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Molecular and functional characterization of multiple aquaporin water channel proteins from the western tarnished plant bug, *Lygus hesperus*

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ABSTRACT

Aquaporins (AQPs) are integral membrane channel proteins that facilitate the bidirectional transfer of water or other small solutes across biological membranes involved in numerous essential physiological processes. In arthropods, AQPs belong to several subfamilies, which contribute to osmoregulation, respiration, cryoprotection, anhydrobiosis, and excretion. We cloned and characterized five novel AQPs from the western tarnished plant bug, *Lygus hesperus*, a polyphagous insect pest of food and fiber crops throughout western North America. The *L. hesperus* AQPs (LhAQP1-5) belong to different phylogenetic subfamilies, have unique transcription profiles and cellular localizations, and all transport water (but not glycerol) when heterologously expressed in *Xenopus laevis* oocytes. Our results demonstrate that multiple AQPs with possible compensatory functions are produced in *L. hesperus* that likely play important roles in maintaining water homeostasis in this important insect pest.

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1. Introduction

Aquaporins (AQPs) belong to the ancient class of major intrinsic proteins (MIPs), which are integral membrane channel proteins found in all kingdoms of life. These proteins facilitate the bidirectional transfer of water or sometimes other small neutral solutes across biological membranes involved in numerous essential physiological processes (Hachez and Chaumont, 2010; Gomes et al., 2009; King et al., 2004). AQPs are best known for their diverse roles in water transport related to cell water balance regulation [reviewed in Agre et al. (1998); Carbrey and Agre, 2009], but they also transport a wide range of solutes, including urea and glycerol, hydrogen peroxide, dissolved gasses (CO_2, NO, NH_3), and certain metalloids (Cohen, 2013; Hachez and Chaumont, 2010). Whereas most solutes permeate through the monomeric channel, AQPs usually assemble as tetramers forming a fifth central pore that in some cases functions to conduct ions (Yool and Weinstein, 2002; Yool, 2007).

The functional diversity of AQPs is facilitated by key modifications to their structure. In general, members of the MIP superfamily share a number of structural features, including six helical transmembrane (TM) domains connected by five alternating extracellular/intracellular loops with intercellular amino and carboxyl termini, two canonical Asp-Pro-Ala (NPA) motifs, and the aromatic/arginine (ar/R) selectivity filter (Zardoya, 2005). Together, the canonical amino- and carboxyl-terminal halves form two tandem repeats, each containing a single NPA motif, forming the 'aquaporin' or 'hourglass' fold (Jung et al., 1994; Murata et al., 2000) and ultimately shaping the selective TM channel. 'Classical' or 'orthodox' AQPs allow water diffusion through an extremely narrow and electrostatically-selective pore that precludes the permeability of large, hydrophobic, or incorrectly charged solutes (Murata et al., 2000). In another functional class of AQPs known as the aquaglyceroporins, the composition of the ar/R constriction site and the corresponding larger, more hydrophobic three-dimensional shape of the pore enables permeation of additional solutes, such as

Abbreviations: AQP, aquaporin; MIPs, major intrinsic proteins; NPA motif, Asparagine-Proline-Alanine motif; ar/R, aromatic arginine; DRIPs, *Drosophila* intrinsic proteins; BiB, Big Brain protein; RNAi, RNA interference; PRIPs, *Pyrocoelia rufa* integral proteins; LhAQP, *Lygus hesperus* aquaporin; RACE, rapid amplification of cDNA ends; cDNA, complementary DNA; CDS, coding sequence; ORF, open reading frame; RT-PCR, reverse transcriptase polymerase chain reaction; Tni cells, cultured *Trichoplusia ni* cells; EGFP, enhanced green fluorescent protein; cRNA, complementary RNA; TM, transmembrane; RPIPs, *Rhodnius prolixus* integral proteins; DVIPs, *Dermacentor variabilis* integral proteins; LHIPs, *Lygus hesperus* integral proteins; ER, endoplasmic reticulum; EST, expressed sequence tag; SIP, short basic intrinsic proteins.

