Identification and Functional Analysis of the First Aquaporin from Striped Stem Borer, *Chilo suppressalis*

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Aquaporins are integral membrane proteins some of which form high capacity water-selective channels, promoting water permeation across cell membranes. In this study, we isolated the aquaporin transcript (*Cs*Drip1) of *Chilo suppressalis*, one of the important rice pests. *Cs*Drip1 included two variants, *Cs*Drip1_v1 and *Cs*Drip1_v2. Although *Cs*Drip1_v2 sequence (>409 bp) was longer than *Cs*Drip1_v1, they possessed the same open reading frame (ORF). Protein structure and topology of *Cs*Drip1 was analyzed using a predicted model, and the results demonstrated the conserved properties of insect water-specific aquaporins, including 6 transmembrane domains, 2 NPA motifs, ar/R constriction region (Phe⁶⁹, His¹⁹⁴, Ser²⁰³, and Arg²⁰⁹) and the C-terminal peptide sequence ending in “SYDF.” Our data revealed that the Xenopus oocytes expressing *Cs*Drip1 indicated *Cs*Drip1 could transport water instead of glycerol, trehalose and urea. Further, the transcript of *Cs*Drip1 expressed ubiquitously but differentially in different tissues or organs and developmental stages of *C. suppressalis*. *Cs*Drip1 mRNA exhibited the highest level of expression within hindgut and the third instar larvae. Regardless of pupae and adults, there were significantly different expression levels of *Cs*Drip1 gene between male and female. Different from at low temperature, the transcript of *Cs*Drip1 in larvae exposed to high temperature was increased significantly. Moreover, the mRNA levels of *Cs*Drip1 in the third instar larvae, the fifth instar larvae, pupae (male and female), and adults (male and female) under different humidities were investigated. However, the mRNA levels of *Cs*Drip1 of only female and male adults were changed remarkably. In conclusions, *Cs*Drip1 plays important roles in maintaining water homeostasis in this important rice pest.

Keywords: *Drosophila* integral protein (Drip), *Chilo suppressalis*, structure, expression, functional assay

INTRODUCTION

Obviously, water is one of the most fundamental molecules for all living organisms. Scientists have confirmed that in addition to simple diffusion, there were high capacity water-selective channels to account for the high water permeability in certain tissues of the animals (Preston et al., 1992; Shakesby et al., 2009). Aquaporins (AQPs), often known as water channels, are integral membrane
proteins that regulate the flow of water driven by osmotic gradients (Campbell et al., 2008; Benga, 2009). AQPs exist as tetramers in the cell membrane with each monomer functioning as a water channel (Agre et al., 1987; van Hoek et al., 1991). These AQPs are present in both prokaryotes and eukaryotes species and play an important role in the water transport system (Heymann and Engel, 1999; Agre, 2006; Campbell et al., 2008). Some AQPs can also transport a number of small solutes, typically glycerol or urea (Rojec et al., 2008). Current research on AQPs primarily focuses on plants and vertebrates (Verkman, 2005; Campbell et al., 2008; Maurel et al., 2008). To date, 13 AQP types have been isolated from mammals, named AQP0-AQP12 (Yasui, 2004; Ishibashi, 2006). However, only a few AQP genes were identified and characterized from insects, among which just four species come from Lepidoptera, Bombyx mori, Grapholita molesta, Spodoptera litura, and Ectropis obliqua (Dow et al., 1995; LeCahérec et al., 1996a,b; Echevarria et al., 2001; Yanochko and Yool, 2002; Duchesne et al., 2003; Kaufmann et al., 2005; Kikawada et al., 2008; Kambara et al., 2009; Kataoka et al., 2009a,b; Shakesby et al., 2009; Goto et al., 2011; Herraez et al., 2011; Mathew et al., 2011; Philip et al., 2011; Azuma et al., 2012; Liu et al., 2013; Fabrick et al., 2014; Ibanez et al., 2014; Li et al., 2016; Van Ekert et al., 2016; Liu and Piermarini, 2017). The phylogenetic analyses of insect AQPs had revealed the existence of five major subfamilies, including the Drip, Prip, Bib, Eglps, and AQP12L, and members of the Drip and Prip subfamilies typically were water selective channels (Chawn and Nicolson, 2004; Kambara et al., 2009; Herraez et al., 2011; Mathew et al., 2011; Drake et al., 2015; Finn and Cerda, 2015; Van Ekert et al., 2016). Meanwhile, according to the database of genome and transcriptome, the only one AQP in Chilo suppressalis, two AQPs in Bombyx mori, three AQPs in Danaus plexippus and Manduca sexta, and five AQPs in Manduca sexta was identified (http://www.insect-genome.com/data/detail.php?id=7) (Yin et al., 2014), and basing on the homology of AQPs, the above species all could possess the water-selective AQPs. Therefore, Drip and Prip are very important to maintain the balance of water in insect.

