Characterization of the First Aquaporin Gene from the Egyptian Cotton Leafworm, *Spodoptera littoralis*

Shimaa M. El-Gamal¹, Sawsan Y. Elateek², S. A. Ibrahim² and Sayed M. S. Khalil¹,³

¹Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Egypt.
²Department of Genetics, Faculty of Agriculture, Ain Shams University, Egypt.
³Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia.

Aquaporins (AQP) are integral membrane proteins belong to the Major Intrinsic Proteins (MIP) superfamily identified in different tissues from all living organisms including mammals, plants, invertebrates and microorganisms. Only few AQP isolated and studied from terrestrial insects. In the present study, we report the cloning of a partial cDNA sequence of AQP1 gene from *Spodoptera littoralis*. The cDNA is 1148bp with an open reading frame of 684bp encoding for 227 amino acids with similarity of 98% and 76% to *Spodoptera litura* and *Grapholita molesta*, respectively. Protein structure analysis showed the presence of conserved features found in all studied AQP such as six transmembrane domains, two conserved motifs of NPA, and the aromatic/arginine (ar/R) selectivity filter. Our study revealed that AQP1 gene is expressed in all insect developmental stages (eggs, larvae, pupae, and adults including males and females). Furthermore, AQP1 expression was detected in tissues of the digestive and excretory system (foregut, midgut, hindgut and Malpighian tubules) of fourth, fifth and sixth larval instars. Gene expression was also detected in the reproductive system of adult females and males. Therefore, we suggest that this AQP1 gene has critical role in maintaining water balance in *Spodoptera littoralis* insects.

**Keywords:** *Spodoptera littoralis*, Lepidoptera, aquaporin, gene expression, water homeostasis.

**INTRODUCTION**

Water regulation is one of the critical physiological mechanisms in all living organisms as water supports all the biochemical processes within the living cells. So it is important to maintain water balance during all developmental stages. Water regulation is maintained by some hormones (diuretic and antidiuretic hormones), their receptors and water channels (aquaporins). Aquaporin proteins are integral membrane proteins belong to the major intrinsic proteins superfamily. They are expressed in different tissues such as Malpighian tubules, hindgut, fat bodies, ovaries, olfactory organs and others. Aquaporins make the membrane 10 to 100 fold more permeable to water than membranes lacking such channels (Krane and Kishore, 2003). Some aquaporins are water-specific and others are aquaglyceroporins that increase glycerol and urea uptake in hindgut in addition to water transport. The first aquaporin was identified from blood cells by Agre et al., (1993), then followed by a number of different AQP which have been identified in all kinds of tissues of mammals, invertebrates, plants, and microorganisms (Gomes et al., 2009). Aquaporin proteins have highly conserved structure among kingdoms (Bansal and Sankararamakrishnan, 2007). Electron crystallography of human AQP1 revealed that aquaporins assemble in tetramer groups (four
identical protein channels) through the cell membrane producing central water impermeable pore (Ren et al., 2000). Each monomer contains six transmembrane α-helices connected by five loops (A, B, C, D and E); three of them (A, C, and E) are extracellular while loops B and D are cytoplasmic (Walz et al., 1997; Cheng et al., 1997 and Li et al., 1997). The N and C termini of AQP1 proved to be intracellular (Smith and Agre, 1991; Nielsen et al., 1993). All MIPs including AQPs have two highly conserved amino acid motifs, Aspargin-Proline-Alanine (NPA) in loops B and E (Park et al., 1996). Jung et al., (1994) proposed the hourglass structural model of AQPs. They suggested that loops B and E may associate physically at the opposite sides of the channel within the membrane bilayer forming the central pore through which water molecules can pass. In insects, water permeability has been investigated deeply in blood sucking (Elvin et al., 1999 and Duchesne et al., 2003) and sap sucking insects (Le Cahérec et al., 1996). AQPcic of the green leafhopper (Cicadella viridis) was the first insect AQP to be isolated from agricultural pest. It was characterized functionally and defined as water selective AQP (Le Cahérec et al., 1996). Regarding lepidopteran insects, some AQPs were isolated and characterized from only five species; Bombyx mori, Spodoptera litura, Grapholita molesta, Ectropis oblique, and chilo suppressalis (Kataoka et al., 2009b; Azuma et al., 2012; Liu et al., 2013; Li et al., 2016 and Lu et al., 2018), most of these AQPs were characterized as water selective channels. Only two AQPs were characterized as aquaglyceroporins, AQP-Bom2 and AQP-Gra2 from B. mori and G. molesta, respectively (Kataoka et al., 2009a and Kataoka et al., 2009b). Spodoptera litoralis (boisduval, 1833) belongs to order Lepidoptera. According to the European and Mediterranean Plant Protection Organization (EPPO 1997), Sp. littoralis is considered one of the most harmful pests in Egypt and Mediterranean basin. It has a wide host range of at least 87 plant species belonging to over 40 plant families. The most harmful state is in Egypt as the insect is active all the year and feeds on and causes damage for many crops. To date, only little is known about Sp. littoralis on the molecular level and no aquaporin genes were isolated from it. Therefore, the aim of this study is to isolate the gene coding for aquaporin1 and to study its expression in different tissues and developmental stages.

