AQUAPORIN IDENTIFICATION AND LOCALIZATION IN THE ASIAN CITRUS PSYLLID (*Diaphorina citri*) AND THE POTATO PSYLLID (*Bactericera cockerelli*)

A Thesis

by

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ABSTRACT

Aquaporin is a family of major intrinsic proteins found in every living organism which play a vital role in channeling water through cellular membranes. These “water channels” have many roles within insects, some of which involve a complete adaptation of the protein to a novel function not associated to the passive movement of water. Particular interest has been given to insects with unique osmotic challenges, such as those that feed on blood or high water content food sources such as xylem. However, there is little knowledge about aquaporins from phloem feeding insects, which are believed to play a vital role in osmoregulation within the insect as it feeds on hyperosmotic phloem.

Because of this, we identified aquaporins within phloem feeding hemipterans by identifying expressed sequence tags from available databases and assembled these into unigenes for analysis. This analyses assessed how many aquaporins were found in phloem feeders and in which organs or tissues those aquaporins were expressed. We then focused on characterizing psyllid aquaporins. We identified four aquaporins in the potato psyllid *Bactericera cockerelli* and two aquaporins in the Asian citrus psyllid *Diaphorina citri*. Using semi-quantitative RT-PCR we investigated the expression pattern of two *B. cockerelli* and two *D. citri* aquaporin candidates. For one of the *B. cockerelli* candidate aquaporin we further assessed its expression by *in situ* hybridization.

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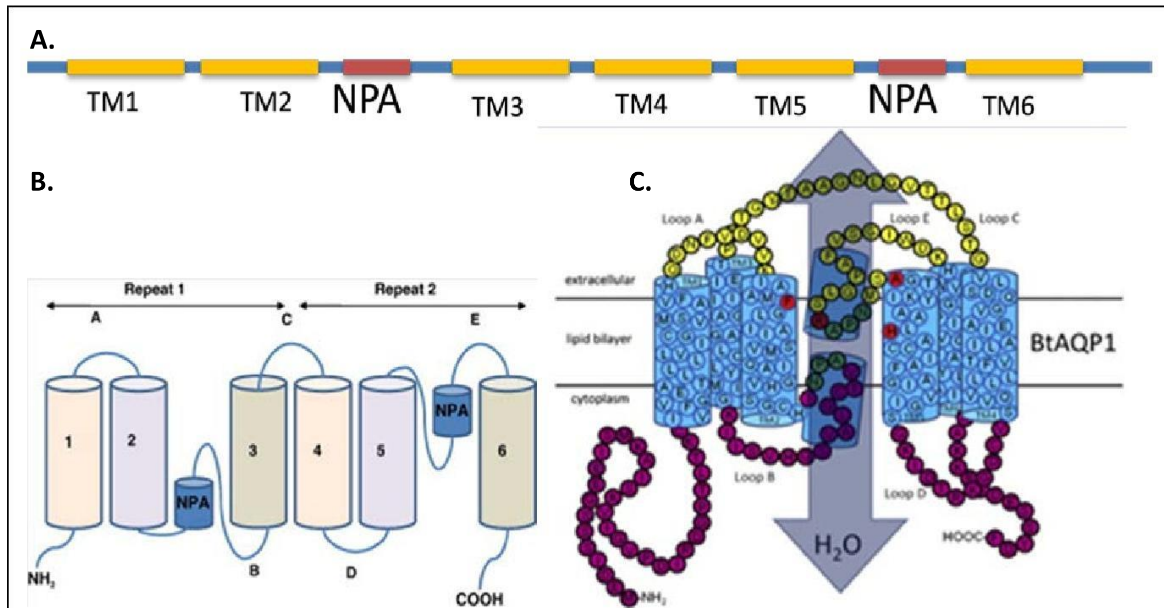
1. INTRODUCTION

1.1 History and Structure of Aquaporins

Until the mid-1980’s, diffusion of water across the cell membranes surpassing normal membrane diffusion rates had been observed in certain cells but could not be explained. This process was elucidated by Peter Agre, who identified a membrane protein in humans, later called “aquaporin”, which functioned as a membrane water channel (Agre et al. 1987; Agre 2009). Aquaporins have since been found in every living organism including plants, animals, fungi and bacteria (Calamita et al. 1995; Johanson et al. 2001; Pettersson et al. 2005) with 2 members identified in *Escherichia coli*, 5 in fungi, 13 found in humans and up to 35 in plants (Johanson et al. 2001; Agre & Kozono 2003; Morishita et al. 2004). In bacteria, only one aquaglyceroporin (see below) and one classical aquaporin exist, but in more complex organisms more members of the aquaporin family can be found.

Aquaporin proteins are highly conserved even among kingdoms. All aquaporins share a distinctive structure: six transmembrane spanning alpha helixes (TM1 to 6) separated by five loops (A-E). Loops B and E contain a “NPA” region comprising a span of relatively hydrophobic amino acids Asparagine-Proline-Alanine, with one NPA motif each (see Figure 1 A and B). Each NPA region is followed by a half-transmembrane helix (Figure 1B). The six alpha helixes form a pore through the membrane with both half-helices folded into the pore where the two NPA regions selectively allow molecules through the pore (Figure 1C). This high conservation probably relates to their critical role in cellular osmotic regulation as well as extensive shuttling of water within tissues (Gomes et al. 2009).

