSINWLISYSKYMRALDDFYDKNFQEFVPLRTKV
Dm-VATPA    AQRKHFPSINWLISYSKYMRALDDFYDKNFPEFVPLRTKV

Pg-VATPA    KEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDL
Ms-VATPA    KEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDF
Of-VATPA    KEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDF
Bm-VATPA    KEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDF
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Aa-VATPA    KEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDF
Dm-VATPA    KEILQEEEDLSEIVQLVGKASLAETDKITLEVAKLLKDDF

Pg-VATPA    LQQNSYSAYDRFCPFYKTVGMLKNIISFYDMSRHAVESTA
Ms-VATPA    LQQNSYSSYDRFCPFYKTVGMLKNIISFYDMSRHAVESTA
Of-VATPA    LQQNSYSAYDRFCPFYKTVGMLKNIISFYDMSRHAVESTA
Bm-VATPA    LQQNSYSSYDRFCPFYKTVGMLKNIITFYDMSRHAVESTA
Aa-VATPA    LQQNSYSAYDRFCPFYKTVGMLRNMIGFYDMARHAVETTA
Dm-VATPA    LQQNSYSSYDRFCPFYKTVGMLRNIIDFYDMARHSVESTA

Pg-VATPA    QSDNKVTWNVIRDAMGPVLYTLSSMKFKDPVKDGEAKIKA
Ms-VATPA    QSDNKVTWNVIRDAMGNVLYQLSSMKFKDPVKDGEAKIKA
Of-VATPA    QSDNKVTWNVIRDAMGNVLYQLSSMKFKDPVKDGEAKIKA
Bm-VATPA    QSDNKVTWNVIRDAHGHVLYQLSSMKFKDPVKDGEPKIKA
Aa-VATPA    QSENKITWNVIRDSMGNILYQLSSMKFKDPVKDGEAKIKA
Dm-VATPA    QSENKITWNVIREAMGNIMYQLSSMKFKDPVKDGEAKIKA

Pg-VATPA    DFDQLLEDMSAAFRNLED
Ms-VATPA    DFDQLLEDMSAAFRNLED
Of-VATPA    DFDQLLEDMSAAFRNLED
Bm-VATPA    DFDQLLEDMSAAFRNLED
Aa-VATPA    DFDQLYEDLQQAFRNLED
Dm-VATPA    DFEQLHEDLQQAFRNLED
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Figure 3: Alignment between deduced amino acid sequence of *Pectinophoragossypiella*(Pg) V-ATPase subunit A with other published amino acid sequence of the same gene in other insects in NCBI;*Manducasexta* (Ms) (X64233.1), *Ostirinafurnacalis*(Of) (HQ434762), *Bombyxmori* (Bm)(NM_001098359), *Aedesalbopictus* (Aa) (AY864912) and *Drosophila melanogaster* (Dm)(U19745) . The identical amino acids are shown in the shaded boxes.

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Pg-VATPD MSGKEKLAIFPSRGAQMLIKGRLAGAQKGHGLLKKKADALQVRFRMILGK
Ms-VATPD MSGKDRLAIFPSRGAQMLMKGRLAGAQKGHGLLKKKADALQVRFRLILSK
Bm-VATPD MSGKDRLAIFPSRGAQMLIKGRLAGAVKGHGLLKKKADALQVRFRMILSK

Pg-VATPD IIETKTLMGEVMKEAAFSLAEAKFTTGDFNQVVLQNVTKAQIKIRSKKDN
Ms-VATPD IIETKTLMGEVMKEAAFSLAEAKFTTGDFNQVVLQNVTKAQIKIRSKKDN
Bm-VATPD IIETKTLMGEVMKEAAFSLAEAKFTTGDFNQVVLQNVTKAQIKIRSKKDN

Pg-VATPD VAGVTLPIFESYQDGSDTYELAGLARGGQQLAKLKKNFQSAVKLLVELAS
Ms-VATPD VAGVTLPIFESYQDGSDTYELAGLARGGQQLAKLKKNFQSAVKLLVELAS
Bm-VATPD VAGVTLPIFESYQDGSDTYELAGLARGGQQLAKLKKNFQSAVKLLVELAS