Otherwise, some studies suggested that AQPs played an important role in the physiological functions of insect (Goto et al., 2011; Benoit et al., 2014b; Fabrick et al., 2014; Drake et al., 2015). For example, freeze tolerance of insects was related to the ability to remove water from cells by AQPs (Philip et al., 2011). The removal of water from the cells could suspend metabolic processes or avoid damaging ice crystal formation (Spring et al., 2009). Freeze tolerance also needed to accumulate glycerol in the cells, a role admirably suited to the aquaglyceroporins (Spring et al., 2009). Down regulation of AQPs in Aedes aegypti enhanced mosquito desiccation resistance (Drake et al., 2015). The female tsetse flies of Glossina morsitans morsitans have been studied to elucidate the role of AQPs in heat tolerance (Benoit et al., 2014a,b).

The striped stem borer, Chilo suppressalis (Walker) (Insecta: Lepidoptera: Pyralidae), an important rice pest widely distributed in Asia, has caused significant damage to rice crops in China, especially to hybrid rice varieties in recent years. In the district of Yangzhou (32.23°N, 119.26°E), Jiangsu province, China, C. suppressalis has two complete and a partial third generation each year, and the larvae enter facultative diapause in winter (Lu et al., 2013). According to our studies, in March of 2010, field-collected larvae could survive at −21°C (Lu et al., 2012). Overwintering larvae of C. suppressalis could acquire freeze tolerance (Tsumuki and Hirai, 2007). Some studies demonstrated AQPs might play very important role in the cold tolerance of C. suppressalis (Izumi et al., 2006, 2007). However, they did not further study any aquaporin gene. It is well-known that C. suppressalis need very high humidity condition in the life cycle (Shang et al., 1979; Lu et al., 2014), and AQPs may help to maintain water homeostasis.

Thus, in this paper we firstly described the characteristics of C. suppressalis Drip1 (C3Drip1), and assayed the abundance of C3Drip1 in different tissues or organs and developmental stages. Secondly, in order to understand the C3Drip1 regulation under different humidities, the C3Drip1 mRNA levels of different developmental stages and sexes of C. suppressalis were investigated under different humidities. Moreover, in order to understand the relationship between C3Drip1 regulation and temperature, we also studied the expression patterns of C3Drip1 mRNA under various temperatures. Last but not the least, to further demonstrate the functions of C3Drip1, functional oocyte swelling assays were executed by water and three kinds of solutes. These studies will help us understand the role of AQPs in C. suppressalis, and also may provide insights in developing strategies for the control of this pest.

**MATERIALS AND METHODS**

**Insects**

The population of C. suppressalis was collected from the suburb of Yangzhou (32.39°N, 119.42°E). The rice stem borers were reared in an environmental chamber at 28 ± 1°C, 16:8 (L: D) and RH = 70 ± 5% (Shang et al., 1979).

**Cloning and RACEs**

Total RNA of the fifth instar larva was extracted by the SV Total RNA Isolation System (Promega Z3100) combined with DNase digestion to eliminate DNA contamination. Total cDNA was synthesized by oligo(dT)18 primer (TaKaRa). Degenerate primers DP-F and DP-R (Table 1) were used to amplify the partial segments of AQP. The full-length cDNA of C3Drip1 gene was determined using 5′- and 3′-RACE (SMART RACE, Clontech). The primers used (VA and VB) were shown in Table 1. The full length sequence of C3Drip1 was confirmed by the template of RACE 5′ cDNA.

**Sample Preparation**

The rice stem borers were reared successively to the third generation in the seedlings. Then, the egg masses, the first, second, third, fourth, fifth instar larvae, pupae (male and female), and 1-day adults (male and female) were randomly selected for the experiment. Each experiment was repeated four times. The fifth instar deep diapausing larvae of C. suppressalis collected from rice stubble were anesthetized on ice before dissection. Heads (HE), epidermis (EP), fat body (FB), foregut (FG), midgut...
TABLE 1 | Primers used in this study.