MATERIALS AND METHODS

Spodoptera littoralis (Egyptian cotton leafworm) colony:

Sp. littoralis larvae were reared to pupation at 26±1°C and 60% RH. Larvae were fed castor bean leaves (Ricinus communis) and adults were fed 10% sugar solution and provided with leaves of Nerium oleander for oviposition (El-Defrawi et al., 1964 and Elbarky et al., 2008).

cDNA cloning of AQP1 gene:

Fourth instar Sp. littoralis larvae were dissected, hindgut and Malpighian tubules were used to isolate total RNA using SV Total RNA Isolation System (Promega, Madison, WI, USA). RQ1 RNase-Free DNase (Promega) was used in treatment of 1µg RNA to remove any DNA residues. First strand cDNA was prepared using the Superscript II cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Degenerate primers were designed based on conserved regions of AQP1 genes isolated from other lepidoptran insects and deposited in the GenBank database (Table 1). These primers were used to amplify the target region of AQP1 gene in a standard PCR conditions using the first strand cDNA as a template. PCR product was cloned into pGEM-T Easy vector (Promega) and transformed into DH10β chemically competent cells (Invitrogen). Following bacterial overnight growth and plasmid DNA purification using Wizard Plus SV Miniprep kit (Promega), the cloned fragment was subjected to sequence analysis by the facility of Macrogen Korea (Seoul, Republic of Korea). A new pair of gene-specific primers (Table 1) was designed based on the sequence of initial fragment and was used to obtain the 3’RACE region of AQP1 gene using cDNA as a template. 3’ end was synthesized using First Choice® RLM-RACE kit (Ambion, Austin, TX, USA). PCR product was treated as explained before. The resulted sequences were assembled together using Contig Express utility of Vector NTI Advance 11 (Invitrogen) to obtain the partial Sp. littoralis AQP1 cDNA sequence.

Sequence and phylogenetic analysis:

The sequence of Sp. littoralis AQP1 cDNA was compared with other sequences deposited in GenBank using the “BLASTN” and “BLASTX” tools at the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Gene coding for aquaporin1 and its expression in different tissues and developmental stages.

