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ORIGINAL ARTICLE

Characterization, expression analysis and RNAi-mediated knockdown of two aquaporin genes in the cotton leafworm, *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Abstract. Aquaporins (AQPs) are integral membrane proteins that can transport water and small molecules across cell membranes in organisms. In a previous report, we identified an AQP (SlAQP1) gene of the cotton leafworm, *Spodoptera littoralis*. In the current study, we identify and characterize two more SlAQP genes and their developmental and tissues expression. Predicted amino acid sequence and phylogenetic analysis revealed that SlAQP2 is a glycerol channel belonging to the insect specific Eglp subfamily, whereas SlAQP3 is a water specific channel belonging to the Prip subfamily. *SlAQP2* expression was detected mainly in the first four larval instars and adult males, whereas *SlAQP3* was detected in all developmental stages. *SlAQP2* gene expression was mainly detected in larval midgut and Malpighian tubules and adult male testes, whereas *SlAQP3* was detected in all the tissues tested. RNAi mediated knockdown of each gene separately resulted in deleterious effects including larval and pupal mortality, deformed pupae and adults and prolonged development. Results were confirmed using qRT-PCR, which revealed downregulation of both genes after injection of larvae with gene specific dsRNA. Our results confirm the presence of Prip and Eglp AQPs in *S. littoralis* and suggest that Eglp has a role in male reproductive ability.

INTRODUCTION

Aquaporins (AQPs) transport water and other small solutes such as glycerol and urea, gases such as NO and CO_2, metals and ions such as sodium and silicon ions through cell membranes (Laloux et al., 2018; Kourghi et al., 2019). First AQP channel was identified and characterized for human red blood cells (Preston & Agre, 1991; Agre et al., 1993) and followed by the isolation of hundreds of AQPs from different organisms including mammals, plants, arthropods and microorganisms (Gomes et al., 2009).

AQPs are small molecular weight proteins (~30kDa) with a conserved structure in all organisms (Jung et al., 1994; Heymann & Engle, 1999). AQPs exist in cell membranes as tetramers in which each unit serves as a separate water channel. An AQP channel consists of six alpha transmembrane helices (transmembrane domains, TMDs) connected by five inter-helical loops (A, B, C, D and E). Three loops (A, C and E) are extracellular while the other two (B and D) as well as amino and carboxy termini are cytoplasmic (Cheng et al., 1997; Li et al., 1997; Waltz et al., 1997). Loops B and E contain two highly conserved hydrophobic amino acid motifs; Asparagine-Proline-Alanine (NPA). Within the cell membrane, the protein folds and loops B and E protrude and are embed halfway in the cell membrane opposite each other where the two NPA motifs meet and form the pore through which water and other small molecules move. A second region, the aromatic/arginine (ar/R) selectivity filter, is located extracellularly close to the entrance of the pore and forms the narrowest part of the channel, which has a role in selecting solutes. The ar/R region is formed by four amino acids distributed over TMD2 (1 acid), TMD5 (1 acid) and loop E (2 acids). These four amino acids determine the type of channel, whether water selective AQP or glycerol permeable (aquaglyceroporin, Glp).

The first AQP identified in insects is the *Drosophila melanogaster* big brain (Bib), which has a function in the nervous system of both embryonic and adult stages and has a role in cell adhesion (Rao et al., 1992; Tatsumi et al., 2009). Bib is not permeable to water but transports monovalent cations (Yanochko & Yool, 2002). Interest in studying insect AQPs started with plant sap sucking and blood sucking insects, as these insects imbibe large amount of fluids during feeding. The first AQP isolated for a sap

sucking insect was the AQPcic of the green leafhopper, *Cicadella viridis*, which is functionally characterized as a water selective AQP (Le Cahérec et al., 1996). On the other hand, the first AQP isolated for a blood sucking insect is the AqpBF1of the adult buffalo fly, *Haematobia irritans exigua*, which is also characterized as a water selective AQP (Elvin et al., 1999). After this a number of AQPs were identified and characterized for different insects such as *Aedes aegypti* (Pietrantonio et al., 2000), *Rhodnius prolixus* (Echevarría et al., 2001), *Acheta domesticus* (Spring et al., 2007), *Polypedilum vanderplanki* (Kikawada et al., 2008) and a few others. Recently, genomics and transcriptomics studies facilitated and accelerated the identification of many AQPs in different pest and model insects such as *Ae. aegypti* (Drake et al., 2010), *Bactericera cockerelli* (Ibanez et al., 2014), *Lygus hesperus* (Fabrick et al., 2014), *Cimex lectularius* (Tsujimoto et al., 2017), *Tribolium castaneum* (Yao et al., 2018) and many others.

Finn et al. (2015) analysed phylogenetically AQPs isolated from insects and those identified in insect genomes and reported the presence of six major subfamilies of AQP; Prip (water and urea channels), Drip (water specific channel), Bib (cation channel), Glp (water and glycerol channel), Eglp (water and glycerol channel, insect specific) and AQP12L (related to vertebrate AQP12).

In Lepidoptera, AQPs are characterized for a few species (*Bombyx mori*, *Grapholita molesta*, *Spodoptera litura* and *Ectropis obliqua*) (Lu et al., 2018). Recently, Ahmed & Kim (2019); Lu et al. (2018, 2019, 2021) reported and characterized some AQPs for important agricultural pests, such as *S. exigua* and *Chilo Suppressalis*. In a previous report, a water restricted AQP (SlAQP1) gene for the cotton leafworm, *S. littoralis* is reported (El-Gamal et al., 2018).

S. littoralis is a wide spread lepidopteran pest in Africa, Middle East and also occurs in southern Europe. *S. littoralis* is a polyphagous insect that attacks more than 80 host plants including vegetables, field crops, ornamentals and orchards. Larvae feed on leaves, young shoots, flowering buds, fruit and other plant parts causing sever loss of yield (https://www.cabi.org/isc/datasheet/51070, visited October 2021).

In this report, we present the full-length sequence of two new AQP genes identified for *S. littoralis*. We reveal their protein structure and compare it with that of other insect AQPs. We studied their tissue-specific, sex-specific and developmental expression. The effect of RNAi on both genes, insect growth and survival were studied and results of RNAi confirmed using qRT-PCR.

MATERIALS AND METHODS

Insect colony

S. littoralis larvae were reared to pupation in plastic containers with castor bean leaves as food. Pupae were transferred to a 1-gal plastic container containing sawdust and covered with one layer of cheesecloth. Adults were fed 10% sugar solution and provided with leaves of *Nerium oleander* for oviposition (Elbarky et al., 2008). The whole colony was kept at 26 ± 1°C, 60% RH and 14L : 10D cycle.