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polyols (Fu et al., 2000; Hub and de Groot, 2008). Members of a recently identified subfamily known as 'superaquaporins' or "S-aquaporins" exhibit low sequence similarity with other AQPs, altered NPA signature motifs, and anomalous subcellular localization (Ishibashi, 2006, 2009; Nozaki et al., 2008; Calvanese et al., 2013).

Whereas vertebrate AQPs have been studied extensively [reviewed in King et al. (2004); Verkman 2008; and Ishibashi et al., 2009], far less attention has been given to invertebrate AQPs. In arthropods, AQPs are involved in regulating the movement of high volume liquid diets, osmoregulation, respiration, cryoprotection and anhydrobiosis [reviewed in Campbell et al. (2008); Spring et al., 2009; and Cohen 2013]. Genome sequencing indicates that insects may contain up to 5-8 genes encoding putative aquaporin-like transcripts (Campbell et al., 2008; Drake et al., 2010), but the complete repertoire from any single species is yet to be fully characterized. Multiple functional AQPs have been found in several insects, including three from *Aedes aegypti* (Duchesne et al., 2003; Drake et al., 2010) and *Bombyx mori* (Kataoka et al., 2009a; Azuma et al., 2012), and two from the oriental fruit moth, *Grapholita molesta* (Kataoka et al., 2009b), the pea aphid, *Acyrthosiphon pisum* (Shakesby et al., 2009; Wallace et al., 2012), the sleeping chironomid, *Polypedilum vanderplanki* (Kikawada et al., 2008), and *Drosophila melanogaster* (DRIP and BiB) (Kaufmann et al., 2005; Yanochko and Yool, 2002). For *A. aegypti*, studies have shown that a water-specific DRIP (AeaAQP) is involved in water movement in Malpighian tubules and tracheoles (Duchesne et al., 2003). RNA interference (RNAi) knockdown further revealed a role in diuresis in Malpighian tubules for three of the six putative AQPs (Drake et al., 2010). Functional AQPs from *B. mori* include, AQP-Bom1 (a water-specific DRIP), AQP-Bom2 (an aquaglyceroporin), and AQP-Bom3 (a water-specific PRIP), all of which play important roles in water reabsorption and recycling within the cryptonephric rectal complex (Kataoka et al., 2009a; Azuma et al., 2012). *G. molesta* has a functional water-specific DRIP (AQP-Gra1) and an aquaglyceroporin (AQP-Gra2) (Kataoka et al., 2009b). In *A. pisum*, ApAQP1 was shown to be a water-specific AQP (Shakesby et al., 2009) whereas ApAQP2 exhibited aquaglyceroporin activities, transporting water and glycerol in *Xenopus* oocytes (Wallace et al., 2012). Two *P. vanderplanki* AQPs (PvAQP1 and PvAQP2) facilitated water permeation, but not transport of glycerol in *Xenopus* oocytes (Kikawada et al., 2008). Among the *D. melanogaster* AQPs characterized are a water-selective AQP called *Drosophila* integral protein or DRIP, which is involved in excretion and osmoregulation (Kaufmann et al., 2005), and the novel AQP known as Big Brain (DmBIB), which does not permeate water, but functions as a cation channel (Yanochko and Yool, 2002). Other arthropods having at least one functionally characterized AQP include: *Cicadella viridis* (La Caherec et al., 1997); *Rhodnius prolixus* (Echevarria et al., 2001); *Anopheles gambiae* (Liu et al., 2011); *Blattella germanica* (Herraiz et al., 2011); *Eurosta solidaginis* (Philip et al., 2011); *Bemisia tabaci* (Mathew et al., 2011); *Belgica antarctica* (Goto et al., 2011); *Anomala cuprea* (Nagae et al., 2013); and the ticks, *Dermacentor variabilis* (Holmes et al., 2008), *Rhipicephalus sanguineus* (Ball et al., 2009), and *Ixodes ricinus* (Campbell et al., 2010).