| Gene   | Primer name | Primer sequences (5’ → 3’) | Tm (°C) | Length (bp) |
|--------|-------------|-----------------------------|---------|-------------|
| RT-PCR |             |                             |         |             |
| CsDrip1 | F           | CACATCAAYCCMCGBGTACAC       | 68.0    | 431         |
|         | R           | GGNCCCRCCCATAMACCA          |         |             |
| RACE PCR|             |                             |         |             |
| CsDrip1 | 3’          | CCACAGACAGCAACACCACGCAAGA   | 68.0    | 666         |
| variant A | 5’     | CCTTCATGCGGCTAACGTACTCCT    | 68.0    | 812         |
| variant B | 3’       | CCTTCATGCGGCTAACGTACTCCT    | 68.0    | 757         |
|         | 5’          | TGCAAGCAGCAGTGCGTCTGTTG     | 68.0    | 1093        |
| FUNCTIONAL ASSAYS |             |                             |         |             |
| CsDrip1 | F           | TCAACTAGTGCCACCTAGAAAAACGGATT ACGCTGT | 774     |             |
|         | R           | TCAAGCCGGCCCGTCTTGAAGCTGAGGC |         |             |
| QPCR    |             |                             |         |             |
| CsDrip1 | F           | GTGGATGAGATGACGAAAGGAG      | 59.4    | 120         |
|         | R           | AAAGCATGTCAGCAACAGGGG       |         |             |
| H3      | F           | CTGACACCAACCACACTGGTTGA     | 56.0    | 184         |
|         | R           | TAGCCGCAGCTGACTGGAGGAAGG    |         |             |
| EF1     | F           | AAAATGGACTCGTACTGGACCCCC    | 56.6    | 137         |
|         | R           | TCTCGTGGCGAACAGAAATA        |         |             |
| 18S     | F           | GTGTGGOAGGCAGCTAGTTTATT     | 62.5    | 258         |
|         | R           | GCTGCTTCTCTGCTGTGGTGG       |         |             |
| ACTIN   | F           | AAGGAGAACAGCAAAAACTGGGGG    | 56.0    | 243         |
|         | R           | GTTCAATGAGGATGGGCTAATGAAA   |         |             |
| TUB     | F           | GAGGGCACTGACGCAAGGATAGA     | 60.4    | 178         |
|         | R           | AAGGACGTAGAGTACAGAGGGG      |         |             |
| UBI     | F           | TCACCGCAGCAGCAGACACT        | 60.2    | 219         |
|         | R           | GGAAGAACCACACCCCCCTCATATA   |         |             |

The qPCR primers used in this study were validated.

(MG), hindgut (HG), Malpighian tubules (MT) and haemocytes (HC) were collected and rinsed with 0.9% NaCl. The samples were frozen immediately in liquid nitrogen and stored at −70°C until the experiment.

Temperature Stress Treatment
The larvae used in experiments were all 5th instars of similar body size and were assigned randomly to each experimental group. Each group of larvae was confined individually in glass tubes (relative humidity, 90 ± 10%) and exposed to a given temperature (including −11, −9, −3, 0, 27, 36, 39, and 42°C) for 2 h in a constant temperature incubator (DC-3010, Jiangnan equipment). The larvae were recovered at 27 ± 1°C for 2 h, and the survived larvae were frozen in liquid nitrogen and then stored at −70°C.

Humidity Treatment
The third instar larvae, fifth instar larvae, pupae (male and female) and adults (male and female) were treated in humidity chamber (HTC-100, SANTN, Shanghai, China) at 27°C, respectively under four different relative humidity levels (RH) (25, 50, 75, and 95%) for 24 h, and additionally the third instar larvae were treated for 12 h. Each treatment was replicated 30 times, and each replicate consisted of one insect. The humidities chosen were based on a prior pilot experiment. At the same time, survival in each treatment was assessed, and the survived larvae were treated as above.

Functional Oocyte Swelling Assays
The vector construction followed previously reported protocol (Chang et al., 2015). Firstly, the entire coding region of CsDrip1 was amplified with a high-fidelity polymerase (PrimeSTARHS DNA polymerase. TaKaRa, Tokyo, Japan) using primers with Kozak sequence and restriction enzyme cutting sites (SpeI and NotI). And the PCR products were digested with SpeI and NotI. The humidities chosen were based on a prior pilot experiment. The vector construction followed previously reported protocol (Lu et al., Identification and Functional Analysis of Aquaporin from Chilo suppressalis, 2009a,b). The PCR products were digested with SpeI and NotI, and subcloned into pT7Ts vector (Invitrogen, Carlsbad, California, USA), and then plasmids were fully linearized with SmaI. The cRNAs of CsDrip1 were synthesized in vitro using mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX, USA). Purified cRNAs were resuspended in nuclease-free water at a concentration of 0.2 µg/µl and stored at −80°C.

Unfertilized stage V and VI oocytes of Xenopus were defolliculated with 2 mg/ml collagenase I (GIBCO, Carlsbad, CA) in washing buffer (96 mM NaCl, 2 mM KCl, 5 mM MgCl2, and 5 mM HEPES [pH 7.6]) to maintain osmotic equilibrium. After being cultured overnight at 18°C, oocytes were microinjected with 27.6 nl CsDrip1 cRNA (5.52 ng) and 27.6 nl nuclease-free water as control. Oocytes were incubated for 3 days at 18°C in 1X Ringer’s solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl2, 0.8 mM CaCl2, and 5 mM HEPES [pH = 7.6]) supplemented with 5% dialysed horse serum, 50 mg/ml tetracycline, 100 mg/ml streptomycin and 550 mg/ml sodium pyruvate.