Figure 1: Aquaporin Structural Motif



A) Structural motif of a typical aquaporin, with six transmembrane regions (TM1-6) and two highly conserved Asparagine-Proline-Alanine motifs located between TM 2-3 and TM 5-6. **B) Diagram of the protein structure of an unfolded aquaporin,** indicating the different protein alpha helix transmembrane regions and protein "loops." Image from Mathew et al. (2011) **C) Diagram of *Bemisia tabaci* Aquaporin 1** indicating the orientation of the transmembrane regions within the pore made by the six transmembrane alpha helixes. Image from Gnomes et al. (2009).

1.2 Mammal Aquaporins

Since the first aquaporin identified was a human aquaporin, and due to their potential clinical importance, the majority of knowledge has been developed studying mammalian aquaporins (Agre et al. 2004). Though initially identified as solely water transporters, aquaporins have also been found to shuttle other molecules through membranes, such as cations, glycerol, and metalloids like arsenic (Anthony et al. 2000; Liu et al. 2002). Based on differences in structure and solute selectivity, the 13 mammalian aquaporins are classified in three subfamilies. The traditional (Class 1) aquaporins are water selective (AQP0, 1, 2, 4,5, 6 and 8) though some have been found to transport anions (AQP6) or free radicals (AQP8) (Ikeda et al. 2002; Bienert et al. 2007). The aquaporins in the

second subfamily (Class 2) are called aquaglyceroporins since they also transport small neutral solutes like urea, glycerol and metalloids (AQP3, 7, 9 and 10) (Bienert et al. 2008). The aquaporins in the third subfamily (Class 3), also known as superaquaporins, include AQP11 and 12 and are classified together based on the presence of only 1 NPA motif and a variant motif; the second motif is NPT for AQP12 (Itoh et al. 2005) and NPC for AQP11 (Yakata et al. 2007). Table 1 lists the aquaporins identified in mammals and the phenotypes associated with the null expression of aquaporins. Not surprisingly, aquaporins are found expressed in tissues or organs in which water transport is important such as in the kidney which must filter more than 180 liters of water per day, the brain, the eye or secretory glands. Aquaglyceroporins are expressed in tissues or organs where glycerol transport is necessary, for example, in adipocytes where triglycerides can be metabolized into fatty acids and glycerol, or in hepatocytes for gluconeogenesis.

Table 1: Aquaporin Classes, Phenotypes in Null Mice and Humans (Ishibashi et al. 2009)

Protein Name	Gene Name	Class	Function
Lens fiber major intrinsic protein	AQP0	1	Expressed in lenses and retina. Might play a role in water, nutrient and oxygen transport in lenses. Disruption produces cataract in mice and humans
Aquaporin-1	AQP1	1	Wide expression pattern including kidney. Mutation causes mild urine concentration defect (mild diabetes insipidus)
Aquaporin-2	AQP2	1	Mainly expressed in kidneys, role on antidiuretic hormone sensitive water uptake. Disruption causes severe diabetes insipidus

Table 1 Continued

Protein Name	Gene Name	Class	Function
Aquaporin-3	AQP3	2	Wide expression pattern. Facilitates water exit in the collecting duct in the kidney and is important for urine concentration. Also involved in skin moisture and regeneration.
Aquaporin-4	AQP4	1	Highly expressed in glia cells in the brain. Also expressed at skeletal myocytes, gastric parietal cells, and cortical collecting duct playing a larger role in urine concentration.
Aquaporin-5	AQP5	1	Widely expressed in exocrine glands.
Aquaporin-6	AQP6	1	Expressed in the kidney, cerebellum and synaptic vesicles.
Aquaporin-7	AQP7	2	Expressed in testis (role not clear), adipose tissue (might serve as an exit pathway for glycerol) and brush border membrane of the proximal tubule in the kidney (potential glycerol reabsorption role).
Aquaporin-8	AQP8	1	Expressed in testis and the pancreas. Role not clear.
Aquaporin-9	AQP9	2	Expressed in liver and leukocytes.
Aquaporin-10	AQP10	2	Expressed in the duodenum and jejunum.
Aquaporin-11	AQP11	3	Widely expressed: highest expression in the testis and thymus, and moderate in the kidney, intestine, and liver. Intracellular localization.
Aquaporin-12	AQP12	3	Expressed intracellularly in the pancreas.

1.3 Insect Aquaporins

Due to their life styles, insects must maintain water homeostasis and osmoregulation (blood or plant sap feeding) as well as overcome challenges like dessication and cryoprotection. This is done in part by effectively shuttling water between compartments. Insect aquaporins have not been as intensely studied as aquaporins in mammals, and very little is still known about aquaporins in insects (Spring 2009). Data mining of sequenced insect genomes yielded between 3 and 8 aquaporins (Table 2), a notably smaller number than the 13 found within mammals (Drake et al. 2010).

Table 2: Repertoire of Aquaporin Genes in Insects

Insect Species	Number of aquaporin genes	References
Drosophila melanogaster Fruit fly	8	(Adams et al. 2000)
Aedes aegypti Yellow fever mosquito	6	(Drake et al. 2010)
Anopheles gambiae Malaria mosquito	7	(Liu et al. 2011)
Pediculus humanus Human lice	6	(Pittendrigh et al. 2006)
Acyrthosiphon pisum Pea aphid	3	(Richards et al. 2010)

Though the individual insect aquaporins are characterized as having functions similar to that of aquaporin subfamilies in mammals (such as behaving like an aquaglyceroporin), insect aquaporins were initially divided in three separate phylogenetic subfamilies: Drosophila Intrinsic Proteins (DRIPs), *Pyrocoelia rufa* Intrinsic Proteins (PRIPs) Big Brain intrinsic proteins (BIBs) (Campbell et al. 2008).