Table 1: Oligonucleotides used in AQP1 cloning, AQP1 3’RACE and gene expression analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligo Name</th>
<th>Oligo Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP1 Cloning primers</td>
<td>SI-AQP1/F1</td>
<td>CCGACAAACAAGCTGATYTG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td></td>
<td>SI-AQP1/R1</td>
<td>GCRGGGACGGGCTGATG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>AQP1 RACE primers (3’-end)</td>
<td>SI-AQP1/3’F01P</td>
<td>TCGGTCTCTTTGTGGTCTCT</td>
<td>Macrogen</td>
</tr>
<tr>
<td></td>
<td>SI-AQP1/3’F11P</td>
<td>TCAAGGTCTCTGAGGCAAC</td>
<td>Macrogen</td>
</tr>
<tr>
<td>AQP1 Expression primers</td>
<td>AQP1-Ex/F</td>
<td>CCTTCAGCTGTGTCCA</td>
<td>Eurofins</td>
</tr>
<tr>
<td></td>
<td>AQP1-Ex/R</td>
<td>GAGGGTTAGACGATGTG</td>
<td>Eurofins</td>
</tr>
<tr>
<td></td>
<td>SI-28s/F3</td>
<td>GAGAGTGCAGCGCCTAGTGG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td></td>
<td>SI-28s/R5</td>
<td>CGCACTATGGCGTAGACGTA</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Alignments of nucleotide and deduced amino acid sequences with selected AQPs from other lepidopteran insects were done using T-Coffee Multiple Sequence Alignment Tools (http://www.tcoffee.org/Projects/tcoffee/index.html #DOWNLOAD). Box shading of conserved regions was done using Sequence Manipulation Suite(http://www.bioinformatics.org/sms2/color_align_cons.html). Transmembrane helices were predicted using TMHMM (v2.0) (Möllel et al., 2001, http://www.cbs.dtu.dk/services/TMHMM-2.0/) software. The phylogenetic tree was constructed based on amino acid sequences using the neighbor-joining method in MEGA X software (Kumar et al., 2018, www.megasoftware.net).

-Developmental and Tissue specific expression:

The expression of AQP1 gene was tested in different developmental stages (eggs, all larval instars, pupae and adult males and females). Tissue specific expression of AQP1 gene was tested in foregut, midgut, hindgut, and Malpighian tubules dissected from 4th, 5th and 6th instars. Testes and ovaries were dissected from adult males and females and were also tested for gene expression. Total RNA from all dissected tissues were isolated and tested by RT-PCR for AQP1 gene expression using gene specific primers (Table 1). 28s ribosomal RNA was used as positive control in all RT-PCR reactions. 28s gene specific primers were designed based on 28s sequence of Sp. littoralis (GenBank Acc. No. HQ178645, Kergoat et al., 2012).

RESULTS AND DISCUSSION

-AQP1 cDNA isolation and sequencing:

Total RNA, isolated from hindgut and Malpighian tubules of 4th instars Sp. littoralis, and AQP1 degenerate primers were used to isolate an initial AQP1 fragment of 510bp in length. The obtained fragment was sequenced and its identity was verified as AQP1 by BLASTX and BLASTN on the GenBank database. This fragment was used to design 3’RACE primers to obtain the 3’ end of AQP1 gene (~1.1kb). Sequences of the initial fragment and the 3’ region were assembled together to obtain a partial cDNA sequence of AQP1 gene from Sp. littoralis of 1148bp (GenBank Acc. No. KX943612). The nucleotide and deduced amino acid sequences are shown in Fig. 1. This partial sequence includes 684bp open reading frame encodes for 227amino acid, TAG stop codon and 464bp 3’untranslated region (UTR). Sp. littoralis AQP1 sequence has 98% similarity to Sp. litora AQP1 variants A and B (Acc. No. AGT50942 and AGT50943), 79% similarity to B. mori (Acc. No. BAD69569) and 76% similarity to G. molesta (Acc.no BAH47554) on the amino acid level.