Osmotic water permeability (P₀) was measured as previously described (Kataoka et al., 2009a,b). Oocytes were transferred to a 3-fold dilution of 1X Ringer’s solution with distilled water and images were acquired every 15 s through a CCD camera DP-72 (Olympus, Tokyo, Japan) attached to a Olympus SZX16 stereomicroscope up to 5 min. The osmotic water permeability (P₀) was calculated as in previous reports (Zhang et al., 1990; Preston et al., 1992; Kataoka et al., 2009a,b). By the following equation: 

\[ P₀ = \frac{V₀ \cdot d(V/V₀)/dt}{[S \cdot V \cdot (Osm_in - Osm_out)]} \]

where \( V₀ \) is the oocyte initial volume, \( V/V₀ = 9 \times 10^{-4} \) cm², \( S \) is the surface area (\( S = 0.045 \) cm²), \( V \) is the molecular volume of the water (\( V_w = 18 \) cm³/mol) and Osm_in is 202 mmol·kg⁻¹ and Osm_out is 39 mmol·kg⁻¹. Relative oocyte volume (\( V/V₀ \)) was calculated from the relative area (\( A/A₀ \)) in the focal plane, \( V/V₀ = (A/A₀)^{3/2} \). Oocytes were transferred in an isotonic solution which containing 140 mM of solutes (glycerol, trehalose or urea) for solute transport assays. To maintain the osmotic equilibrium, the increase in oocyte volume corresponds to the water influx accompanying the solute uptake. The volume changes were recorded for 5 min in the same way as described above. Apparent solute permeability was calculated from the equation:

\[ Psol = \left[ \frac{d(V/V₀)/dt}{(V₀/S)} \right] \] (LeCahérec et al., 1995a,b; Duchesne et al., 2003). Water and solute permeabilities were performed at least for nine different Xenopus oocytes.
**Quantitative Real-Time PCR (qPCR) Analysis**

Total RNA was extracted by the SV Total RNA Isolation System (Promega Z3100), followed by DNase treatment to eliminate DNA contamination. The integrity of the RNA in all samples was verified by comparing the ribosomal RNA bands in ethidium bromide-stained gels. RNA sample purity was estimated using spectrophotometric measurements at 260 and 280 nm (Eppendorf BioPhotometer plus). Total RNA (500 ng) was reverse-transcribed into first-strand cDNA using the SuperScript II reverse transcription (RT)-PCR kit (Bio-Rad). The volume of reaction mixture was 20 µl. Each reaction mixture contained 10 µl of iTaq Universal SYBR Green supermix (2x) (Bio-Rad), 1 µl of each of gene specific primers (Table 1), 2 µl of cDNA templates, and 6 µl ddH₂O. Reactions were carried out on a CFX-96 real-time PCR system (Bio-Rad). The amplification efficiencies of the target and reference genes were similar in this study. Therefore, the quantity of CsDrip1 mRNA was calculated using the 2^(-ΔΔCq) method (Nolan et al., 2006; Schmittgen and Livak, 2008; Bustin et al., 2009). Relative expression levels of CsDrip1 in different tissue or organs were normalized with histone 3 (H3), elongation factor 1 (EF1) for different developmental stages and temperature stress (Xu et al., 2017). And previous tests of stability of the reference gene demonstrated 18S rRNA for the third larvae, ACTIN for the fifth larvae, TUB for the male pupae, UBI for the female pupae and male adults, and EF1 for the female adults under different humidity was most suitable respectively, and corresponding reference genes were selected to normalize (data not shown). Following qPCR, the homogeneity of the PCR products was confirmed by melting curve analysis, which was read 5 s per 0.5°C increment from 65°C to 95°C. Every treatment included four biological replicates, and every repeat was run in triplicate.

**Bioinformatic Analysis**

The open reading frames (ORFs) were identified with the aid of the ORF Finder software (http://www.ncbi.nlm.nih.gov/orffinder/orf.html). The deduced amino acid sequences were aligned using ClustALX software. Sequence analysis tools of the ExPASy Molecular Biology Server of Swiss Institute of Bioinformatics, including Translate, Compute pI/MW, Blast and TMHMM, were used to analyze the deduced CsDrip1 protein sequence. Phosphorylation and kinases sites were predicted using NetPhos 2.0 and NetPhosK 1.0, respectively (Blom et al., 1999, 2004). Amino acid sequences were used to estimate phylogeny with the neighbor-joining, minimum evolution, maximum likelihood and maximum parsimony methods. Phylogenetic trees were constructed with 1000 bootstrap replicates using MEGA version 7.0 (Kumar et al., 2016).

