TARGETING THE VACUOLAR ATPase SUBUNITS B AND C IN PINK BOLLWORM, *Pectinophora gossypiella* (Saunders) (Lepidoptera; Gelechiidae)

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Cotton is grown commercially in many countries around the world including Egypt. The pink bollworm (PBW), *Pectinophora gossypiella*, causes yield reduction of cotton with average of 20 to 30 percent (Ahmed, 1980). The first published record of the pest was in 1842 by W. W. Saunders in India and it is believed to have been introduced into Egypt from India about 1906 or 1907 (Pearson, 1958). The larvae burrow into cotton bolls to feed on the cotton seeds destroying the cotton lint. This feeding damage causes secondary infection with other insects and fungi. When the larva exits the cotton boll it leaves a perfectly round and clean cut exit hole which diagnose of pink bollworm damage. In heavy infestations the entire boll may be so damaged that pickers leave it on the plant while partly damaged bolls are picked. The grade and staple of the lint are reduced due to the staining and cut fibers caused by the feeding of the worm.

The vacuolar proton pumps, V-ATPases, are ubiquitous among eukaryotes (Dow, 1999). 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. The V-ATPase holoenzyme generates energy to pump protons across plasma membranes by hydrolyzing ATP molecule to ADP and phosphate (Jefferies *et al*., 2008). V-ATPase is composed of two functional domains, V_1 and V_0. The V_1 domain which is responsible for ATP hydrolysis comprised eight different subunits (A-H), is located on the cytoplasmic side of the membrane. The V_0 domain is the membrane-bound protein, composed of five subunits (a-e) and functions in proton-conductivity (Forgac, 1998). In the midgut of the tobacco hornworm (*Manduca sexta*), they are localized in the apical membrane of goblet cells where they exclusively energize all secondary active transport processes across the epithelium (Wieczorek *et al*., 2000). The disruption of the V-ATPase complex either by selective gene inactivation or dsRNA leads to insect lethal effect as in the fruit fly (Davies *et al*., 1996), the corn plant hopper (Yao *et al*., 2013) and three coleopteran species; western corn root-

worm, southern corn rootworm and Colorado potato beetle (Baum *et al*., 2007).

RNA interference (RNAi) was first discovered in the nematode, *Caenorhabditis elegans* (Fire *et al*., 1998). Later on, it has emerged as a powerful tool for the rapid analysis of gene function and use in biotechnology. Relevant applications include the capacity to inactivate target genes. Direct microinjection is the most commonly used procedure for delivery of double-stranded RNA (dsRNA) into organisms (Bucher *et al*., 2002; Tomoyasu *et al*., 2004). However, different methods have been exploring more simple and convenient means of dsRNA delivery, including soaking (Tabara *et al*., 1998), oral feeding (Turner *et al*., 2006) and transgenic plant expression (Baum *et al*., 2007). RNAi could be deployed as a powerful tool in entomological research and for insect pest management (Zu, 2013). In 2007, two groups made major progress in the exploitation of transgenic plants engineered to express insect dsRNAs for entomological research and field control of insect pests (Baum *et al*., 2007; Mao *et al*., 2007). In the following years more dsRNA-expressing plants were developed resistant to different species of insect pest (Zha *et al*., 2011; Zhu *et al*., 2012; Mao *et al*., 2013; Mao and Zeng, 2013).

In this study, we used RNAi to determine the efficacy of dsRNA on silencing V-ATPase B and C subunits in PBW. The synthesized dsRNA was delivered via injection and we observed increased mortality level of injected larvae compared to control.

MATERIALS AND METHODS

Insect culture

Neonate larvae of pink bollworm (*Pectinophora gossypiella*. Leipdoptera: Gelechiidae) were reared individually in 35 ml glass vial on a synthetic diet at 26°C±2, until pupation. Pupae were collected and allowed to emerge as adult moths in glass jars supplied with 10% sugar solution. The eggs were harvested on filter papers.

First strand cDNA synthesis

The PBW guts were dissected in insect physiological saline according to Ghanim *et al*. (2001). Total RNA was extracted from guts using the Triazol® reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was prepared from the total RNA using the Superscript II cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction.

Cloning and Sequencing of V-ATPase subunits B and C

Degenerate primers were designed on the conserved regions of V-ATPase subunit C sequences published in the Genebank database of; *Bombyx mori* (DQ311199), *Helicoverpa armigera* (AF337638), *Manduca sexta* (AJ249388) and *Drosophila melanogaster* (AF006655). One degenerate primer set

was used to amplify 140 bp fragment; VATPC873F and VATPC1013R (see primer list in Table 1). The template cDNA was denatured at 95°C for 5 min followed by 25 cycles of 95°C for 30 sec and annealing temperature at 55°C for another 30 sec followed by 30 sec extension time at 72°C, the PCR reaction was ended at 72°C for 7min. The PCR product was cloned into a pGEMT-easy vector (Promega, Madison, WI). The cloned fragments were subjected to sequence analysis using the Big TriDye sequencing kit (ABI Applied Biosystems) by the facility of Macrogen, Korea. The VATPC873F was redesigned as specific primer; VATPC873FS and was used with another degenerate reverse primer VATPC1233R to amplify 360 bp fragment.