Table 1. Nucleotide sequences of primers used in cloning, RACE, dsRNA synthesis and real time PCR analysis.

Primer	Sequence 5'–3'
Cloning primers	
AQP3-Clon-F	ATCAAAAACGCGCRACAGYCGGCT
AQP3-Clon-R	GAGAATCCRTGSACGTACA
3' RACE primers	
AQP3-Outer	GCTCGTCGCCAACGTGTGGA
AQP3-Inner	CATAGCGGCTGCACTATTGT
Expression analysis	
AQP2-Exp-F	ACGGAACTGAAGATGACAC
AQP2-Exp-R	GGACTTACTTCCCCAGATTACTG
AQP3-Exp-F	GTCTCGATGAGCTGACTGGA
AQP3-Exp-R	GAGAACGAATCCGAGGAAGAA
28S-Exp-F	GAGAGTGCAGCCCTAAGTGG
28S-Exp-R	CGCACTATGGCGTAGACGTA
dsRNA primers	
AQP2-ds-F	TAATACGACTCACTATAGGGGCCATCGATTCCCCAAATACT
AQP2-ds-R	TAATACGACTCACTATAGGGGTGGGTATATGGTTAAGTTA
AQP3-ds-F	TAATACGACTCACTATAGGGCCTAGGTCTGGCTTCAAC
AQP3-ds-R	TAATACGACTCACTATAGGGCCGAGCGTCACCGTCAGG
AMPF	TAATACGACTCACTATAGGGCCCAGTGCTGCAATGATACC
AMPR	TAATACGACTCACTATAGGGTTCTGACAACGATCGGAGGAC
qRT-PCR primers	
AQP2-qpcr-F	GAAGATGACACAGGATGA
AQP2-qpcr-R	ACGAGCATCATTGTGGAT
AQP3-qpcr-F	CGTGTGAAGATTGCGCGT
AQP3-qpcr-R	AGGGTTCCAGCAGCTCGG
28S-qpcr-F	AGGGGTAAACCTGCGAAACT
28S-qpcr-R	CGGGTCGCGATGTATTTACT

cDNA cloning of *SlAQP3* and transcriptomic identification of *SlAQP2*

Hindgut and Malpighian tubules of fourth instar larvae (L4) of *S. littoralis* were dissected and used to isolate total RNA using the SV Total RNA Isolation System (Promega, Madison, WI, USA). RQ1 RNase-Free DNase (Promega) was used to treat 1 µg 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 instructions. Degenerate cloning primers (Table 1) were designed based on conserved regions of AQP3 genes isolated from other lepidopteran insects and deposited in GenBank. These primers were used to amplify the target region of AQP3 gene in 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 sequenced by Macrogen Korea (Seoul, Republic of Korea). 3' RACE was done using 3' RACE primers (Table 1) and First Choice® RLM-RACE kit (Ambion, Austin, TX, USA) to obtain the 3' end. PCR products were treated as explained before. The sequences were assembled using ContigExpress utility of Vector NTI Advance 11 (Invitrogen) to obtain the full-length *SlAQP3* cDNA sequence.

SlAQP2 sequence was obtained from a transcriptome made for the whole body of the fifth instar larvae (L5) of *S. littoralis* (unpubl. data).

Sequences and phylogenetic analysis

The sequence of *SlAQP2* and *SlAQP3* cDNAs were compared with other *AQP* sequences in GenBank using the ''BLASTN'' and ''BLASTX'' tools at the National Center for Biotechnology Information (NCBI) web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments of nucleotide sequences and deduced amino acid sequences were done using Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The molecular weight (MW) and isoelectric point (pI) were determined using Compute pI/Mw tool (https://web.expasy.org/compute_pi/). The

deduced amino acid sequence was scanned for motifs against the PROSITE database (http://prosite.expasy.org). Possible signal peptides were searched for using SignalP-5.0 server (http://www.cbs.dtu.dk/services/SignalP/). Transmembrane helices were predicted using CCTOP server version s.1.00 (http://cctop.enzim.ttk.mta.hu/). Phosphorylation sites were detected using NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/). Glycosylation sites were identified using NetNGLyc 1.0 software (http://www.cbs.dtu.dk/services/NetNGlyc/) and the YinOYang program (http://www.cbs.dtu.dk/services/YinOYang/). The phylogenetic tree was constructed using the neighbour-joining method in MEGA version 7.0.

Developmental and tissue-specific expression

The expression of SlAQP2 and SlAQP3 genes was determined at different developmental stages (eggs [2 g], all six larval instars [20 larvae for L1–L2 and 5 larvae for L3–L6], pupae and adult males and females [5 each]). Tissue-specific expression of *SlAQP2* and *SlAQP3* was recorded in foregut, midgut, hindgut and Malpighian tubules dissected from L4, L5 and L6 (10 larvae each). Testes and ovaries were dissected from adult males and females (10 adults each) and were also tested for gene expression. Total RNA samples from different developmental stages and dissected tissues were isolated and cDNAs were synthesized as explained in the cDNA cloning of *SlAQP3* and transcriptomic identification of *SlAQP2* section. *SlAQP2* and *SlAQp3* expression was determined using PCR and synthesized cDNAs using gene specific primers (Table 1). PCR included 1 µl cDNA, 1 µl of each primer (10 pmol/µl) and 10 µl of 5 × FIREPol Master Mix (Solis BioDyne, Tartu, Estonia) in a final volume of 50 µl. The thermocycling conditions were one cycle at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, annealing for 30 s (based on the optimal temperature for each primer pair) and 72°C for 2 min. *28S* ribosomal RNA was used as a positive control in all RT-PCR reactions. *28S* gene specific primers were designed based on the *28S* sequence for *S. littoralis* (GenBank Acc. No. HQ178645).

dsRNA synthesis

The dsRNA fragments were obtained by in vitro transcription using MEGAscript® RNAi Kit (Invitrogen) according to manufacturer's instructions. A unique target region of each gene was selected after alignment of *SlAQP* gene sequences including *SlAQP1* (KX943612, El-Gamal et al., 2018) and gene specific primers designed (Table 1). Ampicillin resistance gene specific dsRNA (dsAmpR) was prepared using AMPF and AMPR primer set (Table 1) to be used as a control. Each primer contains the T7 promoter sequence necessary for dsRNA synthesis (TAATACGACTCACTATAGGG) at the 5′ end. The dsRNA was eluted in ddH_2O, quantified using a spectrophotometer and stored at –20°C till injection.