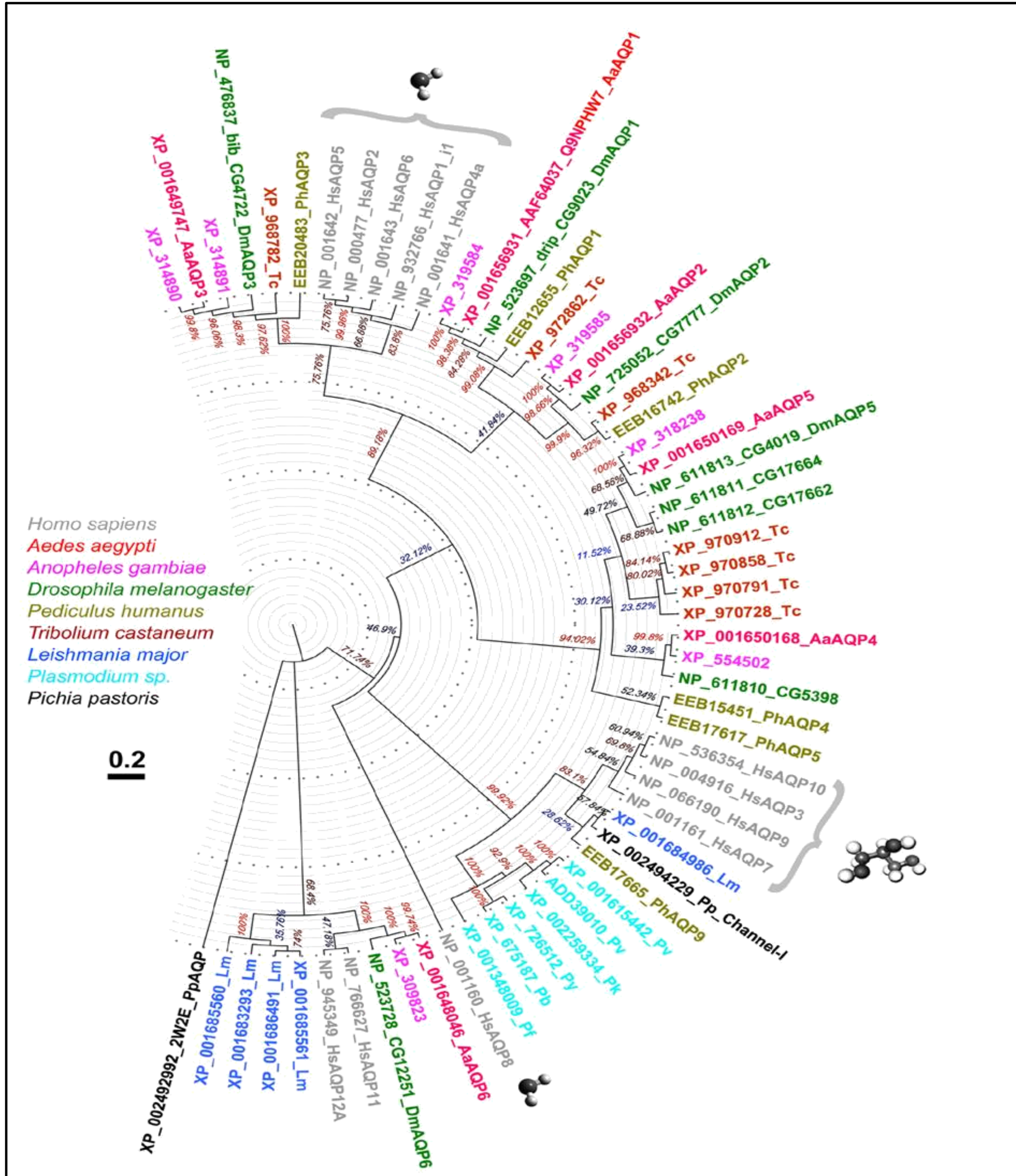
DRIPs and PRIPs are considered “classic” aquaporins in that they are believed to function largely as water channels and assist in osmoregulation within insects (Campbell et al. 2008). BIBs are neurogenic aquaporins that are similar to the other aquaporins, yet have a primary role in neural development through cell-cell communication (Doherty et al. 1997). The annotation of full sequenced genomes for several insects has expanded the list of insect aquaporins, and today it is becoming evident that the initial classification on BIB, PRIP and DRIP needs to be updated.

Dipteran aquaporins

Studies of insect aquaporins have been mainly carried out in dipterans, in the model insect *Drosophila melanogaster* and in mosquitoes (*Aedes aegypti, Anopheles gambiae*) (Pietrantonio, Jagge et al. 2000; Drake et al. 2010). Due to wide range of genomic data available to study *D. melanogaster*, this was the first insect where an aquaporins could be easily identified and characterized. Eight putative aquaporins have been identified in *D. melanogaster*, two of which were quickly characterized and are the template for the subfamily separation of insect aquaporins: DRIP and BIB. Among *D. melanogaster* putative aquaporins, only 4 encode both NPA boxes: DRIP, Aqp4019, Aqp7777 and BIB. Figure 2 shows a dipera aquaporin protein neighbor joining tree.