Subunit B was obtained by designing one degenerate primer set (VATPB705F and VATPB979R) on the conserved region between 705 and 979 bp of the subunit B of the following insects; *H. armigera* (GU370066), *M. Sexta* (X64354), *D. melanogaster* (X67839) and *Spodoptera littoralis* (AY169409). PCR conditions, cloning and sequencing were as mentioned above.

5′ and 3′ Rapid amplification of cDNA ends (RACE)

The full length cDNA of *Pectinophora gossypiell*a V-ATPase (PgV-ATPase) subunits B & C was obtained by identifying both 5' and 3' ends using FirstChoice®RLM-RACE kit (Ambion life technologies, Austin, TX) according to manufacturer's procedures. To amplify the 5'end, 10 µg total RNA treated with both Calf Intestine Alkline Phosphatase (CIP) and Tobacco Acid Pyrophospatase (TAP) was ligated to 5' RACE adaptor for one hour at 37°C. The ligated RNA was then reverse transcribed using M-MLV Reverse Transcriptase for one hour at 42°C. Two rounds of PCR were used to amplify the 5' end of Subunit C; firstly, the 5' RACE outer primer supplied with the kit and specific primer VATPC970R were used for the first round. Secondly, the nested PCR was performed using 5' RACE inner primer and another closer specific primer VATPC912R. The 3'end was essentially synthesized as 5'end procedures but treated RNA was ligated to 3' RACE adaptor using different primer sets. The 3' RACE outer primer supplied with the kit and specific primer VATPC873F were used for the first PCR round. Secondly, the nested PCR was performed using 3' RACE inner primer and another closer specific primer VATPC1074F. The 5' RACE was synthesized for subunit B using VATPB828R and VATPB798R primers for the PCR rounds while VATPB806F and VATPB940F were used to amplify the 3' end. The PCR conditions were standard and the annealing temperatures varied between 57°C and 65°C depending on the primer melting point. The PCR products were cloned into a pGEMT-easy vector and then sequenced.

Sequence Analysis

Annotation, comparison and alignment of sequences were performed using the National Center for Biotechnology Information (NCBI) BLAST search services (Altschul *et al*., 1990) and Vector NTI® software (Life Technologies).

Preparation of dsRNA fragments

Two dsRNA fragments covering 266 and 524 bp were prepared according to the sequence of the PgV-ATPase subunits B and C, respectively. The T7 promoter-containing primers were used to generate transcription template for both strands of the dsRNA. Two fragments were amplified bearing T7 promoter sequences on both ends using primer set VATPB713F/VATPB979R for subunit B and VATPC372F/VATPC896R for subunit C (Table 1). Previously cloned cDNA fragment was used as a template. The template was heated to 95°C for 5 min followed by 95°C for 30 sec, annealing temperature at 60°C for another 30 sec and at 72°C for 30 cycles and ended at 72°C for 7min. The dsRNA fragmens were generated using MEGAscript®RNAi Kit (Ambion) according to manufacturer's instructions. The final dsRNA products were eluted by dd H_2O and stored at -20°C for injection.

dsRNA injection

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 (0.1 mM $NaPO_4$ pH 6.8, 5 mM KCl) to final concentration of 1 µg/µl and used to inject larvae with 0.2 µl between meso and metathoracic segments. Control larvae were injected with dsRNA-free buffer and treated under the same conditions as experimental individuals. Injected larvae were transferred to the diet. Larvae died within the first 24 hours were removed and excluded from the experiments. The mortality was recorded five days post injection.

RESULTS AND DISCUSSION

Sequence analysis of the PgV-ATPase cDNAs B and C

The sequence of the entire cDNA corresponding to the PgV-ATPase was obtained by sequencing DNA fragments resulted from PCR reactions using cDNA prepared from midgut tissue as the template and degenerate primers covering most of the ORF as well as by 5'- and 3'-RACE. Full length sequence of subunit C is 1835 nucleotides containing an open reading frame of 1145 nucleotides (Fig. 1) coding for 382 amino acids. Calculation of the protein's molecular mass and its isoelectric point (pI) revealed values of 43.58 kDa and pH 8.46, respectively. The deduced protein is 91.5% identical and 94.8% similar to the silkworm C; it was also 89.9% identical and 94.3% similar to the tobacco hornworm C (Fig. 2).