Bioassay of dsRNA fragments

The effect of RNAi was evaluated by injecting 1 µg of each AQP gene dsRNA (*SlAQP2* and *SlAQP3*) into the haemocoel of larvae using a Hamilton Syringe model SYR75N (Hamilton, Reno, Nevada, USA). Synchronized day 1 L3 larvae were injected once with dsRNA dissolved in 1 µl physiological saline solution. Larvae were kept on ice to reduce their movements before injection. A group of 50–60 day 1 L3 larvae was injected in each treatment. Three replicates for each dsRNA were used and each replicate was injected on a different day. Control larvae were injected with 1 µg of dsAmpR. Each injected replicate was placed in a new cup and kept at the same rearing conditions as the main colony. Larvae that died within 24 h post-injection were removed and not counted. Mortality was recorded every day during the larval and pupal stages. Adult emergence was recorded every day until the emergence of the last adult and the total number of adults emerging in each treatment was calculated.

Quantitative real time PCR (qRT-PCR)

SlAQP2 and *SlAQP3* mRNA levels were quantified using qRT-PCR of *S. littoralis* larvae injected with 1 µg dsRNA specific to each AQP gene. Injection of dsRNA was done as explained in the previous section. The RNA was extracted from three to five injected larvae 24, 48, 72 and 96 h post-injection. The extracted total RNA was quantified and cDNA was synthesized using SuperScript II cDNA synthesis kit (Invitrogen) according to the manufacturer's instruction. qRT-PCR was carried out using a final volume of 20 µl containing 10 µl Maxima SYBR Green qPCR master mix (Thermo scientific cat no. K0251), 1 µl cDNA and 10 µM each gene specific forward and reverse primers (Table 1). *S. littoralis 28S* gene was used as a reference control for normalizing gene expression data (*28S* specific primers are listed in Table 1). qRT-PCR reactions were carried out in Mx3000P Real-Time Thermocycler (Stratagene, La Jolla, CA, USA). The amplification conditions were: 10 min at 94°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The reaction was followed by dissociation curve analysis by heating at 95°C for 60 s; 55°C for 30 s and a 0.2°C increase per cycle till 95°C. There were three technical replicates for each biological replicate.

Statistical analysis

Larval and pupal mortality, adult emergence and relative gene expression were analysed using a one-way analysis of variance with $\alpha = 0.05$ and Fisher's least significant difference test to determine significant differences between means using Statistix 8.1 statistical software (Analytical Software, Tallahassee, FL, USA). For the qRT-PCR results, relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

RESULTS

SlAQP2 and *SlAQP3* sequence analysis

Two new AQPs of *S. littoralis* were identified in this study; *SlAQP2* and *SlAQP3*. *SlAQP2* (GenBank Acc. No. MN883562) was identified from a transcriptome generated from L5 larvae of *S. littoralis*. *SlAQP2* cDNA consists of 5,354 bp with 759 bp open reading frame (ORF) coding for 253 amino acids (Fig. 1). The length of the 5' untranslated region (UTR) is 175 bp while that of 3' UTR is relatively long; 4,420 bp including the TAA stop codon. *SlAQP3* cDNA sequence (GenBank Acc. No. MN883561) was identified by sequencing 2 DNA fragments obtained in PCR reactions using cDNA prepared from hindgut and Malpighian tubules of L4 larvae as the template. Initial RT-PCR using degenerate primers produced a 894 bp fragment. Analysis of this fragment showed a 5' UTR, a start codon and ORF belonging to *AQP3*. Another fragment of 1,340 bp was obtained using 3' RACE. *SlAQP3* obtained by compiling fragments is 2,134 bp including an ORF of 807 bp, coding for 269 amino acids, 227 bp 5' UTR and 1,100 bp 3' UTR including the TGA stop codon. No signal peptide was detected in the predicted amino acid sequences of both SlAQP2 and SlAQP3. Calculated molecular weight (MW) and isoelectric point (pI) of SlAQP2 protein are 27.588 kDa and 6.80, respectively, while those for the SlAQP3 protein are 27.112 kDa and 6.50. Domain search and comparison with other AQPs revealed the presence of six transmembrane domains (TMD1–6), five inter-domain loops (A–E),