DRIP (DmAQP1) was the first *D. melanogaster* aquaporin identified. It was cloned from an adult Malpighian tubule cDNA library (Dow et al. 1995). DRIP is most similar to mammalian water-specific AQPs, and is most closely related to human AQP4 (44% sequence similarity). It was identified as a water exclusive transporting protein highly expressed in the Malpighian tubule of the fly. The Malpighian tubule is a blind ended organ that connects with the hindgut and enables fluid excretion. Data obtained in *Rhodnius prolixus* showed that DRIP aquaporin was highly expressed in the Malpighian tubule which supported its major function in insect homeostasis (Maddrell 1969; Echevarria et al. 2001; Kaufmann et al. 2005b). Later, analysis of DRIP gene expression in *D. melanogaster* showed a dynamic pattern throughout development, but always in organs in which high water transport was expected (Kaufmann et al. 2005a). Today, it is believed that DRIP plays a critical role in fluid homeostasis in the fly

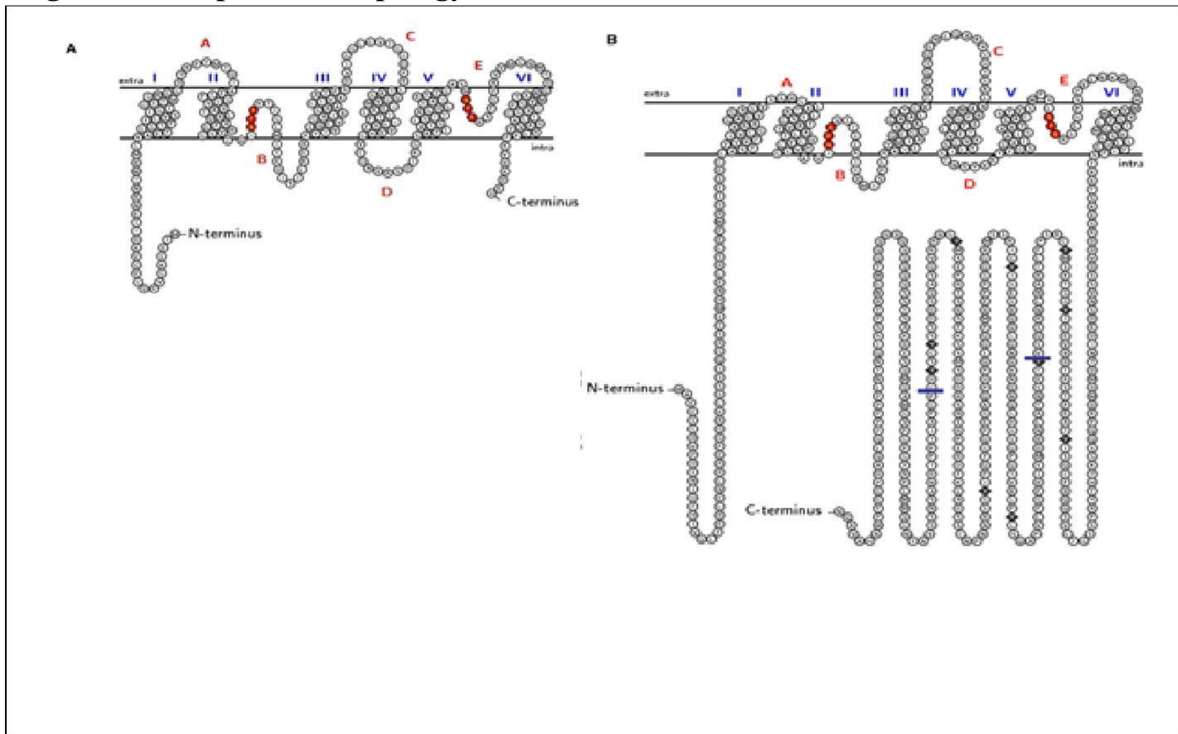
Figure 2: Evolutionary Relationships of AQPs from Selected Organisms



Evolutionary relationships of AQPs from selected organisms with available sequenced genomes. The evolutionary history was inferred using the Neighbor-Joining method.The bootstrap consensus tree inferred from 5000 replicates represents the evolutionary history of the taxa analyzed. Evolutionary distances were computed using the Poisson correction method. Image provided by Drake et al. 2010**.**

embryo and adult. CG7777 (DmAQP2) is expressed in the brain and in the segmental ganglia in embryos (Kaufman 2005), salivary glands and digestive system of larvae and adults, as well as in the germarium and early egg chambers of females and accessory gland of males. CG4019 (DmAQP5) is expressed in the embryo body wall and visceral muscles, in the Malpighian tubule and fat body of larvae and adults, and the adult salivary gland and female spermatheca.CG17664 is highly expressed in the adult digestive system, in particular in the Malpighian tubules, and in the male accessory gland. This gene only encodes the first NPA, the second box encodes NPV. CG17662 is only expressed at low levels in the midgut of third instar larvae. This gene only encodes the first NPA, the second conserved region encodes for NPT.CG5398 is moderately expressed in the L3 imaginal discs and highly expressed in male testis. This gene only encodes the second NPA box, the first conserved motif being NPC. CG12251 (DmAQP6) is expressed in the adult head, hindgut, crop, and in the male reproductive system accessory gland. This gene does not encode any of the NPA boxes. DmAQP6 is most similar to mammalian AQP11.BIB, or DmAQP3, was identified as a major intrinsic protein which has a notably longer C-terminal protein region that extends into the cytoplasm of the cell (Figure 3). The BIB protein appeared to transport cations, but little to no water (Yanochko & Yool 2002). Instead, the protein is seen as having important functions in intercellular communication between neural cells in the developing insect (Lehmann et al. 1983; Brand & Campos-Ortega 1988).