The sequence of transcript encoding subunit B is 1701 nucleotides contain-

ing 1014 nucleotides of open reading frame (Fig. 3). The encoded protein is 490 amino acids in length with approximate molecular weight of 53.75 kDa and pI of 5.25. The deduced protein shows 89.1 and 88.3% identical to the silkworm and the cotton bollworm, respectively (Fig. 4). Peptide sequence motifs were identified using the PROSITE data base. The conserved motif "PPVNVLPSLS" is present at 370 as in other B subunits in different insects. It is thought that this motif is essential for ATPase function (Davies *et al*., 1996).

dsRNA injection

In the dsRNA bioassays, PBW third instar larvae were injected with 200 ng dsRNA targeting genes encoding V-ATPase subunit B or C in separate experiments. The mature larvae are about 12.7 mm long. Therefore, a new syringe adaptor kit specialized for nerve injection was used to avoid sever damage of injected larvae. The larval mortality within 24 hours post-injection ranged between 20% and 28% within control and experiment, this is more likely due to injection and handling processes. Therefore, the ceased larvae within this period were excluded from the assay and the larvae were monitored for 120 hours. The dsRNAs targeting the V-ATPase B and C, each caused a relative larval mortality of 30.77% and 25.68%, respectively (Table 2). On the other hand, the mortality rate of control larvae was below 5%. Larvae were kept on diet to observe larval development and pupation (Fig. 5). The low mortality levels of PBW larvae were unexpected. Only one dsRNA fragment was used for each subunit, additional dsRNA covering different transcript region(s) may confer more effective target(s). Another reason, small sized-larvae may be problematic for successful injection because some of the dsRNA-buffer is extruded after injection reducing the amount of injected dsRNA.

A major problem of using RNAi approach against insects is that adequate amount of dsRNA is needed to effectively block the targeted expression, since dsRNA itself cannot replicate inside the insects (Kumar and Sarin, 2013).

Different studies demonstrated the feasibility of crop protection using RNAi against wide range of potential targets for gene suppression in agricultural pests. The first knock-out of V-ATPase in an animal (*Drosophila melanogaster*) was developed by Davies *et al*. (1996). The *vha55* gene encoding the V-ATPase subunit *B* was identified in the fruit fly and was mutated with *P* element insertions. Deletion of the *B* subunit locus was shown to be lethal, whereas point mutations gave varying phenotypes that ranged from lethal to surviving flies. Baum *et al*. (2007) screened a total of 290 dRNAs against western corn rootworm (WCR), this number was reduced to 67 that showed significant mortality within WCR larvae. Of these, 14 dsRNAs including dsRNA targeting genes encoding V-ATPase subunit A, D and E, were the most active relative to their median lethal concentration (LC_{50}) values. Moreover, WCR dsRNA targeting subunit

A and E demonstrated significant mortality against other two coleopteran species, southern corn rootworm and Colorado potato beetle. Transgenic corn expressing V-ATPase A-dsRNA showed root protection from WCR feeding damage. Yao *et al*. (2013) conducted different delivery methods of RNAi in the corn planthopper, *Peregrinus maidis*, using oral feeding and microinjection of dsRNAs targeting V-ATPase B and D. Quantitative real-time PCR revealed reduction of 27-fold of V-ATPase transcripts two days post injection, while ingestion of dsRNA resulted in two fold reduction after six days of feeding (Yao *et al*., 2013).

The effectiveness of RNAi is promising as a new tool in crop-protection strategies. Identification of target genes and utilizing these multiple targets will soon lead to the application of RNAi-based technologies in insect pest management.

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SUMMARY

The vacuolar proton pump, V-ATPase, is located in the apical cell membranes of the goblet cells within the midgut of lepidopteran larvae. It plays a role in amino acid absorption, by energizing the plasma membrane through pumping H^+ ions/proton into the goblet lumen. The full transcripts of V-ATPase subunit B and C were sequenced from the midgut of pink bollworm larvae. We used RNAi to determine the efficacy of dsRNA on silencing genes encoding B and C subunits. Larval injection with dsRNAs targeting subunits B and C caused larval mortality of 30.77% and 25.68%, respectively.

REFERENCES

Ahmed, Z. (1980). Incidence of major cotton pests and diseases in Pakistan with special reference to pest management. Proceeding of First International Consultation on Cotton Production Research with focus on the Asian Region. Manila, Philippines, 17-21 November, 156-179.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, (1990). Basic local alignments search tool. J. Mol. Biol., 215: 403-410.

Baum, J. A., T. Bogaert, W. Clinton1, G. R. Heck1, P. Feldmann, O. Ilagan, S. Johnson, G. Plaetinck, T. Munyikwa, M. Pleau, T. Vaughn and J. Roberts (2007). Control of coleopteran insect pests through RNA interference. Nat. Biotechnol., 25: 1322-1326.

Bucher, G., J. Scholten and M. Klingler (2002). Parental RNAi in *Tribolium* (Coleoptera). Curr. Biol., 12: R85-R86.