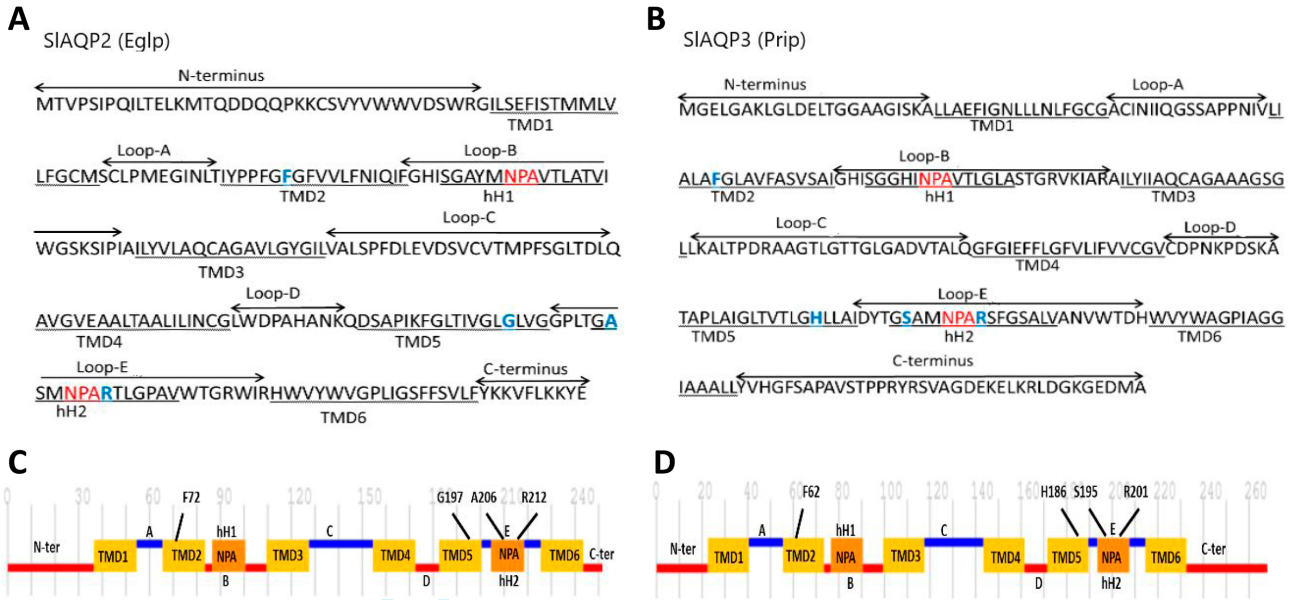

Fig. 1. Predicted amino acid sequence of SlAQP2 (A) and SlAQP3 (B) showing different protein domains. Amino acids of the NPA motifs are shown in red font while those of the ar/R selectivity region are shown in blue font. Predicted topology of SlAQP2 (C) and SlAQP3 (D) obtained from CCTOP online software showing different AQP domains. Yellow rectangles represent the transmembrane domains, orange squares the hemihelices carrying the NPA motifs, horizontal blue lines the extracellular loops and the horizontal red lines the intracellular loops as well as the N- and C-termini. The four amino acids of each ar/R selectivity region and their locations are shown. C-ter – C-terminus, hH – hemihelix, N-ter – N-terminus, TMD – transmembrane domain.

two NPA domains found on loops B and E, and the ar/R selectivity region (Fig. 1). N-terminus, C-terminus, loops B and D are intracellular and loops A, C and E are extracellular. Loops B and E contain hemi helices each carrying an NPA domain that come together in the cell membrane to form a pore. The ar/R region of SlAQP2 consists of the four amino acids F72-G197-A206-R212, indicating a glycerol channel whereas that of SlAQP3 consists of F62-H186-S195-R201, indicating a water specific channel. A total of 16 possible phosphorylation sites were detected in SlAQP2 at positions 2(T), 5(S), 11(T), 16(T), 26(S), 40(S), 90(Y), 99(T), 104(S), 132(S), 140(S), 144(T), 213(T), 220(T), 229(Y) and 240(S), and 17 in SlAQP3 at positions 14(T), 21(S), 50(S), 91(S), 92(T), 103(Y), 122(T), 129(T), 133(T), 170(S), 193(T), 195(S), 202(S), 213(T), 244(T), 248(Y) and 250(S). Only one N-glycosylation site was detected for SlAQP2 at position 63(N) with 3 O-glycosylation sites detected at positions 5(S), 96(T) and 204 (T). On the other hand, no N-glycosylation sites were detected for SlAQP3 while 8 O-glycosylation sites were detected at positions 49(S), 86(T), 92(T), 170(S), 193(T), 213(T), 243(S) and 244(T).

The comparison of the amino acid sequence of SlAQP2 with that of other AQPs from the GenBank revealed the highest similarity is to AQP2 (Eglps) of lepidopteran insects, such as *S. litura* XP_022825447 (99%), *S. frugiperda* API68430 (91%) and *Heliothis virescens* PCG62502 (72%). SlAQP3 has 100% similarity to *S. litura* XP_022823668, 92% similarity to *H. virescens* PCG79886 and 91% similarity to *Helicoverpa armigera* XP_021196925. Phylogenetic analysis of different AQP protein sequences from other insects revealed six clusters of AQPs representing the six AQP types found in insects. Each AQP protein clustered in a different branch with SlAQP2 clustered with Eglps and SlAQP3 with Prips from other lepidopteran insects confirming their identities (Fig. 3).

Developmental and tissue-specific expression

Using RT-PCR and gene specific primers, the developmental and tissue specific expression of *SlAQP2* and *SlAQP3* were recorded in all developmental stages (eggs and whole body of the six larval instars, pupae, male and female adults). Expression was also recorded in internal tissues including foregut, midgut, hindgut and Malpighian tubules of larvae, testes and ovaries of adults. *SlAQP2* expression was detected in whole body RNA extracted from all larval instars with the highest expression recorded in L3 and lowest (not seen in the gel picture) in L5 and L6 (Fig. 4A). *SlAQP2* expression was detected in the whole body of adult males but not adult females, pupae and eggs (Fig. 4A). Regarding tissue expression, that of *SlAQP2* was detected in tissues from the last three larval instars, especially in the midgut and Malpighian tubules (Fig. 4B). *SlAQP2* expression was detected in adult male testes but not in adult female ovaries (Fig. 4B). *SlAQP3* expression was detected in all developmental stages including eggs, larvae (six instars), pupae, adult males and females, especially in the first 4 larval instars (Fig. 4A). *SlAQP3* expression was detected in foregut, midgut, hindgut and Malpighian tubules of the last three larval instars with a relatively low expression in the foregut (Fig. 4B). *SlAQP3* was detected in both ovaries and testes, especially in ovaries (Fig. 4B).

Bioassay of dsRNA

To study the effect of RNAi-mediated knockdown of AQPs, L3 larvae were injected with 1 µg of each of the