**Computational Molecular Modeling**

Homology models were generated using Protein Homology/analogy recognition engine V 2.0 (http://www.sbg.bio.ic.ac.uk/~phyre2/html) (Kelley and Sternberg, 2009). Briefly, the CsDrip1 amino acid sequence was aligned by the Phyre2, and the best model of bovine AQP1 X-ray A* structure (PDB ID: 1J4N) was used for modeling analyses (Sui et al., 2001). The Chimera Tool was used to visualize the three-dimensional coordinates for the atoms of the model (Pettersen et al., 2004).

**Statistical Analysis**

The data were tested for normality using the Shapiro-Wilk’s test. Homogeneity of variances among different groups was evaluated by the Levene test. All the data was log-transformed when necessary. Then, the significance of differences between treatments was identified with either a Tukey’s test (Homogeneity of variances) or Dunnett’s T3 test (Non-homogeneous) for multiple comparisons. In the experiments with two groups, significant differences were determined by Student’s t-test. The data were analyzed using SPSS16.0 software, and denoted as means ± SE (standard error) (Pallant, 2005).

**RESULTS**

**Isolation, Cloning, Sequencing, and Structure of CsDrip1**

Degenerate primers based on conserved regions from several insect aquaporins were used to amplify a 431 bp partial fragment from C. suppressalis cDNA. The cloned fragment was sequenced, and a BLAST analysis of its deduced amino acid fragment revealed apparent sequence homology with insect Drip1 (data not shown). The 1,416 and 1,825 bp full-length of variant A and variant B, including the UTRs was obtained, respectively through 5’and 3’ RACE (GenBank accession no. JQ011314 and JQ011315). Two Drip1 types of C. suppressalis (CsDrip1_v1 and CsDrip1_v2) were obtained in the present study. Variant A and variant B possessed the same open reading frame (ORF) of 258 amino acids with a predicted molecular weight of 26.9 kDa and an isoelectric point of 6.5. The protein sequence that we referred to as CsDrip1 possessed the hallmarks of the aquaporin family, including the classical "NPA" boxes (residue 89–91; residue 206–208) and 6 putative transmembrane region s (Figures 1A,B). According to the hydrophathy analysis, CsDrip1 contained cytoplasmic N- and C-terminal domains and the C-terminus ended with “SYDF”. In the amino acid sequence, seven potential phosphorylation sites (Tyr5, Ser15, Ser16, Ser176, Ser210, Ser250, and Ser253) and three potential protein kinase C-specific sites (Thr3, Ser21, Ser255) were also identified (Figures 1A,B). To investigate the potential structure-function relationship of CsDrip1, we generated its homology model with Phyre using bovine aquaporin-1 (1J4N) as a template (Sui et al., 2001). The structure of CsDrip1 model was very similar to that of aquaporin-1 (confidence, 100% statistically and identity, 36% based on sequence alignment). CsDrip1 models showed two tandem structural repeats, each consisting of three transmembrane helices (TM1–3 and TM4–6) and a short α-helix in loops B and E, each containing an NPA motif predicted to line one side of the pore (Figure 1C), which was called the "aquaporin fold" (Murata et al., 2000). Conserved NPA motifs formed the canonical structure in the center of the pore (Fu et al., 2000), and the structural model provided the defining force that orients water as it passed through the midpoint of the channel (Hoa et al., 2009). Residues
FIGURE 1 | Sequence analysis, predicted topology and structure of CsDrip1. (A,B) Nucleotide and deduced amino acid sequence of CsDrip1 variant A (JQ011314) and variant B (JQ011315). Six transmembrane domains (TMs) are also shown by the red underline and C-terminal “SYDF” sequence found in insect AQPs with dark underline. The NPA boxes are shown with gray highlight. Orange indicates amino acid residues constituting the ar/R constriction region. Blue dots and green dots, respectively are potential phosphorylation sites and protein kinase C-specific sites. (C) Homology modeling of the CsDrip1 is compared with bovine aquaporin-1 (PDB ID: 1J4N) as template using Phyre2. Intracellular (B,D) and extracellular (A,C,E) loops and transmembrane helices are shown (TM1 in blue, TM2 in red, TM3 in yellow, TM4 in green, TM5 in purple, TM6 in orange). (D) The structure depicted is from the extracellular side of the membrane. The classical NPA motifs are shown in gray sphere, and Ar/R selectivity residues regions (Phe-69, His-194, Ser-203, and Arg-209) are shown in gray stick.

that comprised the Ar/R constriction (Phe$^{69}$, His$^{194}$, Ser$^{203}$, and Arg$^{209}$) were found in CsDrip1 and predicted to establish water selectivity (Figures 1C,D).