Figure 3: Comparative Topology of DRIP and BIB Proteins



Comparative topology prediction of unfolded (A) AeaAQP (DRIP-like) and (B) AeaBiB in the cell plasma membrane**.** Of note is the large C-terminal region of the BiB aquaporin, though other structural portions of the protein are relatively unchanged (Campbell et al. 2008).

Due to the apparent importance of insect aquaporins in homeostasis, these proteins have been studied in detail in mosquitoes. Female mosquitoes ingest a blood meal for yolk protein production. When the insect intakes a blood meal, the weight of the meal makes flight difficult and leaves the insect susceptible to predation if it is unable to fly, thus the rapid excretion of the water from the blood meal is evolutionarily advantageous to the mosquito (Sungvornyothin et al. 2001). Forty percent or more of the water and sodium found in the blood meal can be discharged within the first hour of feeding (Clements 1992). Thus, aquaporins are believed to play a crucial role in female mosquito water excretion after a blood meal. Several studies have characterized the expression of the six aquaporins in the yellow fever

mosquito *Aedes aegypti* and have shown that aquaporins in this insect have a wide range of expression in different tissues (Pietrantonio, Jagge et al. 2000, Drake et al. 2010). Table 3 summarizes the findings for *A. aegypti*, and it comparisons with *D. melanogaster*.

Table 3: *Aedes aegypti* Homology

***Aedes aegypti* AQP**	***Drosophila melanogaster*-like AQP**	**Expression in Mosquito**
AaAQP1	*Drosophila* DRIP	Expressed in all organs, highly expressed in tracheolar cells
AaAQP2	DmAQP2	Strong expression in the MTs, midgut, and ovaries
AaAQP3	*Drosophila* BiB	Weak expression in Malpighian tubule, ovaries, midgut
AaAQP4	DmAQP4	Malpighian tubule
AaAQP5	DmAQP5	All organs except ovaries
AaAQP6	DmAQP6	Foregut

Homology of *Aedes aegypti* aquaporins to *Drosophila melanogaster* aquaporins, and their localization within the mosquito. Homology is based upon the similarity of sequences using the Neighbor-Joining method shown in Figure 3. Localization of aquaporins was completed by quantitative real time PCR (Pietrantonio, Jagge et al. 2000, Drake et al. 2010).

Hemipteran aquaporins

Hemiptera is the only order containing species that can feed exclusively from plant phloem. Phloem feeding poses two barriers that animals have to overcome, first, low abundance of essential amino acids (overcome by presence of endosymbionts), and second, a high osmotic pressure caused by the sugar rich diet. Phloem osmotic pressure is two to five times greater than the osmolarity of insect tissues. Therefore, without adaptations to decrease the osmotic pressure of the phloem ingested, insects would dehydrate as they feed (Douglas 2006). The anatomical structure of hemipterans' gut, where the hindgut is in close contact with the foregut, is believed to allow water to be shuttled from the hindgut into the foregut to dilute incoming phloem and thereby avoid insect dehydration (Shakesby et al. 2009) hindgut is in close contact with the foregut, is believed to allow water to be shuttled from the hindgut into the foregut to dilute incoming phloem and thereby avoid insect dehydration (Shakesby et al. 2009). The "water shuttling" across the epithelium foregut and hindgut linings is thought to be done by an aquaporin (Shakesby et al. 2009). Among phloem feeding insects, two DRIP-like aquaporins have been characterized, one in the pea aphid *A. pisum* (ApAQP1) and one in the whitefly *Bemisia tabaci* (BtAQP1) (Shakesby et al. 2009; Mathew et al. 2011). The first study of the role of aquaporins in phloem feeding was published in 2009 and was conducted in *Acyrthosiphon pisum*, the pea aphid (Shakesby et al. 2009). In 2011 the first aquaporin in whiteflies was reported (Mathew et al. 2011) and in 2012 a second aquaporin was identified in *A. pisum* (Wallace et al. 2012).

ApAQP1 expression was detected by *in situ* hybridization of RNA within aphid embryo gut and salivary glands, and the adult gut (Shakesby et al. 2009). The *B. tabaci* aquaporin protein was shown through immunofluorescence of the protein in dissected gut tissue, only giving us information of expression within the gut (Mathew et al. 2011). In both insects, however, the aquaporin expression was localized in a specialized region of the gut where proximal and anterior gut regions come in close contact with each other. This indicates that those aquaporins might play a role in water cycling in the gut and that other hemipterans might also encode an aquaporin involved in water cycling in the gut.

The second aphid aquaporin, ApAQP2, that has recently been studied was found highly expressed within the *A. pisum* bacteriocyte (Wallace et al. 2012). The bacteriocyte is an important organ that houses the primary endosymbiont (*Buchnera aphidicola* in the case of aphids). This aquaporin was found to transport water and neutral polyol substrates; therefore, it might play a role in the interaction of aphids and endosymbionts (Wallace et al. 2012).