Pg-VATPD LQTSFVTLDEVIKITNRRVNAIEHVIIPRLERTLAYIISELDELEREEFY
Ms-VATPD LQTSFVTLDEVIKITNRRVNAIEHVIIPRLERTLAYIISELDELEREEFY
Bm-VATPD LQTSFVTLDEVIKITNRRVNAIEHVIIPRLERTLAYIISELDELEREEFY

Pg-VATPD RLKKIQDKKKIIKDKAEAAKAAKIAAGSMDIRDVIANILLNEGDDDLLF-
Ms-VATPD RLKKIQDKKKIIKDKAEAKKAALRAAG---QDLRDSANLLDEGDEDLLFM
Bm-VATPD RLKKIQDKKKIIKDKAEAKKAALLAAGN--DLRGGVTNLLDEGDEDLLF-

Pg-VATPD --------------------------------------------------
Ms-VATPD SGKDRLAIFPSRGAQMLMKGRLAGAQKGHGLLKKKADALQVRFRLILSKI
Bm-VATPD --------------------------------------------------

Pg-VATPD --------------------------------------------------
Ms-VATPD IETKTLMGEVMKEAAFSLAEAKFTTGDFNQVVLQNVTKAQIKIRSKKDNV
Bm-VATPD --------------------------------------------------
```

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Pg-VATPD ------------------------------------------------
Ms-VATPD AGVTLPIFESYQDGSDTYELAGLARGGQQLAKLKKNFQSAVKLLVELASL
Bm-VATPD ------------------------------------------------

Pg-VATPD ------------------------------------------------
Ms-VATPD QTSFVTLDEVIKITNRRVNAIEHVIIPRLERTLAYIISELDELEREEFYR
Bm-VATPD ------------------------------------------------

Pg-VATPD ------------------------------------------
Ms-VATPD LKKIQDKKKIIKDKAEAKKAALRAAGQDLRDSANLLDEGDEDLLF
Bm-VATPD ------------------------------------------
```

Figure 4: Alignment between deduced amino acid sequence of *Pectinophoragossypiella* (Pg) V-ATPase subunit D with other published amino acid sequence of the same gene in other insects in NCBI; *M. sexta* (Ms) (AJ251992) and *B. mori* (Bm)(DQ311428). The identical amino acids are shown in the shaded boxes.

dsRNA Injection

The third larval instars were injected by 200 ng dsRNA in between thoracic segments. Three injection experiments were performed targeting the regions 756-1155 and 347-753 for VATPase subunit A and 221-703 fragment of subunit D. Three replicas were used for each treatment. The mortality within the first 24 hours was neglected to avoid handling damage and injection injury that might be occurred during the injection procedure. The larval mortalities were recorded after four, six, eight and ten days from initial injection time. The percentages of larval mortalities after 10 days werescored as 46.3%, 43.9%and 25% for subunits A 756-1155, 347-753 and subunit D 221-703,respectively (**Table2**).

Furthermore, the larvae treated with the two dsRNA fragments targeting V-ATPase A were morphologically compared to the control. Shrinkage of the bodies and slower development of treated larvae (**Figure5**) clearly indicates the starvation effect of treatment with dsRNA. Some of the treated larvae were unable to pupate due to their incapability of normal food absorption. Furthermore, larvae which were able to pupate were less weight and size compared to control individuals (**Figure6**).

Table 2: The effect of dsRNA fragmnetsVATPA756-1155, VATPA347-753andVATPD221-703on the V-ATPase subunit A and D transcripts. The larval mortality was detected throughtwo days intervals for ten days.The larval mortality within the first 24 hours was neglected.

	Total No. of injected larvae	Mortality				
		24 hrs	4 days	6 days	8 days	10 days
			Dead/Alive (%)	Dead/Alive (%)	Dead/Alive (%)	Dead/Alive (%)
Control	120	89	5/84 (5.6%)	5/84 (5.6%)	9/80 (10.1%)	11/78 (12.3%)
V-ATPase A 756-1155	129	69	24/45 (34.7%)	41/28 (40.5%)	31/38 (44.9%)	32/37 (46.3%)
Control	98	67	2/65 (2.9%)	4/63 (5.9%)	8/59 (11.9%)	10/57 (14.9%)
V-ATPase A 347-753	101	66	19/47 (28.7%)	26/40 (39.4%)	27/39 (40.9%)	29/37 (43.9%)
Control	95	72	1/71 (1.3%)	3/69 (4.1%)	4/68 (5.5%)	5/67 (6.9%)
V-ATPase D 221-703	120	68	12/56 (17.6%)	16/52 (23.5%)	17/51 (25%)	17/51 (25%)

Figure 5: Starvation symptoms of the larvae injected with dsRNAs. Control and treated third larval instars were injected in the same days with either buffer or dsRNAs. Larvae injected with dsRNA-free buffer (C) showed normal development and average sizes while larvae injected with dsRNAs (T) showed slower development of larvae and shrinkage of their bodies.