Phylogenetic Analysis

We used CLUSTALX and MEGA 7.0 phylogenetic analysis to compare CsDrip1 with other insect aquaporins. Because neighbor-joining, minimum evolution, maximum likelihood, and maximum parsimony methods constructed the similar phylogenetic tree, Figure 2 only illustrated the phylogenetic tree constructed by the neighbor joining method. The result exhibited the phylogenetic tree include two clusters: Drip1 and Prip. And CsDrip1 (C. suppressalis Drip1) was most closely related to BmDrip1 (B. mori Drip1) and DpDrip1 (Danaus plexippus Drip1), to which they were 79% identical at the amino acid level. Our phylogenetic tree showed BmDrip1, DpDrip1, and CsDrip1 belonged to the same group, which was water-specific Drip subfamily. All the Lepidoptera except BmPrip (B. mori Prip) fell into the well-supported cluster, which was consistent with a previous publication about BmPrip classification in the Prip subfamily (Azuma et al., 2012).

Functional Assay

In order to confirm further that CsDrip1 was a water-selective aquaporin, the permeability of CsDrip1 to water, glycerol, trehalose and urea transport was performed using the Xenopus oocyte expression system, respectively. Compared to control oocytes, oocytes expressing CsDrip1 showed an 11-fold increase in the osmotic water permeability coefficient ($P_f$) ($N = 10$) (Figure 3A), and as a result of continuous uptake, even some...
tested oocytes broke. For glycerol, trehalose and urea uptake in *X. laevis* oocytes expressing CsDrip1, no significant (*p > 0.05*) uptakes were observed in oocytes expressing CsDrip1, and these oocytes showed a slight shrinkage. For example, the *P* of CsDrip1 oocytes in glycerol, trehalose and urea was 5.031e−6 ± 5.766e−7 cm/s (*N = 9*), 5.670e−6 ± 6.0758e−7 cm/s (*N = 10*), and 5.029e−6 ± 3.542e−7 cm/s (*N = 11*), respectively (*Figures 3B–D*). These results clearly indicated that CsDrip1 was a specific water-selective aquaporin.

**Expression of CsDRIP1 in Tissues or Organs of C. Suppressalis Larvae**

Real-time PCR verified that CsDrip1 mRNA was expressed in head, epidermis, fat body, foregut, midgut, hindgut, and...
Malpighian tubules $\left[F\left(6, 14\right) = 4.441, P = 0.010\right]$, but there were not expression level in haemocytes. The highest level of CsDrip1 mRNA was observed within hindgut, which was 19.23-fold higher than that in the foregut. And CsDrip1 mRNA in Malpighian tubules also exhibited the second high abundance. Interestingly, the head was one of the organs that expressed a high level of CsDrip1 mRNA (Figure 4).

**Expression of CsDRIP1 in Developmental Stages of C. suppressalis**

We investigated the expression patterns of CsDrip1 transcripts over the life cycle of *C. suppressalis*, including eggs, larvae (1st, 2nd, 3rd, 4th, and 5th instars larvae), pupae (male and female) and adults (male and female). The results demonstrated that the highest mRNA level of CsDrip1 was observed in the third instar larvae, which was 117.89-fold higher than that of male pupae, which followed by the first instar larvae, male adults, and egg mass. CsDrip1 transcript of the male pupae was the least. In addition, CsDrip1 mRNA exhibited significantly different level in male pupae and male adults than those of female pupae and female adults, respectively $\left[F\left(9, 19\right) = 23.954, P < 0.001\right]$ (Figure 5).

**Expression of CsDrip1 under Various Temperatures**

Although the survival rate of larvae of *C. suppressalis* only reached to 55.56% at $-11^\circ$C and 100% at $42^\circ$C, respectively (Lu et al., 2014), CsDrip1 mRNA in larvae exposed to low temperatures for 2 h was not different significantly $\left[F\left(7, 16\right) = 39.957, P = 0.560\right]$ (Figure 6). However, CsDrip1
displayed a different expressional pattern under heat stress. For example, CsDrip1 mRNA reached maximum at 36°C, which was 3.75-fold of that at control temperature (27°C) \( F(7, 16) =39.957, P < 0.001 \). Subsequently, the abundance of CsDrip1 mRNA in larvae decreased by the elevated temperature (Figure 6).