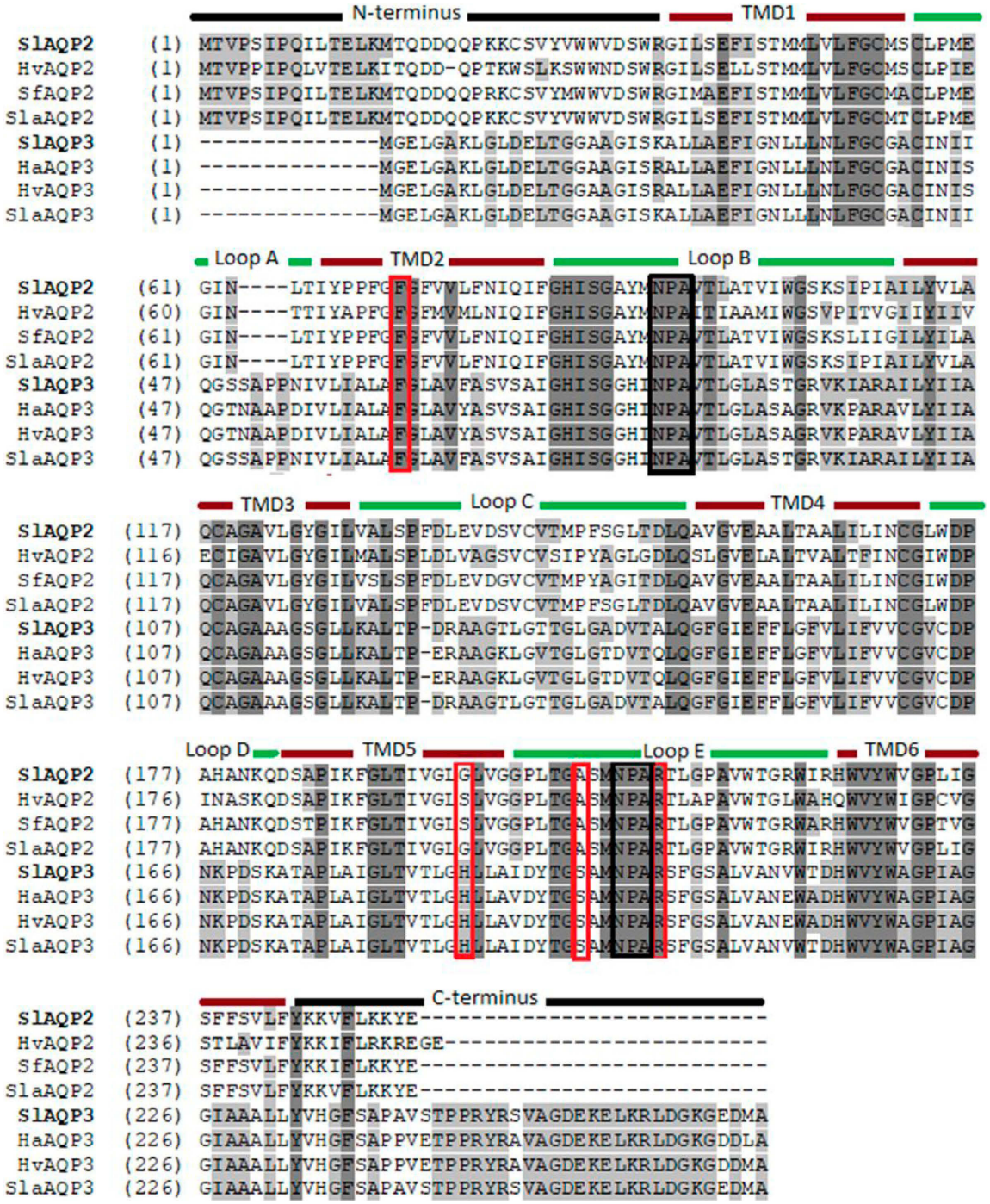

Fig. 2. Amino acid sequence alignment of the *Spodoptera littoralis* aquaporins (SlAQP2 and SlAQP3) with selected AQP2 and AQP3 proteins of other Lepidoptera. NPA motifs are shown in black frames while the four amino acids of the ar/R selectivity region are shown in red frames. Note the presence of uncharged amino acid (G or S) in TMD5 of AQP2 (Eglp) and a charged amino acid (H) at the corresponding position in AQP3 (Prip). Ha – *Helicoverpa armigera*, Hv – *Heliothis virescens*, Sf – *Spodoptera frugiperda*, Sl – *Spodoptera littoralis*, Sla – *Spodoptera litura*, HaAQP3 – XP_021196925, HvAQP2 – PCG62502, HvAQP3 – PCG79886, SfAQP2 – API68430, SlaAQP2 – XP_022825447, SlaAQP3 – XP_022823668.

AQP gene dsRNA (*SlAQP2* and *SlAQP3*) and *AmpR* as a control. Mortality was recorded every day in larval and pupal stages. AQPs dsRNA caused a higher mortality, which differed significantly from that due to dsAmpR (Fig. 5). dsSlAQP2 treatment resulted in 19.6% ± 3.266 and 11.08% ± 1.41 mortality while dsSlAQP3 resulted in 14.1% ± 3.6 and 6.85% ± 2.41 mortality in larval and pupal stages, respectively (Fig. 5A). dsAmpR mortality (6.2% ± 0.667 for larvae and 3.1% ± 1.0 for pupae) was lower than that due to both *SlAQP2* and *SlAQP3*. Adult emergence was recorded every day until the emergence of the last adult when the total adult number and emergence period were recorded. Total percentage that emerged as adults in the *SlAQP2* treatment was 53.53% ± 11.77 and in the *SlAQP3* treatment 59.72% ± 10, both of which are lower than from the *AmpR* treatment (81.38% ± 2.08).