However, because their essential role in water transport, aquaporins might also be involved in other functions such as reproduction or salivation. Both functions are essential for insect survival and/or proliferation and represent target functions to disrupt in order to control pest populations. Salivation is also of particular importance for pathogen transmission, since plant inoculation usually happens when the insect salivates in the plant tissues during feeding.

1.4 Study System

The order Hemiptera comprises many ecologically and economically important insect species, including aphids, cicadas, leafhoppers and shield bugs. This includes a wide diversity of liquid feeders, including plant sap (phloem and xylem) as well as blood feeders. The defining feature of this group is the presence of piercing sucking mouthparts that are used for uptaking liquid from their food source. These insects include many important plant pests, such as insects in the suborders Sternorrhyncha (Aphids, psyllids), Auchenorrhyncha (cicadeas), and Heteroptera (shieldbugs), since they are highly damaging to crop plants, have a high reproductive rate, and some can transmit harmful pathogens from plant to plant (Rocha-Pena et al. 1995; Bundy et al. 2000; Ahmad et al. 2002).

The hemipteran phloem feeders Asian citrus psyllid (*Diaphorina citri*) and the potato psyllid (*Bactericera cockerelli*) are both economically important insect pests due to their ability to act as a vector several bacterial plant pathogens, as well as their destructive feeding on their crops of choice (Halbert & Manjunath 2004b; Hansen et al. 2008).

Diaphorina citri selectively feeds on citrus plants, and can cause discoloration of the leaves of the plant as well as stunting of the plant if in high enough numbers (Catling 1970). *D. citri* more importantly transmits a disease, called "citrus greening" that causes millions of dollars in damage and/or expensive control measures by citrus growers. Citrus greening causes the greening of ripe fruit on the tree and the eventual death of the tree after a few years. (Gasparoto et al. 2012).

The potato psyllid, *B. cockerelli*, feeds on solanaceous crops, and is a notable pest on potatoes. It also vectors "*Candidatus* Liberibacter solanacearum" which causes the abnormal growth of the plant, early death of plants, and in potatoes it induces the stripping of tubers upon frying, hence the name of the disease "zebra chip" (Abad et al. 2009).

1.5 Objectives

The overall objective of this thesis is to conduct an exploratory study of psyllid aquaporins. Because of the scarcity of genomic resources for hemipterans in general and psyllids in particular, first, using the available hemipteran genomic information from NCBI, we identified Expressed Sequence Tags (ESTs) with sequence similarity to insect aquaporins. These ESTs were then assembled into unigenes in order to draw a catalog of hemipteran aquaporins and to have an estimate of how many aquaporins it could be expected in psyllids. Secondly, the full coding sequence of candidate psyllid (potato psyllid and Asian citrus psyllid) aquaporins was obtained by Rapid Amplification of cDNA Ends (RACE). The full coding sequence of the aquaporins was used for *in silico* analyses to predict particular features of these aquaporins. Thirdly, using semiquantitive RT-PCR we evaluated the expression of four candidate aquaporins in different life stages and tissues.

2. IDENTIFICATION OF AQUAPORIN CANDIDATE GENES IN HEMIPTERA

2.1 Introduction

Few aquaporins have been identified within insects. The majority of insect aquaporins have been characterized within Diptera (Campbell et al. 2008) due to the availability of fully sequenced genomes and other genomic resources that provided the sequence of candidate aquaporins for analysis (Kaufmann et al. 2005b; Drake et al. 2010). Transcriptomic resources have been extensively developed in some dipteran species and could also be used as a resource to identify aquaporins and other genes in the respective species. As previously described, the analysis of the Diptera genomes identified around eight putative aquaporins per species, of which several have been characterized (Kaufmann et al. 2005b; Drake et al. 2010).

Hemiptera, though being one of the largest insect orders with over 50,000 species, has very few well characterized aquaporins, in part because genomic information for insects in this order are not as developed as in other orders. For instance, in 1995, a *Cicadella viridis* aquaporin was cloned from a cDNA library (Beuron et al. 1995) and later named AQPcic. AQPcic RNA and protein expression was shown in the filter chamber (Beuron et al. 1995), where the protein is believed to be involved in the water-shunting complex that rapidly transfers large excess of water ingested by xylem feeders from the initial midgut to the terminal midgut (Hubert et al. 1989). In 2010 the genome of the first Hemiptera, *Acyrtosiphon pisum*, was released and annotated (Richards et al. 2010). An important effort to produce cDNA libraries was performed by the aphid community in order to help with the annotation of this genome (Sabater-Munoz et al. 2006; Shigenobu et al. 2010). The first aphid aquaporin ApAQP1 was published in 2009 (Shakesby et al. 2009) and is expressed in the insect gut and salivary glands. Since the *A. pisum* genome has been annotated, new aquaporins can be easily identified. Recently, a new aquaporin has been characterized (Wallace et al. 2012) and shown to be expressed in the bacteriocyte and fat body. However, no global analysis of aphid aquaporins has ever been performed; therefore, it remains unclear how many

aquaporins are encoded in hemipterans, or how aquaporins relate between the various species within Hemiptera. The other hemipteran aquaporin studied was *Bemisa tabaci* BtAQP1, which was shown to be expressed in the insect filter chamber. With the development of transcriptomic platforms, the decreasing costs of sequencing and the advances in technology allowing the use of minute starting material, Expressed Sequence Tags (ESTs) collections have been developed for many hemipterans which can be used to identify candidate genes. ESTs are sequenced portions of transcribed genes (Parkinson & Blaxter 2004). These EST libraries are readily available sources of genomic information that can be used for aquaporin identification (NCBI 2004). Due to the diversity of Hemipera and the lack of understanding of how many aquaporins exist in hemipterans, our initial work focused on the discovery of aquaporins within Hemiptera as a whole to better understand what numbers and types of aquaporins exist in the order.