Structural and Phylogenetic analysis:

Deduced amino acid structure analysis revealed that Sp. li AQP1 protein includes all conserved features of the water selective AQPs such as the six transmembrane α-helices, five interhelical loops (three extracellular and two cytoplasmic). Fig. 2. shows the conservation of this structure in aquaporins from different insects belonging to orders Lepidoptera (Sp. litora, B. mori. and Ch. Suppressalis) and Diptera (Aedes aegypti and Eurosta solidaginis) using the TMHMM2.0 software. Two highly conserved Asn-Pro-Ala motifs and the aromatic/arginine selective filter for water permeability (phenylalanine F39, histidine H163, serine S172, and arginine R178) were detected and showed in Fig.3. The amino acids of the selectivity filter suggest that this protein is mostly water specific AQP as it has the same amino acids in the selectivity filter of structurally confirmed water selective aquaporin such as Chilo suppressalis AQP1, B. mori AQP-Bom1, and Grapholita molesta AQP Gra-1 (Lu et al., 2018, Azuma et al., 2012 and Kataoka et al., 2009b).
Figure 1. Nucleotide and deduced amino acid sequence of isolated AQP1 gene partial sequence. Gray boxes show the conserved Asn-Pro-Ala signature motifs, red underlines show the six transmembrane domains, green underline shows the conserved SYDF motif at the end of C-terminus and red dots show the TAG stop codon of the AQP1 protein.

Figure 2. Predicted structure of *Sp.litoralis* AQP1 and some selected AQPs identified from other insects using TMHMM-2.0. *Sp.li* AQP1 topology shows 6 transmembrane domains present in all AQP1 proteins (red color). Blue lines represent the intracellular domains while pink lines represent the extracellular ones. *Sp.li*: *Spodoptera litoralis* (APY26693), *Sp. lu*: *Spodoptera litura* (AGT50942), Bom-1: *Bombyx mori* AQP-Bom1 (BAD69569), Ch.sp: *Chilo suppressalis* (AFC34081), Ae.ae: *Aedes aegypti* (AAF64037) and Es.so: *Eurosta solidaginis* (ACT34032).
El-Gamal et al., Gene coding for aquaporin1 and its expression in different tissues and developmental stages.

Figure 3. Amino acid alignment of Sp.Ii AQP1 with some selected AQPs from other lepidopteran insects showing the two conserved Asn-Pro-Ala signature motifs (NPA, red underlined). The water selective aromatic/arginine sites indicated with red arrows. Identical residues are shown in black background while conserved residues are shown in grey background. Alignment was done with T-Coffee Multiple Sequence Alignment Tools: http://www.tcoffee.org/Projects/tcoffee/index.html#DOWNLOAD. Box shading of conserved regions done using Sequence Manipulation Suite: http://www.bioinformatics.org/sms2/color_align_cons.html. Sp. Ii AQP: Spodoptera littoralis (APY26693), G. molesta: Grapholita molesta (BAH47554), B. mori: Bombyx mori (BAD69569), Sp. litura var A: Spodoptera litura variant A (AGT50942), Sp. litura var B: Spodoptera litura variant B (AGT50943), Ch. suppressalis var A: Chilo suppressalis variant A (AFC34081) and Ch. suppressalis var B: Chilo suppressalis variant B (AFC34082).

The structure of water selectivity filter was studied intensively in vertebrates and found to be Phe, His, Cys and Arg and in aquaglyceroporins amino acids are Trp, Gly, Phe and Arg (Fu et al., 2000). The water selectivity filter of water specific aquaporins isolated from different invertebrates has the conserved residues Phe, His, Ala/Ser and Arg where alanine or serine replaces cysteine found in vertebrate aquaporins. In lepidopteran insects and in the present study, water specific aquaporins were found to have serine residue (Sui et al., 2001 and Lu et al., 2018). Phylogenetic analysis of Sp. Ii AQP1 partial protein sequence with respect to AQPs from some lepidopteran (Sp. litura AGT50942 and AGT50943, Chilo suppressalis AFC34081 and AFC34082, and B. mori BAD69569), dipteran (Aedes aegypti AAF64037 and Drosophila melanogaster AFA35131) and Coleoptera (Tribolium castaneum CM000285) insects is shown in Fig.4.