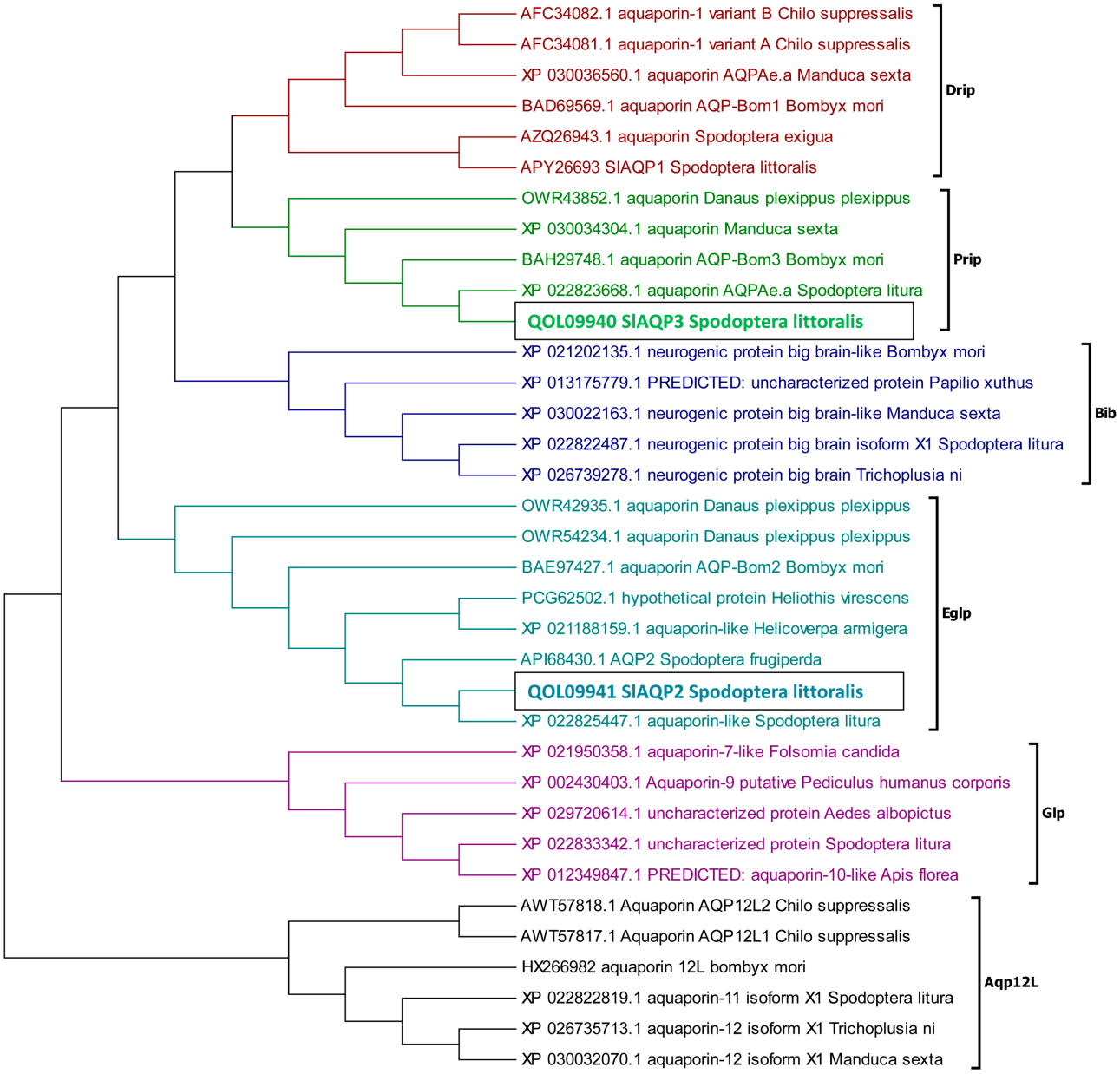

Fig. 3. Phylogenetic analysis of AQP proteins from insects showing the main six AQP classes present in insects. SlAQP3 clustered with Prip proteins while SlAQP2 clustered with the insect specific Eglp proteins of other insects. Tree was generated using MEGA 7.0 and neighbour-joining method based on amino acid sequences.

Emergence period also differed between treatments. In the *AmpR* treatment, adults started to emerge d17 post-injection and continued for 6 days. For the *SlAQP2* treatment, adult emergence started on d20 post-injection and continued for 7 days while for the *SlAQP3* treatment it started on d18 and continued for 9 days (Fig. 5B).

Quantitative real time PCR (qRT-PCR)

The time course effect of dsRNA injection on the level of *SlAQP* gene expression was analysed using qRT-PCR and *28S* mRNA to normalize the data (Fig. 6). The qPCR analysis indicated a reduction in the expression of the *SlAQP* gene 24 h post-injection, which lasted for up to 4 days. The expression of *SlAQp2* decreased significantly to 0.85 ± 0.0025 on d2, then decreased gradually to 0.36 ± 0.14 on d4 (Fig. 6A). Although the decrease in the expression of *SlAQP3* was not significant up to two days post-injection, it decreased from 0.71 ± 0.36 on d2 to 0.29 ± 0.07 on d4 (Fig. 6B).

DISCUSSION

AQPs are integral membrane proteins that have a vital function in the transport of water and small molecules through cell membranes in organisms (Gomes et al., 2009). In this study, we report the identification of two new AQP genes of the cotton leafworm, *S. littoralis*, a polyphagous lepidopteran pest that attacks more than eighty plant hosts. Structural analysis of both SlAQP2 and SlAQP3 proteins revealed features common to all known AQPs, such as: two standard NPA motifs, one ar/R restriction region, six TMDs, five inter domain loops and cytoplasmic N- and C-

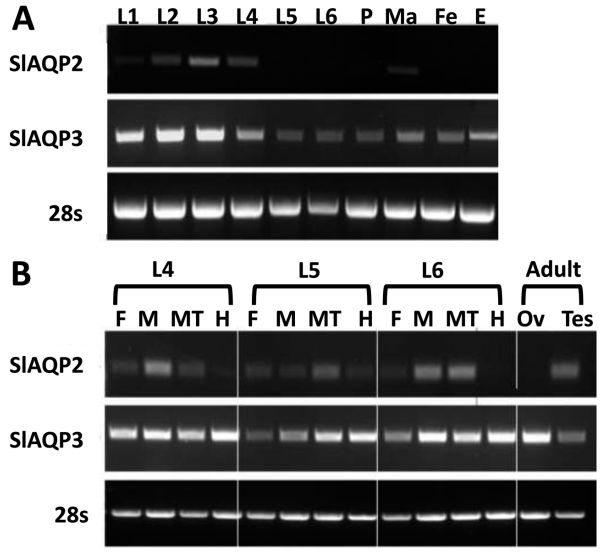

Fig. 4. Agarose gel electrophoresis showing RT-PCR results using *SlAQP2*, *SlAQP3* and *28S* specific primers. A. developmental expression showing *SlAQP2* mRNA in the first four larval instars (L1–L4) and adult males, while *SlAQP3* was detected in all developmental stages. B. Tissue expression of *SlAQP2* and *SlAQP3* showing *SlAQP2* mRNA detected mainly in midgut and Malpighian tubules of the larval stages and in testes of adult males. *SlAQP3* was detected in all the tissues tested, and *28S* was detected in all developmental stages and tissues. E – egg, F – foregut, Fa – female, H – hindgut, L – larval instar, M – midgut, Ma – male, MT – Malpighian tubules, Ov – ovary, Tes – testes.

termini. The two NPA domains along with the ar/R constriction region control the passage of water and other molecules through channels. Based on the four amino acids forming the ar/R constriction region, water channels are classified into two major categories; water specific AQPs and aquaglyceroporins (Glps). Finn et al. (2015) analysed insect AQPs available in different databases and noticed that the ar/R region of glycerol channels (Glps and Eglps) have an uncharged amino acid on TMD5 whereas water restricted AQPs have in the corresponding position a charged amino acid. Moreover, they mutated the charged amino acid on TMD5 of some water restricted AQPs to an uncharged amino acid and found that this single point mutation switched the water restricted AQP to a glycerol channel. Similar results are reported by Beitz et al. (2006) who transformed the mammalian water specific AQP1into a glycerol permeable channel by inducing mutations in the ar/R region. Finn et al. (2015) report the presence of two types of glycerol channels in insects; aquaglyceroporins (Glps) and Entomoglyceroporins (Eglps). Glps exist in different life forms including insects (reviewed in Abascal et al., 2014; Finn & Cerda, 2014; Finn et al., 2015). On the other hand, Eglps are insect specific channels reported only for species such as *B. mori* (Kataoka et al., 2009a), *T. castaneum* (Yao et al., 2018), *Bemisia tabaci* (Van Ekert et al., 2016), *Ae aegypti* (Drake et al., 2010), *Apis mellifera* (de Souza et al., 2020a, b) and *Dendroctonus armandi* (Fu et al., 2019). SlAQP2 ar/R region consists of the four amino acids F72-G197-A206-R212 and SlAQP3 ar/R region of the four amino acids F62-H186-S195-R201. Based on this amino acid arrangement, it is concluded that SlAQP2 is a glycerol channel and SlAQP3 a water restricted channel. Phylogenetic analysis of different AQP proteins from insects revealed the presence of two different clusters of glycerol channels (Glps and Eglps) and that SlAQP2 clustered with Eglps (Fig. 3). Based on these results, it is concluded that SlAQP2 is a glycerol channel of the insect specific Eglps and confirmed that SlAQP3 is a water specific channel of the Prip subfamily. Alignment of AQP2 (Eglps) and AQP3 (Prips) from insects (Fig. 2) revealed some differences with uncharged amino acid (G or S) on TMD5 in AQP2 proteins (Eglps) and a charged amino acid (H) at the corresponding position on AQP3 proteins (Prips).