**Expression of CsDrip1 in Developmental Stages of C. suppressalis under Different Humidities**

According to prior experiments, C. suppressalis could tolerate these humidity treatments. The expression levels of CsDrip1 mRNA of the third instar larvae, fifth instar larvae, pupae (male and female) and adults (male and female) of C. suppressalis under different humidities have been determined. The results demonstrated that after exposure of the third instar larvae, fifth instar larvae, pupae (male and female) to 25–95% RH, CsDrip1 mRNA levels were not significantly different from each other \( F(3, 12) = 1.309, P = 0.317; F(3, 8) = 1.285, P = 0.324; F(3, 8) = 3.453, P = 0.072; F(3, 8) = 0.964, P = 0.455 \) (Figures 7A–D). However, the CsDrip1 mRNA of female and male adults exposed to different humidities was changed significantly, and the CsDrip1 mRNA of female adults was up-regulated remarkably, which was contrary to male adults \( F(3, 8) = 4.272, P = 0.045; F(3, 10) = 3.816, P = 0.047 \) (Figures 7E,F).

**DISCUSSION**

Water-selective aquaporins are integral membrane proteins belonging to a large family of water channel proteins that assist in rapid movement of water across cellular membranes. This report was the first extensive analysis of Drip1 and its functions in C. suppressalis, an important rice pest of Lepidoptera. We isolated and characterized the first AQP from the C. suppressalis (CsDrip1). Deduced amino acid sequence of CsDrip1 resembled the features of other insect Drip1 (Ibanez et al., 2014). For example, two inverted hemi-helices on loops B and E that project opposing NPA motifs, which regulate the conductance of water (Wree et al., 2011). Additionally, it had seven potential phosphorylation sites (Tyr5, Ser15, Ser16, Ser176, Ser210, Ser250, and Ser253), and three potential protein kinase C (PKC) specific sites (Thr3, Ser21, and Ser255) which somewhat contrasted to those of B. mori Drip1 (BmDrip1) that had only one PKC specific sites (Thr3) and one...
tyrosine kinase phosphorylation site (Tyr\textsuperscript{250}) (Kataoka et al., 2009a). Although CsDrip1 variant A and variant B possessed the same ORF, the full-length sequence of variant A was 409 bp shorter than variant B (Figures 1A,B). Similar phenomenon was found in Prip of Anopheles gambiae, which included two variants, but two variants encoded different amino acids (Tsujimoto et al., 2013). It was also found that in Belgica antarctica there were three types of Prip (variants A-C) derived from the same gene, which was suggested to be due to alternative splicing (Goto et al., 2011). Hydrophobicity and structural prediction indicated that CsDrip1 possessed the conserved feature of water-specific AQPs. The pore of vertebrate AQPs is restricted by four residues (Phe\textsuperscript{58}, His\textsuperscript{182}, Cys\textsuperscript{191}, and Arg\textsuperscript{197}) that comprise the Ar/R constriction site (Sui et al., 2001; Horsefield et al., 2008; Hoa et al., 2009). Although the Ar/R constriction residues are generally conserved, Cys\textsuperscript{191} in vertebrate AQP is replaced by serine (Ser\textsuperscript{203} in TM5) in CsDrip1 (Figures 1C,D). Further comparative analysis of the vertebrate and insect AQPs revealed that either alanine or serine in all known insects Drip1 was substituted for Cys\textsuperscript{191} in vertebrate AQP1, and all Drip1 from Lepidoptera had a serine residue.

Aquaporins are most highly expressed in tissues where water movement is frequent and/or physiologically important.
CsDrip1 transcripts showed specific expression patterns in various tissues (Figure 4). Water homeostasis in insects is achieved by a two-part system composed of Malpighian tubules and hindgut, and Malpighian tubules are the primary excretory and osmoregulatory organ in insects, analogous to the vertebrate renal tubules (Chawn and Nicolson, 2004). CsDrip1 mRNA was expressed abundantly in hindgut and Malpighian tubules, which was similar to the results observed in several other insects (Kambara et al., 2009; Kataoka et al., 2009a,b; Drake et al., 2010, 2015; Goto et al., 2011; Liu et al., 2011; Nagae et al., 2013; Fabrick et al., 2014). Fat body was one of the most cold-resistant tissues in the C. suppressalis because glycerol was accumulated in the fat body of cold acclimated and diapause larvae (Izumi et al., 2006, 2007). And our results revealed that CsDrip1 mRNA in fat body was high (Figure 4). However, low abundance or absence of Drip1 in the fat body had also been observed in several insect species (Kambara et al., 2009; Liu et al., 2011, 2013; Philip et al., 2011). Fat body was the site of glycerol biosynthesis (Kukal et al., 1988), which likely requires other kind of aquaporins, aquaglyceroporins for glycerol transportation as suggested in B. mori and G. molesta (Kataoka et al., 2009a,b). Therefore, CsDrip1 in the diapausing larvae of C. suppressalis couldn't contribute its cold hardiness. In summary, an unexpected diversity of AQPs was found in insect, and different AQPs contributed various functions. For example, there were six AQP genes in Ae. aegypti. However, AQP 1 and AQP 2 were the strict water channels, which raised the osmotic pressure of midgut and Malpighian tubules. And AQP 5 demonstrated significant solute permeability for trehalose, which was important for insect temperature and dehydration tolerance (Drake et al., 2010, 2015; Van Ekert et al., 2016).