Analysis showed that lepidopteran AQPs are divided into two clusters. Sp. Ii AQP1 protein clustered with AQP1 variants A and B from Sp. litura while the Ch. Supressalis AQP1 variants A and B were closer to B. mori AQP 1 and clustered together in different branch as shown before by Lu et al., (2018). Aquaporins from Diptera (Aedes aegypti and Drosophila melanogaster) and Coleoptera (Tribolium castaneum) were closer to each other than other insect orders and clustered together in a third branch.

Developmental and Tissue expression of AQP1:
Aquaporins play critical roles in water homeostasis in living organisms, excretion process in insects and maintaining the appropriate aqueous environment during egg maturation (Spring et al., 2009 and Maruyama et al., 2015). To investigate AQP1 importance for Sp. littoralis,
total RNA isolated from all developmental stages (eggs, 1st, 2nd, 3rd, 4th, 5th, and 6th instars larvae, pupae, male and female adults) was used to test AQP1 expression by RT-PCR (Fig.5). Obtained data showed that the transcript of Sp. li AQP1 gene was detected in all developmental stages studied but with different levels of expression based on the obtained bands intensities. Expression was low in eggs and 1st larval instars, increased in 2nd, 3rd and 4th instars then decreased again in 5th and 6th instars. Expression stayed low in pupae and then increased again in adult stages (male and female). Similar results were found in C. suppressalis where AQP1 expression was detected in all developmental stages but with different levels (Lu et al., 2018). They found the highest expression level in third instars and the lowest level in 4th instars larvae and male pupae. We also investigated the Sp. li AQP1 gene expression in different tissues of digestive and excretory systems (foregut, midgut, Malpighian tubules and hindgut) in 4th, 5th, and 6th instars larvae, and in the reproductive tissues (ovaries and testes) in adult females and males. RT-PCR results revealed that AQP1 gene expression detected in all tested tissues but with different band intensities, which might indicate different levels of expression in different tissues (Fig.6).

Figure 4. Phylogenetic analysis of Sp. li AQP1 partial protein sequence with AQP from selected lepidopteran (Lep), dipteran (Dip) and coleopteran (Col) insects. Tree was constructed with Neighbor-joining method using MEGA X software. Spodoptera littoralis (APY26693), Spodoptera litura variant A (AGT50942) and variant B (AGT50943), Chilo suppressalis variant A (AFC34081) and variant B (AFC34082), Bombyx mori (BAD69569), Aedes aegypti (AAF64037), Drosophila melanogaster (AFA35131) and Tribolium castaneum (CM000285) insects.

Figure 5. Developmental expression profile of Sp.li AQP1 gene. RNA was isolated from different developmental stages; 1st, 2nd, 3rd, 4th, 5th, 6th larval stages, pupa (P), adult Male (M), adult Female (F), eggs (E) and negative control has 1µl water as template (-ve). 28s mRNA was detected in all stages used.
El-Gamal et al., Gene coding for aquaporin1 and its expression in different tissues and developmental stages.

Figure 6. Tissue expression profile of Sp.l AQP1 gene. RNA was isolated from different larval tissues. F: foregut, M: midgut, MT: Malpighian tubules, H: hindgut, Ov: ovaries, tes: testes and –ve: negative control has 1µl water as template. 28s mRNA was used as positive control on Sp. littoralis aquaporin genes, their number, function and level of expression in different developmental stages and tissues to understand how important terrestrial pest such as Sp. littoralis regulates its water balance.

CONFLICT OF INTEREST
The present study was performed in absence of any conflict of interest.

ACKNOWLEDGEMENT
The authors would like to thank Dr. AMA Mohammed, Agricultural Genetic Engineering Research Institute, for his help and support.

AUTHOR CONTRIBUTIONS
All authors contributed equally in all parts of this study.

REFERENCES
Agre, P., Preston, G. M., Smith, B. L., Jung, J. S.,


Smith, B. L. and P. Agre (1991). Erythrocyte Mr 28,000 Transmembrane protein exists as a multisubunit oligomer similar to channel proteins. J. Biol. Chem. 266:6407-6415