Analysis of SlAQP2 and SlAQP3 amino acid sequences confirmed the presence of several phosphorylation and glycosylation sites. Similar results are reported for other insects such as *D. armandi* (Fu et al., 2019) and *B. tabaci* (Van Ekert et al., 2016). Several studies indicate that the regulation of the function of AQPs is mainly by two post translation mechanisms; gating (opening and closing the pore) and trafficking (movement of protein between cellular compartments) (Maurel et al., 2015; Nesverova &

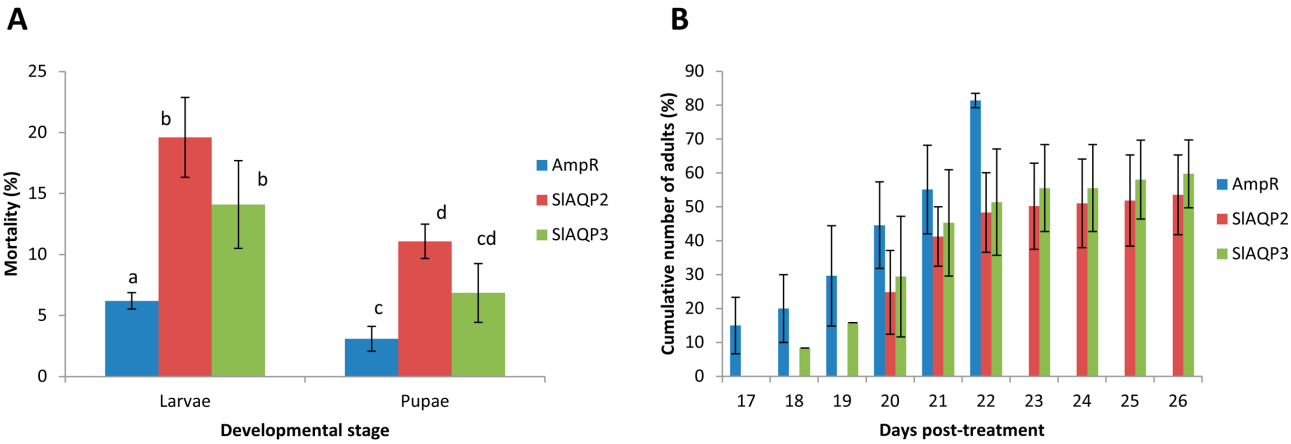

Fig. 5. Results of dsRNA bioassay showing larval and pupal mortality (A) and adult emergence over time (B) of *Spodoptera littoralis* after the larvae were treated with dsRNA for *SlAQP2*, *SlAQP3*, and the ampicillin resistance gene (*AmpR*) as a control. Adult emergence was calculated as a percentage of total larvae used in the bioassay. The data are presented as the mean ± SE. The different letters above the columns in (5A) indicate a significant difference.

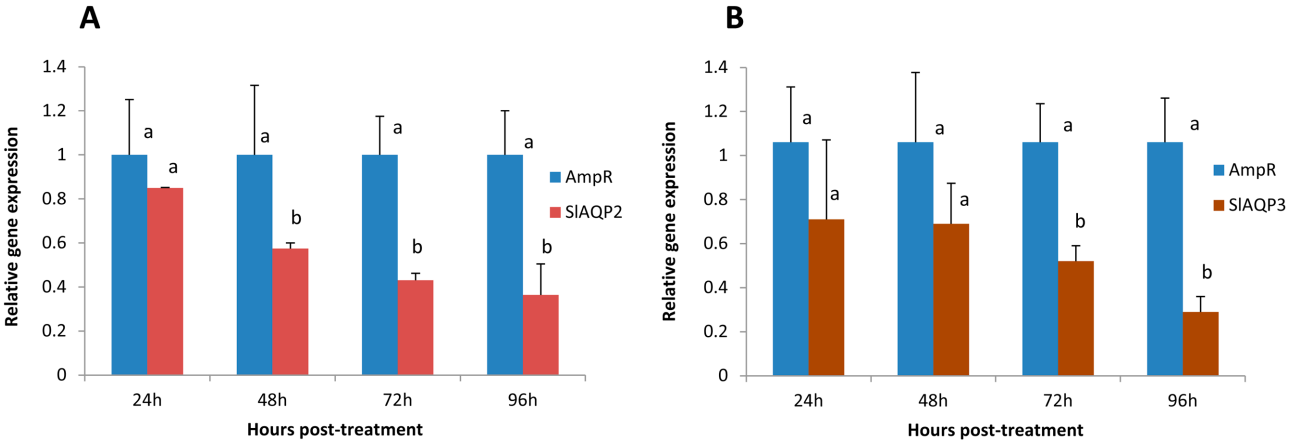

Fig. 6. Time course of relative transcription levels of *SlAQP2* (A) and *SlAQP3* (B) after larval injection with dsRNA for the two target genes and the *AmpR* as a control. The expression of *S. littorallis 28S* gene (HQ178645) was used as an endogenous reference to normalize the data and to calculate the fold change using the $2^{-\Delta\Delta C_T}$ method. The data are presented as the mean ± SE. The different letters above the columns indicate a significant difference.

Törnroth-Horsefield, 2019). Phosphorylation of both mammalian and plant AQPs has a major role in both regulation mechanisms (Offringa & Huang, 2013; Sachdeva & Singh, 2014; Singh et al., 2020). Glycosylation in plant AQPs is important for subcellular redistribution (Singh et al., 2020). Although not studied in insects, the presence of several phosphorylation and glycosylation sites in insect AQPs may indicate a similar role to that in plant and mammalian AQPs.