Davies, S. A., S. F. Goodwin, D. C. Kelly, Z. Wang, M. A. Sözen, K. Kaiser and J. A. T. Dow (1996). Analysis

and inactivation of *vha55*, the gene encoding the vacuolar ATPase B-subunit in *Drosophila melanogaster* reveals a larval lethal phenotype. J. Biol. Chem., 271: 30677-30684.

Dow, J. A. T. (1999). The Multifunctional *Drosophila melanogaster* V-ATPase is encoded by a multigene family. J. Bioenetgetics and Biomembranes, 31: 75-83.

Fire, A., S. Q. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabtidis elegans*. Nature, 39: 806-811.

Forgac, M. (1998). Structure, function and regulation of the vacuolar (H+)-ATPases. FEBS Lett., 440: 258-263.

Ghanim, M., S. Morin and H. Czosnek (2001). Rate of tomato yellow leaf curl virus translocation in the circulative transmission pathway of its vector, the whitefly *Bemisia tabaci*. Phytopathology, 91: 188-196.

Jefferies, K. C., D. J. Cipriano and M. Forgac (2008). Function, structure, and regulation of the vacuolar (H+)-ATPases. Arch. Biochem. Biophys, 476: 33-42.

Kumar, A. and N. B. Sarin (2013). RNAi: A promising approach to develop transgenic plants against geminiviruses and insects. J. Plant Physiol. Pathol., 1: 1-6.

Mao, Y. B., W. J. Cai, J. W. Wang, G. J. Hong, X. Y. Tao, L. J. Wang, Y. P. Huang and X. Y. Chen (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat. Biotechnol., 25: 1307-1313.

Mao, Y. B., X. Y. Xue, X. Y. Tao, C. Q. Yang, L. J. Wang and X. Y. Chen (2013). Cysteine protease enhances plant-mediated bollworm RNA interference. Plant Mol. Biol., 83: 119-129.

Mao, J. and F. Zeng (2013). Plant-mediated RNAi of a gap gene-enhanced tobacco tolerance against the *Myzus persicae*. Transgenic Res. [Epub ahead of print].

Pearson, E. O. (1958). The insect pests of cotton in tropical Africa. Empire Cotton Growing Corp. and Common wealth Inst. of Ent. London, pp 355.

Tabara, H., A. Grishok and C. C. Mello (1998). RNAi in C. elegans: Soaking in the genome sequence. Science, 282: 430-431.

Tomoyasu, Y. and R. E. Denell (2004). Larval RNAi in *Tribolium* (Coleoptera) for analyzing adult development. Dev. Genes Evol., 214: 575-578.

Turner, C. T., M. W. Davy, R. M. MacDiarmid, K. M. Plummer, N. P. Birch and R. D. Newcomb (2006). RNA interference in the light brown apple moth, *Epiphyas postvittana* (Walker) induced by double-stranded RNA feeding. Insect Mol. Biol., 15: 383-91.

Wieczorek, H., G. Grber, W. R. Harvey, M. Huss, H. Merzendorfer and W. Zeiske (2000). Structure and regulation of insect plasma membrane H(+)V-ATPase. J. Exp. Biol., 203: 127-35.

Yao, J., D. Rotenberg1, A. Afsharifar, K. Barandoc-Alviar1 and A. E. Whitfield (2013). Development of RNAi Methods for *Peregrinus maidis*, the Corn Planthopper. PLoS One., 8: e70243.

Zha, W., X. Peng, R. Chen, B. Du, L. Zhu and G. He (2011). Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the hemipteran insect *Nilaparvata lugens*. PLoS One, 6: e20504.

Zhu, K. Y. (2013). RNA interference: A powerful tool in entomological research and a novel approach for insect pest management. Insect Science, 20: 1-3.

Zhu, J. Q., S. Liu, Y. Ma, J. Q. Zhang, H. S. Qi, Z. J. Wei, Q. Yao, W. Q. Zhang and S. Li (2012). Improvement of pest resistance in transgenic tobacco plants expressing dsRNA of an insect-associated gene EcR. PLoS One, 7: e38572.

Table (1): A listing of oligonucleotide primers that were used in cloning, RACE and dsRNA preparation.