2.2 Methods

EST identification

NCBI EST were searched using nine *D. melanogaster* aquaporin sequences (Figure 4) using the tblastn program (Al-Jabr 1999; Gertz et al. 2006) in 2010 (2 isoforms are encoded by DmAQP1). The search query was limited to the EST database within Hemiptera (taxid:7524). EST sequences with an E-value lower than 1e-10 was selected for further analysis.

EST assembly and annotation

All ESTs were then filtered and assembled using ArthropodEST (http://arthropodest.ksu.edu/) (Chellapilla 2012). The analysis pipeline involved four steps (seen also in Figure 4):

1) Trimming: poly A/T and undetermined bases were removed.
2) Cleaning: vector contaminants were removed using the Seqclean program, sequences shorter than 100 base pairs or of low quality were discarded.
3) RepeatMasking program was used to screen for repeats and low complexity

sequences against *Drosophila*

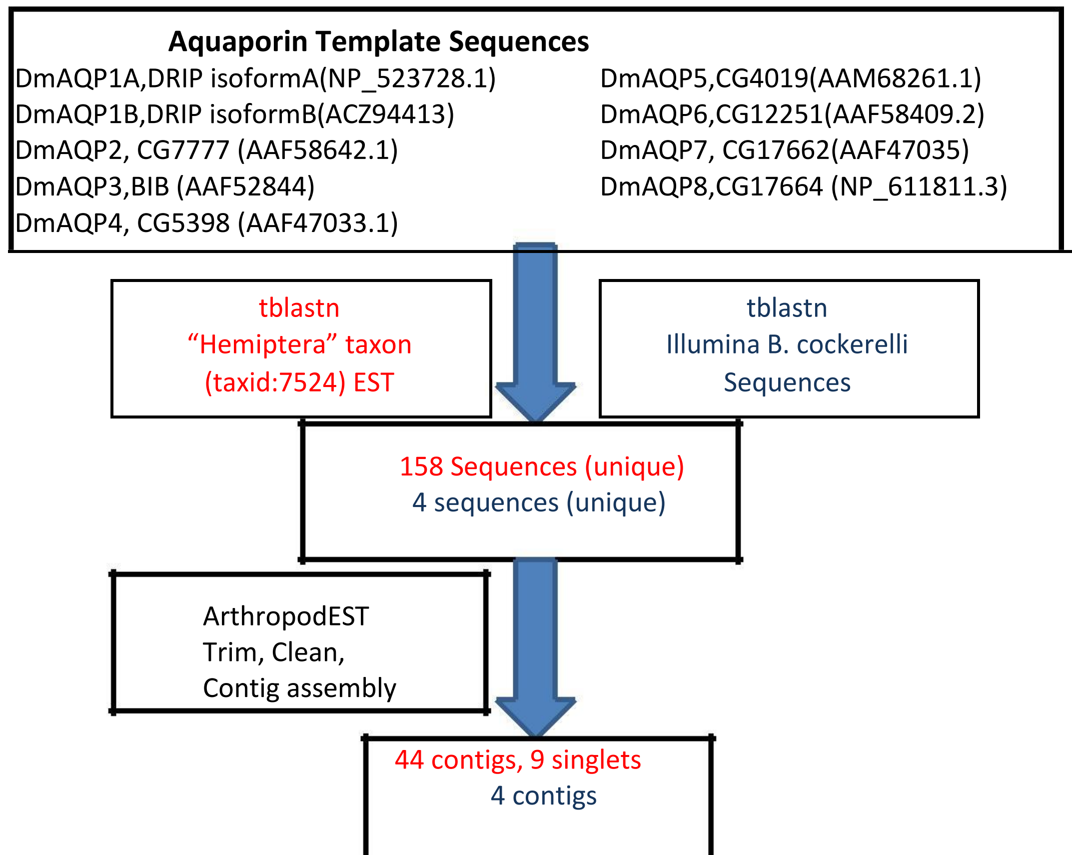
4) Contig assembly involved using the default clustering values of 94% overlap of at least 30 base pairs, as well as the default assembly values.

The resulting unigenes (contigs and singlets) were then analyzed using blastx for sequence similarity to the entire database of NCBI.

B. cockerelli sequences

A *B. cockerelli* transcriptomic resource was generated in 2011. This resource was obtained by Illumina sequencing cDNA from full body adult insects (Nachappa et al. 2012). This resource was queried and candidate aquaporins identified in the same manner as the other hemiptera sequences.

Figure 4: Diagram Showing the EST Analysis Pipeline



2.3 Results

The searchable quantity of Hemiptera ESTs in NCBI included 688,516 sequences. A total of 158 sequences were identified in the Hemiptera EST database with a high sequence similarity to aquaporins (Table 4 shows EST information for the hemipteran species for which ESTs with similarities to aquaporins were identified). After processing those sequences using the ArthropodEST pipeline, 111 ESTs were retained and were assembled into 28 unigenes (contigs and singletons). BlastX searches confirmed similarities with aquaporins for 25 of those unigenes.