The levels of CsDrip1 transcript are high at early stages of development, and the expression of CsDrip1 was quite dynamic throughout development (Figure 5). CsDrip1 mRNA was highest in the third larvae of C. suppressalis, which just entered into the galled stage. The eggs of insect generally require high moist environment condition for hatching. High abundance of CsDrip1 mRNA in eggs of C. suppressalis was propitious to maintain the water balance of the embryonic development. However, BrDrip1 transcripts were most highly expressed in 2nd instar nymphs and least present in eggs (Mathew et al., 2011). AgDrip1 mRNA was higher in female adults than that in male adults (Liu et al., 2011), whereas CsDrip1 mRNA in male adults was significantly higher than in female adults. In addition, CsDrip1 mRNA was also greater in adults than that in pupae (Figure 5). We speculated that CsDrip1 expression might be related to reproduction.

When exposed to extreme temperatures, insects may respond in different ways: they can adopt behaviors to avoid or escape extreme temperatures, or they may regulate various proteins in response to adverse temperatures (Tursman et al., 1994; Rinehart and Denlinger, 2000; Tyshenko et al., 2005; Huang et al., 2007; Cui et al., 2010). In E. solidaginis by increasing the number of AQPs, cells likely improved their ability to rapidly redistribute water, better protecting themselves against the build-up of osmotic pressures across the membrane during freezing in winter (Philip et al., 2008, 2011). In Megaphorura arctica, up- or down-regulation of AQPs were contributed to exploit cryoprotective dehydration to enhance its cold tolerance (Clark et al., 2009). The AQPs of B. antarctica also play very important roles in its freeze tolerance (Yi et al., 2011). And the studies suggested that the aquaporin might contribute to the cold tolerance of C. suppressalis (Izumi et al., 2006, 2007). The CsDrip1 might play the important role in the cold hardiness and diapause initiation of C. suppressalis (Lu et al., 2013). However, the CsDrip1 transcript in our studies was not up-regulated significantly under low temperature stress, but the CsDrip1 transcript increased significantly under high temperature stress (Figure 6). At the same time, our results confirm that CsDrip1 is a strict water channel. Therefore, C. suppressalis could utilize the up-regulation of CsDrip1 to exchange water in order to escape heat stress, but survival at low temperature they might largely depend on the ability of cells to accumulate the cryoprotectants among cellular compartments. Maybe, other AQPs existing in C. suppressalis could be coordinated to resist to the low stress.

Although the X. oocyst expression system clearly demonstrated that CsDrip1 allowed water, but not glycerol, trehalose or urea, to pass through the cell, CsDrip1 mRNA of the third instar larvae, fifth instar larvae, and pupae (male and female) of C. suppressalis after exposure to 25–95% RH was not significantly regulated. However, CsDrip1 mRNA in female adults was greatly induced while expression of CsDrip1 mRNA in male adults was suppressed (Figure 7). Our data are inconsistent with that from a previous study in C. pipiens, which found that Prip mRNA level was significantly down-regulated in response to a low relative humidity (Liu and Piermarini, 2017). In the ovary of B. antarctica under water stress, no significant differences were observed in the levels of BaPrip mRNA (Goto et al., 2011). In response to dehydration, expression of PvDrip1 of Polypedilum vanderplanki larvae was greatly induced (Kikawada et al., 2008). Therefore, when confronted with water stress, different kinds of insects could possess different strategies by regulating different AQPs.

It is widely known that most insects possess multiple AQP genes. At least seven putative AQPs in A. gambia, six in Ae. aegypti, eight in Drosophila, five in Lygus hesperus, eight in Bemisia tabaci, and seven in Tribolium castaneum had been identified (Adams et al., 2000; Holt et al., 2002; Drake et al., 2010; Fabrick et al., 2014; Van Ekert et al., 2016). And three AQPs in B. mori and two in G. molesta from Lepidoptera also had been isolated (Kataoka et al., 2009a,b; Azuma et al., 2012). The fat body of C. suppressalis could contain aquaglyceroporins related to the diapause and cold hardness (Izumi et al., 2006, 2007). Therefore, it’s significant to identify and further analyse the other AQPs in C. suppressalis. We expect that the research of CsAQPs will be key in revealing the plot underlying mechanism of development, temperature tolerance, diapause and distribution of C. suppressalis in the future.

**AUTHOR CONTRIBUTIONS**

Y-ZD and G-RW conceived and designed the experiments. M-XL, YL, D-DP, and JX preformed the experiments. M-XL and Y-ZD analyzed the data and wrote the manuscript.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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