Figure 6: The effect of dsRNAs on larval development. Control pupae (C) had normal size while treated pupae (T) was smaller in size.

DISCUSSION

RNAi occurs widely in eukaryotic organisms and previous studies have demonstrated an induction of RNAi response in several insect species(Tereniu*set al*. 2011). RNAi has proven the capability to be used as a tool in crop protection and its efficacy was recorded against many species belonging to different insect orders. In this study, we demonstrated larval mortality of the lepidopteran *P.gossypiella* when the midgut gene V-ATPase is suppressed by dsRNA.The 3rd larval instars were injected with a concentration of 20ng dsRNA/mg insect weight. This ratio is frequently used in injection experiments because it usually achieves high silencing results (Tereniu*set al*. 2011). Injection of subunit A-specific dsRNA demonstrated moderate larval mortality. On the other hand, lower mortality resulted from dsRNA targeting subunit D. A similar rate of mortality was induced by knockingdown subunits B and C in *P. gossypiella*(Mohammed 2013). The four subunits A, B, C and D belong to the V_1 domain of the PgV-ATPase. Nevertheless, the mortality results due to interfering effect could be assorted into two groups; firstly subunit A with mortality about 46% and secondly lethality ranging between 25 and 31% for B, C, and D subunits. Unfortunately, none of these transcripts was quantified post dsRNA injection so the reduction ratio was not calculated. However, the relatively higher effect of subunit A RNA interference maybe due to its primary role in ATP hydrolysis, while subunit B plays a secondary role. Subunits D and C represent the center and peripheral stalk, respectively(Beyenbachand Wieczorek2006andMa *et al.* 2011). V-ATPase was targeted with either dsRNA or siRNA in different insect species.dsRNA targeting genes encoding V-ATPase subunits A, D and E, are active against*Diabroticavirgiferavirgifera.*

Transgenic corns expressing V-ATPase A-dsRNA conferred root protection from

feeding damage by coleopteran insect pests(Baum *et al*. 2007). Transgenic tobacco lines expressing double strand RNA reduced up to 62% of V-ATPase A transcript level in *Bemisiatabaci* (Thakur *et al.* 2014). However, the percentage of mortality caused by oral feeding of dsRNA was97.5% in *B.tabaci* (Upadahay*et al*. 2011), 27.3:54.5% in*Tetranychusurticae*(Kwon *et al*. 2013) and 35% in *Bactrocera dorsalis* (Li *et al.* 2011a). The silencing effect of V-ATPase A- dsRNA was only 2.5 fold reduction of the transcript in *Aedesaegypti* (Coy *et al.* 2012). Other subunits were also targeted and showed variable RNAi effect. Oral feeding and microinjection of dsRNAs targeting V-ATPase B and D showed significant difference in reduction of the transcripts in*Peregrinusmaidis* (Yao *et al.* 2013). In *M.sexta,* low silencing level was detected due to V-ATPase subunit E dsRNA (Whyard*et al.*2009). No response was observed in *Nilaparvatalugens*after the feeding of V-ATPase E dsRNA (Li *et al.* 2011b).Similar negative response to dsRNAs of V-ATPase subunits A, B, D or H was reported in *Spodopterafrugiperda* (L. M., unpublished data).

The aforementioned significant variability of insects' response towards RNAi of V-ATPase is more likely due to different factors. Kitzmann*et al.* (2013), suggested that this variability could be due to differences in genetic backgrounds .While, Roignant*et al*. (2003) and Miller *et al*. (2008) conferred it to whether RNAi processing machinery exists or not, how the cell uptake dsRNA and allow its propagation of signal.Possible degradation of dsRNA by native enzymes could be another mechanism (Arimatsu*et al*. 2007).

Despite of low mortality achieved in the current study, V-ATPase is a potential target for RNA interference to be deployed in insect control of the *P.gossypiella*, and further investigation is required to enhance its effect.

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