Primer	Sequence
Cloning primers	
Subunit C	
VATPC873F	5'-GACAAGAAGAAGCANTTYGGDCC-3'
VATPC1013R	5'-CTGGAAGTTHACVGGCARHCC-3'
VATPC873FS	5'-GACAAGAAGAAGCATTTTGGTCCG-3'
VATPC1233R	5'-ACRTASGGGWARTAYTCSGAYTGRCC-3'
Subunit B	
VATPB705F	5'-GCTATGGGTGTBAAYATGGARAC-3'
VATPB979R	5'-GGRAAACCACGWCGHCCDGGHAC-3'
RACE primers	
Subunit C	
VATPC970R	5'-ACGCGGAGAGCTTTGACATGTATCC-3'
VATPC912R	5'-ACCTTCAGCCATCGCACCAGC-3'
VATPC873F	5'- GACAAGAAGAAGCATTTTGGTCCG-3'
VATPC1074F	5'-CTCTACGCGCACCTCGACCACT -3'
Subunit B	
VATP B828R	5'- GATGAATTCTCTCGATGGTGGGA-3'
VATPB798R	5'-CCAAGTTCAAGAACAGGCACACG-3'
VATPB806F	5'-TCCCACCATCGAGAGAATTCATC-3'
VATPB940F	5'-CCGCCGCCCGTGAGGAGGTA-3'
dsRNA primers	
Subunit C	
VATPC372F	5’-TAATACGACTCACTATAGCGCTTTCAGTGGGACATGGCC-3’
VATPC896R	5'- TAATACGACTCACTATAGACCTTCAGCCATCGCACCAGC-3’
Subunit B	
VATPB713F	5'-TAATACGACTCACTATAGTGTAAATATGGAAACTGCTCGG-3'
VATPB979R	5'-TAATACGACTCACTATAGGGAAACCACGTCGCCCGG-3'

Table (2): The effect of dsRNA fragemnets on the V-ATPase subunits B & C transcripts. The larval mortality was detected 120 hours post injection.

Control/ Sub-unit	Total number of injected lar-vae	Number of larvae after 24 hours	Dead/ Alive after 120 hours	Mortality
Control	72	52	2/50	3.85%
V-ATPase C	97	74	19/55	25.68%
Control	102	81	1/80	1.23%
V-ATPase B	98	78	24/54	30.77%

GAAACCGCATCACGTGAATTTTCATGGCGCTGAGGGGCTAGTCCCGATTGTCTTGTGAT
TTCACCAAATTACAGCTGTTTTGAATTATTTGTTGTTGAACAGCACCAAATTATAAGTT
TTGGTGAAAATGTCAGAATACTGGTTGATTAGCGCCCCTGGCGACAAAACTTGCCAGCA
GACATGGGACAACCTGAACAATGCCACCAAATCTGGCAACCTCAGCGTCAACTACAAAT
TCCCAATCCCAGACTTGAAGGTGGGGACTCTGGACCAGCTGGTGGGGCTGTCTGATGAC
CTCGGCAAGTTGGACACCTTCGTCGAGAGCGTCACTCGGAAGGTCGCGCAATATCTCGG
AGAGGTTCTCGAAGATCAACGCGACAAGCTTCATGAGAACTTGATGGCAAATAACAGCG
ACCTGCCGACATACCTCACCCGCTTTCAGTGGGACATGGCCAAGTATCCCATCAAGCAG
AGCCTCCGTAACATCGCTGATATCATCAGTAAACAGGTGGGTCAAATTGACGCCGACCT
GAAATCCAAGCCTTCGGCCTACAACGCACTCAAGGGCAACCTACAAAACTTGGAGAAGA
AACAAACTGGAAGCCTGTTGACCCGCAACCTAGCGGACTTGGTGAAGAAGGAGCACTTC
ATTCTGGACAGCGAGTACCTCACCACGCTGCTCGTCATCGTGCCCAAGTCGATGTTCAA
TGAGTGGAACGTCAACTACGAGAAGATCACCGACATGATCGTGCCTCGCTCCACGCAGC
TCATCTATCAGGACAACGACTACGGGCTCTACAGCGTCACGCTCTTCAAGAAGGTGGCA
GACGAGTTCAAGCTACACGCTCGTGAGCGCAAGTTCGTCGTGCGTGAGTTCTCGTACAA
TGAAGCTGACTTGGCCGCCGGCAAGAATGAGATCACCAAGTTGGTCACCGACAAGAAGA
AGCAGTTCGGTCCGCTGGTGCGATGGCTGAAGGTGAACTTCTCCGAGTGCTTCTGTGCC
TGGATACATGTCAAAGCTCTCCGCGTGTTCGTGGAGTCGGTGCTAAGATACGGTCTGCC
CGTCAACTTCCAGGCGGCGGTGCTGGTCCCGTCGCGCAAGAGCATGAAGAAGCTGCGCG
ACGTGCTGCACTCGCTCTACGCGCACCTCGACCACTCCGCCAACGCCGGCGCACAGGCC
GAGGGCGCGGAGCTGGCGGGGCTCGGGTTCGGGCAGTCGGAGTACTTCCCGTACGTGTT
CTACAAGATCAACATCGACATGATCGAGAAGGCTTAAGCTAGCGTCGCCACGACACGCA
TACATAGGTAAATGTTACCCAGGCCTTGAACATGCGGCCGCGCCGCTAGCCATACGCAA
TAATATCCTGTACATCTTACTTTACTCCAGCCAATCAATTTATAATTTATTTTCTCCCA
CACCTTTGTAAAATAAAATCAATAATTTCCGACAACTATATTTTTCTATGTACAGATTT
TTTGAAATTTTATTTTAATTATATTTTTTCGTGTTTAACTAATCGATGCAGGTTGTTTG
TTACATAAATGTATTTATGTATATTATTTATCAATAAGCAGTTTGAATAGAAATTTTGA
TTAATGTTTGGTGCGCTTTCACATTTTTCTTTAAACGCAACAATCCTTTCAGTATTATG
TGTTGCGACTAGAGTAGTGTAATATACGTTATAGCCACCAGTTGAAGCCTGTATTATAT
AAATGTTCAATAAATATATTGGAGTTATGAAATGCAAAAGCTATGTAAATAAGAACATT
CCTCATTATAACTCGTGTAGTACATAATAAAAATATTATTGAAGTTACTGTCAAAAAAA
AAAGAA