SlAQPs expression varied during the life cycle of *S. littoralis* with each transcript of *AQP* differing in abundance. In the immature stages, *SlAQP2* was expressed in the first four larval instars with highest level recorded in L3. *SlAQP2* was not expressed in eggs or pupae. *SlAQP2* was expressed mainly in the midgut and MTs of larval stages. In general, Eglps and Glps of other insects are highly expressed in both organs (midgut and MTs). For example, *AQP2* is mainly expressed in the midgut and MTs of *B. mori* (Kataoka et al., 2009a) and in the MTs of *G. molesta* (Kataoka et al., 2009b). In *A. aegypti*, Eglps (*AQP4* and *AQP5*) are mainly expressed in the MTs of larvae (Marusalin et al., 2012) and midgut and MTs of adult females 72 h post-emergence (Drake et al., 2015). *Anopheles gambiae AQP3* (Eglp) has a similar expression pattern with the highest level in the gut and MTs of 7 days old females (Liu et al., 2016). In the adult stage, the transcript of *SlAQP2* is reported in the testes of males, but not in females (Fig. 4). A few reports for other insects indicate a higher expression of some Glps or Eglps in males than in females, such as *DaEglpA1-v1* in *D. armandi* (Fu et al., 2019), *Eglp1* in *Culex pipiens* (Yang & Piermarini, 2017), *ClGlp1* and *ClGlp2* in *C. lectularius* (Tsujimoto et al., 2017) and *AgAQP3* in *Anopheles gambiae* (Liu et al., 2016). Taken together, this indicates that SlAQP2 and glycerol channels in general are most likely to have a specific role in male reproduction. The pattern of expression of *SlAQP3* differed from that of *SlAQP2* in that it was detected in all developmental stages with highest levels recorded in the first four larval instars (Fig. 4). Regarding tissue expression, *SlAQP3* was detected in gut and MTs with the highest levels recorded in the midgut, MTs and hindgut of the last three larval instars. The pattern of expression of *SlAQP3* is similar to that of *SlAQP1* reported by El-Gamal et al. (2018). Similar results are reported by Azuma et al. (2012) with the coexistence of two water specific AQPs (Drip and Prip) in the cryptonephric rectal complex of the larvae of *B. mori* where they facilitate the recycling of water into the haemolymph. Lu et al. (2021) also report high expression of *CsPrip* in the hindgut and MTs of *C. supperssalis* where it facilitates the transport of water and other small molecules. They also report a higher level of expression of *CsPrip* in adult males than adult females of *C. supperssalis,* but did not compare expression in ovaries and testes. In the current study, *SlAQP3* was also detected in both adult males and females and in testes and ovaries, with higher expression in ovaries. However, Fabrick et al. (2014) report higher expression of *LhAQP4* (Prip) in the ovaries than the testes of *L. hesperus*. In general, the expression of *AQPs* in insects is reported in the ovaries of some adult females, such as *B. germanica* (Herraiz et al., 2011), *Ae. aegypti* (Drake et al., 2010), *An. gambiae* (Liu et al., 2011), *B. mori* (Maruyama et al., 2015) and *Apis mellifera* (Medeiros-Santana et al., 2021), where they are thought to have a role in oocyte development. In addition, *AQPs* are expressed in different parts of vertebrate male and female reproductive systems with important functions in reproduction and their malfunction is linked to diseases such as infertility, polycystic ovaries and carcinoma (summarized in Zhang et al., 2012; Carrageta et al., 2020).

RNAi is a mechanism of post-transcription gene regulation discovered first in the nematode *Caenorhabditis elegans* by Fire et al. (1998). RNAi mediated knockdown has been used in many insect species as a tool to study gene function and also for pest management (Liu et al., 2020; Zhu & Palli, 2020). RNAi of *AQPs* has been successfully used against many agricultural pests and disease vectors and to study AQPs gene function and the effect of their silencing on insect life. For example, in the female mos-

quito *Ae. aegypti*, RNAi-mediated silencing of AQP genes expressed in gut and MTs resulted in reduced excretion and helped this mosquito to conserve water in dry environments (Drake et al., 2010, 2015). Similarly, RNAi of *AgAQP1* in *An. gambiae* females resulted in higher tolerance of desiccation while RNAi of *AgAQP3* reduced survival at a high temperature (39°C) (Liu et al., 2011, 2016). RNAi of *AQP1* of *Bemisia tabaci* through an artificial diet resulted in 70% reduction in gene expression and 84% mortality by d6 post-feeding (Vyas et al., 2017). Similarly, RNAi of AQPs was successfully achieved in other insects such as *Acyrthosiphon pisum* (Shakesby et al., 2009), *Myzus persicae* and *Bactericera cockerelli* (Tzin et al., 2015), *Glossina morsitans morsitans* (Benoit et al., 2014) and few others. Regarding Lepidoptera, we found only one study reporting RNAi of *Se-AQP1* in *S. exigua* (Ahmed & Kim, 2019). RNAi of *Se-AQP1* impaired haemocyte immunity functions such as phagocytosis and nodule formation. Also, *Se-AQP1* RNAi affected larval growth, period of development, body weight, pupation and adult emergence. Similar to previous reports, our qRT-PCR analysis revealed significant reductions in the expression of both *SlAQP* genes and the bioassay resulted in deleterious effect on insect life including larval and pupal mortality, pupal and adult deformation, prolonged development and low survival. On the other hand, Herraiz et al. (2011) downregulated *BgAQP* in the ovary of adult *Blattella germanica*, but the oocytes developed normally. They report that the presence of other AQPs in the same tissue may compensate for the silenced gene function. Yao et al. (2018) report similar findings in *T. castaneum* when different AQP genes are silenced.

In conclusion, two new AQP genes of the cotton leafworm, *S. littoralis*, were identified. The predicted amino acids of SlAQP2 and SlAQP3 showed characteristics of all known AQPs. Phylogenetic analysis and ar/R selectivity region structure confirmed that SlAQP2 belongs to the insect specific Eglp subfamily and SlAQP3 to the Prip subfamily. *SlAQP2* is expressed mainly in the first four larval instars, pupae and adult males while *SlAQP3* is expressed in all developmental stages. *SlAQP2* was expressed mainly in the midgut and Malpighian tubules of larvae while *SlAQP3* was expressed in all dissected tissues. *SlAQP2* expression was detected in testes, but not ovaries, whereas *SlAQP3* was detected in both organs with a higher expression in ovaries. RNAi-mediated knockdown of each gene separately resulted in deleterious effects such as mortality, deformed pupae and adults, and prolonged development time.

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