Numerous species within the hemipteran family Miridae are important agricultural pests causing crop damage from feeding with specialized piercing-sucking mouthparts (Wheeler, 2001). One such mirid bug is *Lygus hesperus* Knight (the western tarnished plant bug), a highly polyphagous pest causing economic losses in numerous cropping systems in western North America (Wheeler, 2001). Lygus bugs are "cell rupture" feeders (previously termed "lacerate-and-flush") that first puncture host tissue and inject saliva containing digestive enzymes (Miles, 1987; Strong and Kruitwagen, 1968; Shackel et al., 2005; Backus et al., 2007). The pre-digested fluid is then pumped through the food canal for absorption of nutrients primarily within the midgut epithelium of the alimentary tract (Wheeler, 2001; Habibi et al., 2008). Some hemipterans use specialized biochemical processes or gut morphology (e.g. filter chambers) to abate osmotic stress and remove excess dietary fluid (Douglas, 2006; Gullan and Cranston, 2005; Lehane and Billingsley, 1996; Hubert et al., 1989; Mathew et al., 2011). Lygus bugs lack the specialized filter chamber or gastric cecae of sap feeding hemipterans, and instead have a simple, cimicomorph type alimentary tract typical of other mesophyll feeders (Goodchild, 1966; Habibi et al., 2008).

Here, we characterize five AQPs (LhAQP1-5) from *L. hesperus*. Based on sequence conservation and phylogenetics, bioinformatic predictions, cellular and tissue localization, temporal expression, and functional analysis, the LhAQPs belong to different subfamilies but all function as water-specific channel proteins. Localization and transcription profiles for LhAQPs provide clues about the putative roles these functionally redundant AQPs may play in maintaining *L. hesperus* water homeostasis. The combination of standard molecular cloning techniques and the mining of transcriptome data for AQPs provides an unprecedented opportunity to comprehensively identify and characterize AQPs within *L. hesperus*.

2. Materials and methods

2.1. Insects

A laboratory colony of *Lygus hesperus* was maintained at the USDA-ARS U.S. Arid Land Agricultural Research Center in Maricopa, AZ, USA. The colony was fed green beans and artificial diet at 25 °C under 20% humidity and with an L14:D10 photoperiod (Debolt, 1982; Patana, 1982).

2.2. cDNA isolation and 5′-RACE of LhAQPs

Total RNA was extracted from 100 mg of *L. hesperus* adults and 2nd–3rd instar nymphs using TRIzol® reagent (Invitrogen-Life Technologies, Carlsbad, CA). cDNA was produced using random hexamer primers and SuperScript III First-strand Synthesis System (Invitrogen-Life Technologies) according to manufacturer's recommendations. PCR primers 1LhAQP5-8LhAQP3 (Table 1) were designed using Primer3Plus (Untergasser et al., 2007) from four putative *Lygus lineolaris* AQPs obtained from a *L. lineolaris* transcriptome sequencing project (O.P. Perera, unpublished). Four partial LhAQPs (named LhAQP1-4) were PCR amplified using ExTaq DNA polymerase premix (Takara-Clontech, Palo Alto, CA) and primer pairs (Table 1). Products were electrophoresed on a 1% agarose gel and visualized using SYBR Safe (Invitrogen-Life Technologies). Bands were gel-purified using Montage DNA Gel Extraction Kit (EMD Millipore, Billerica, MA) and ligated into pCR2.1-TOPO using TOPO TA Cloning Kit (Invitrogen-Life Technologies). Plasmid DNA was propagated in OneShot TOP10 chemically competent *Escherichia coli* and purified using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Inserts were sequenced with T7 and M13 Reverse vector primers by the Arizona State University DNA Core Lab (Tempe, AZ).

The 5′ and 3′ ends of LhAQP1-4s were identified by rapid amplification of cDNA ends (RACE) using the SMARTer RACE cDNA Amplification Kit (Clontech). At least two sense and two antisense primers were designed for each partial LhAQP consensus sequence (Table 1). The primers were used with Universal Primer A (Clontech) in fully nested PCR to amplify 5′ and 3′ ends (Table 1). PCR products were sub-cloned into pCR2.1-TOPO (Invitrogen-Life Technologies) and sequenced as indicated above.