Fig. (1): Full length of PgV-ATPase subunit C mRNA for *P. gossypiella*. The full sequence is 1835 nucleotides, both initiation and termination codons are black shaded .

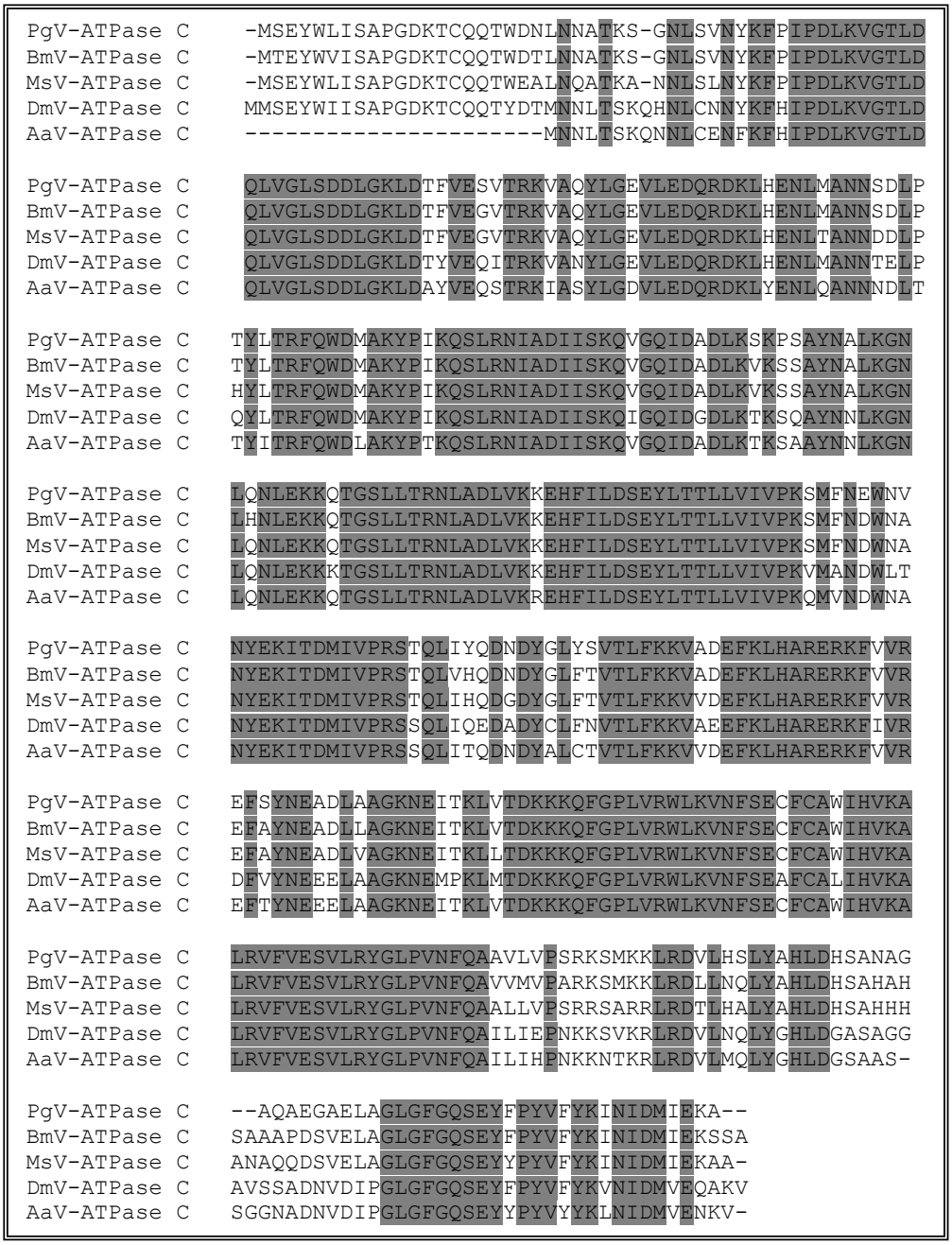

Fig. (2): Deduced amino acid sequence of PgV-ATPase subunit C cDNA and alignment with number of insect V-ATPases subunit C; *Bombyx mori* (Gene Bank™ accession number NP_001040138) (BmV-ATPase C), *Maduca sexta* CAB55498 (MsV-ATPase C), *Drosophila melanogaster* AAB62571 (DmV-ATPase C) and *Aedes aegypti* ABF18462 (AaV-ATPase C). The identical amino acids are shaded in black boxes.

CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATGAAATTGAGCGGACTGCCAAAGAGT
GAAAACTGAAAAATGGCAGATAGCATGGGAGCAAGACAGGCTGCCAGGGAGCATGTTTT
GGCTGTCTCCAGGGATTTCATATCGCAGCCGCGATTGACCTACAAGACAGTATCTGGTG
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CAACTGAAGCTTGCCGATGGCACGCTCCGTTCCGGTCAGGTGCTGGAGGTCAGCGGCTC
CAAGGCCGTGGTGCAGGTGTTCGAGGGCACCTCCGGTATCGACGCCAAGAACACGCTCT
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ATCTTCTCAGCCGCTGGGTTGCCGCATAACGAAATTGCCGCGCAGATCTGTCGTCAGGC
CGGCCTCGTCAAGCTCCCAGGCAAGGGCGTGCTGGACTCGCACGAAGACAACTTCGCCA
TCGTATTCGCGGCCATGGGTGTCAACATGGAGACTGCTCGGTTCTTCAAGCAGGACTTC
GGAGAGAATGGCTCCATGGAGAACGTGTGCCTGTTCTTGAACTTGGCCAACGATCCCAC
CATCGAGAGAATCATCACCCCACGTCTCGCTTTGACCGCTGCTGAGTTCTTGGCTTACC
AGTGTGAGAAACACGTGCTGGTAATCTTGACTGACATGTCTTCGTACGCCGAGGCCCTG
CGTGAGGTGTCCGCCGCCCGTGAGGAGGTACCCGGGCGACGTGGTTTCCCAGGTTACAT
GTACACCGATTTGGCCACCATCTACGAGCGCGCTGGGCGAGTGGAGGGCCGCAACGGGT
CCATCACCCCAGATCCCCATCCTGACTATGCCCAATGACGACATCACCCCATCCCGTCC
CCGATTTGACTGGTTACATTACTGAGGGACAGATCTACGTAGATCGTCAGCTGCACAAC
AGACAGATCTACCCTCCGGTGAACGTGCTCCCGTCCCTGTCTCGTCTCATGAAGTCCGC
CATCGGCGAGGGCATGACCCGCAAGGACCACTCCGACGTCTCCAACCAGCTGTACGCGT
GCTACGCCATCGGCAAGGACGTGCAGGCGATGAAGGCCGTGGTGGGTGAGGAGGCGCTC
ACGCCCGACGACCTGCTCTACCTCGAGTTCCTCACCAAGTTCGAGAAGAACTTCATCAC
CCAGGGCTTTTGTGACCGGGACTGCACCCTGGTTGTCGACCGACCAGCAGAAGTCACGA
CTTGCCGCTCTGCCGCTGCTACACCCCTACTGCTGTCCACCATCGGGGCCGTTTTGGAG
TTCGTCGCGCTCCGACCACGGTAGGGAAGTGGTACTCCTTTTGGAAGTGTTTCAAGTGA
CCCTCAAAGTCTCGATCGAAAACTCGATCTCCACAACCTCGAGTTGTTCTACCGTGATT
TCTCTGGTTCTTGATAATAATATAAGTTACTTTGTTACAAAAAAAAAA

Fig. (3): Full length of PgV-ATPase subunit B mRNA for *P. gossypiella*. The full sequence is 1701 nucleotides, both initiation and termination codons are black shaded .

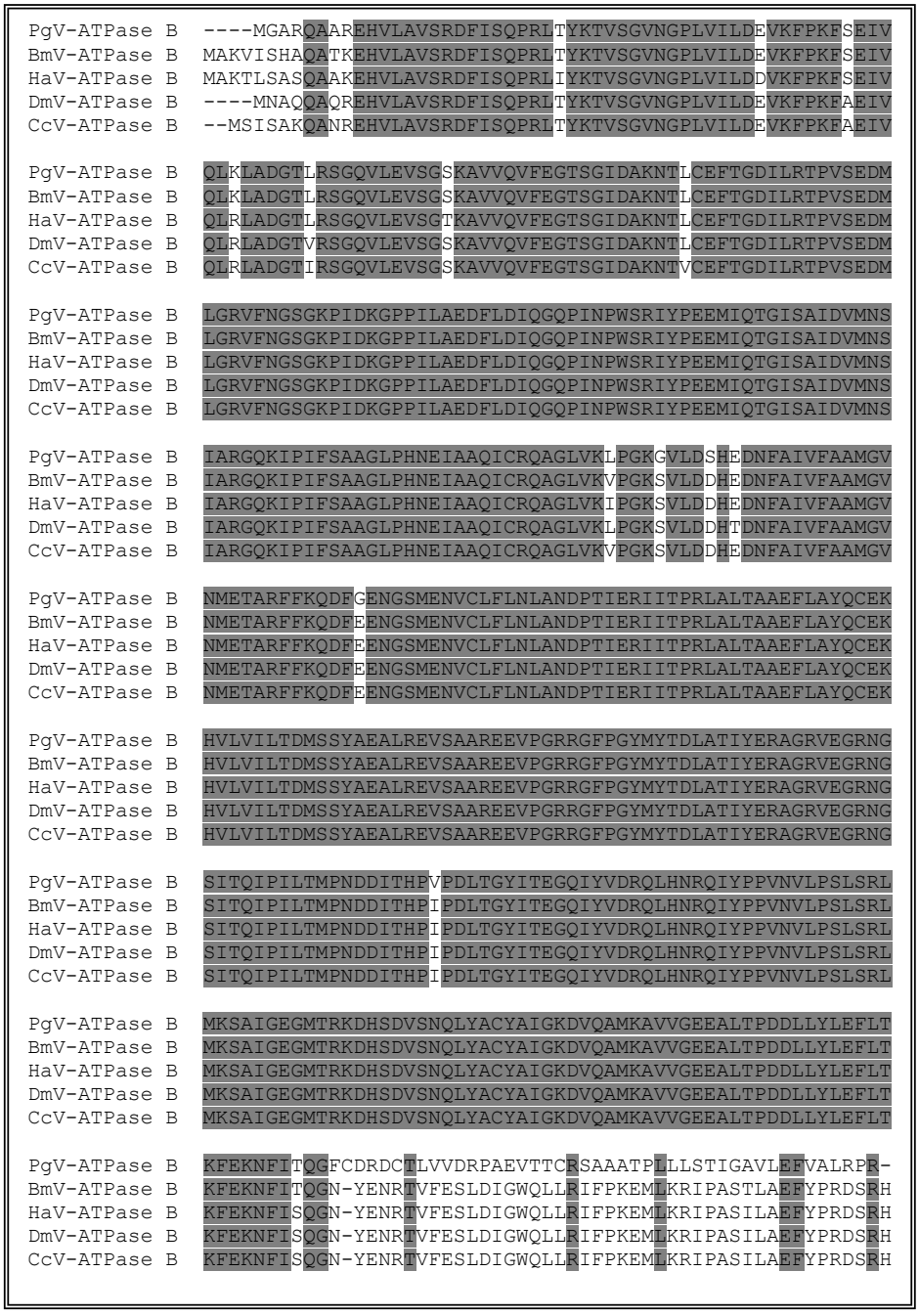

Fig. (4): Deduced amino acid sequence of PgV-ATPase subunit B cDNA and alignment with number of insect V-ATPases subunit B; *Bombyx mori* (Gene Bank™ accession number ACE78271) (BmV-ATPase C), *Helicoverpa armigera* GU370066 (HaV-ATPase B), *Ceratitis capitata* XP_004523388 (CcV-ATPase B) and *Drosophila melanogaster* AAF54837 (DmV-ATPase B). The identical amino acids are shaded in black boxes.

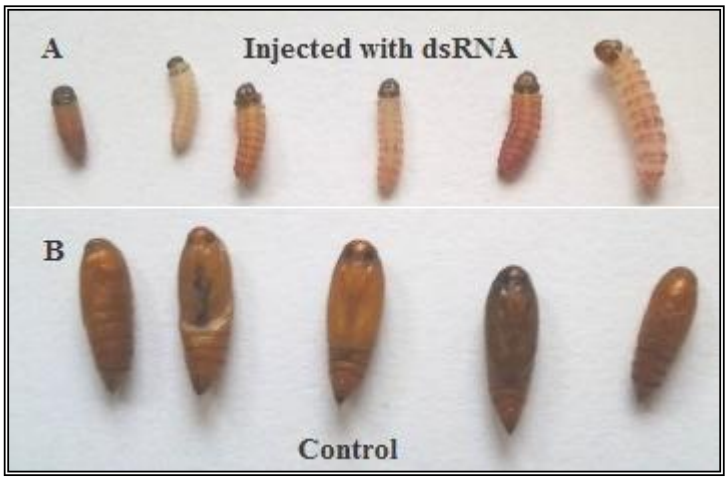

Fig. (5): Bioassay results after 15 days of injection. A) Larva injected with 200 ng dsRNA targeting genes encoding V-ATPase subunit showing symptoms of starvation. B) Puape resulted from larvae injected with buffer.