The analysis of the potato psyllid Illumina dataset yielded 4 contigs showing similarities to aquaporins. Between 1 and 4 unigenes showing similarities to aquaporins were identified among the hemipteran species for which transcriptomic resources were available (Table 5). Four unigenes with similarities to aquaporins were identified for *A. pisum.* However, 2 of those unigenes (3 and 4) showed similarity to the same predicted aquaporin (neurogenic protein big brain-like) without sequence overlap, indicating that these two unigenes might in fact represent a single gene. *In silico* analysis of *A. pisum* genome identified a total of 3 putative aquaporins encoding for 4 different proteins (NM_001145904.1 encodes 2 splicing forms). Therefore, for *A. pisum* we identified each putative aquaporin but we only identified one of the isoforms encoded by NM_001145904.1. Similarly for *A. gossypii* we identify 3 putative aquaporins and only 2 for *M. persicae*. Interestingly, 4 unigenes with similarities to different aquaporins were identified for the *B. cockerelli*. It is therefore possible that aphids are not good models for other hemipteran species.

Among the identified unigenes, twelve seemed to be full length (*in silico* translation included putative 5'UTR region, methionine, coding sequence with a stop codon and 3'UTR sequence). Those sequences included 3 *B. cockerelli,* 2 *A. pisum*, *H. vitripennis*, and 1 *A. gossypii*, *M. persicae*, *D. citri*, *N. lugens*, *P. maidis* putative aquaporin sequences.

Table 4: Summary of Hemipteran EST Collections Found in NCBI from which Putative Aquaporin Candidates were Identified

Species	Common name	Number ESTs	Tissues	References
Acyrthosiphon pisum	Pea aphid	214,834	1-Salivary gland 2- Head 3-Antenna 4-Digestive tract 5-Bacteriocyte 6-Whole body	(Sabater-Munoz et al. 2006)
Myzus persicae	Green peach aphid	27,728	1-Whole insect 2- Salivary gland 3-Digestive tract 4-Head	(Ramsey et al. 2007)
Aphis gossypii	Cotton aphid	88,851	1-Gut 2-Mixed tissues (whole insects, midgut, nervous system)	
Diaphorina citri	Asian citrus psyllid	19,598	1-Testes 2-Midgut 3-Whole body	(Hunter et al. 2009)
Bactericera cockerelli	Potato/tomato psyllid	47,399	Whole Body	(Nachappa et al. 2012)
Nilaparvata lugens	Brown planthopper	118,020	Whole body	(Xue et al. 2010)
Maconellicoccus hirsutus	Pink hibiscus mealybug	7,669	Whole body	
Rhodnius prolixus		16,105	1-Midgut 2-Folicular epithelium 3-Central nerve system 4-Whole body	(Ursic-Bedoya & Lowenberger 2007; Medeiros et al. 2011)
Homalodisca vitripennis	Glassy-winged Sharpshooter	20,030	1-Salivary gland 2-Midgut 3-Whole body	

Table 4 Continued

Species	Common name	Number ESTs	Tissues	References
Peregrinus maidis	Corn Planthopper	20,678	1-Gut	(Whitfield et al. 2011)

Table 5: Summary of Candidate Aquaporins Found in Hemipteran EST Collections

Species	Uni-gene	Best Blastx similarity hit (E value)	Sequence coverage	Tissue used for cDNA production
Acyrthosiphon pisum	1	ref|NP_001139377.1| aquaporin isoform 2 [Acyrthosiphon pisum] (6e-148)	Full length (250AA)	Adults
Acyrthosiphon pisum	2	ref|NP_001232971.1| uncharacterized protein LOC100168499 [Acyrthosiphon pisum] (0000)	Full length (275AA)	Salivary gland, whole body
Acyrthosiphon pisum	3	ref|XP_001948407.1| neurogenic protein big brain-like [Acyrthosiphon pisum] (e-78)	1 to 243 (470)	Adults
Acyrthosiphon pisum	4	ref|XP_001948407.1| neurogenic protein big brain-like [Acyrthosiphon pisum] (e-131)	257 to 470 (470)	Adults
Aphis gossypii	5	ref|NP_001139377.1| aquaporin isoform 2 [Acyrthosiphon pisum] (1e-155)	Full length (250)	Whole insect
Aphis gossypii	6	ref|XP_001948407.1| neurogenic protein big brain-like [Acyrthosiphon pisum] (1e-62)	109 to 245 (470)	Whole insect
Aphis gossypii	7	ref|XP_001948407.1| neurogenic protein big brain-like [Acyrthosiphon pisum] (2e-57)	327 to 470 (470)	Whole insect

Table 5 Continued

Species	Uni-gene	Best Blastx similarity hit (E value)	Sequence coverage	Tissue used for cDNA production
Aphis gossypii	8	ref|NP_001232971.1| uncharacterized protein LOC100168499 [Acyrthosiphon pisum] (4e-78)	32 to 275 (275)	Whole insect
Myzus persicae	9	ref|NP_001139377.1| aquaporin isoform 2 [Acyrthosiphon pisum] (1e-168)	Full length (250)	Salivary gland, gut, whole body
Myzus persicae	10	ref|NP_001232971.1| uncharacterized protein LOC100168499 [Acyrthosiphon pisum] (1e-45)	187 to 275 (275)	Salivary gland, whole body
Bactericera cockerelli	7752	ref|XP_002138558.1| GA24838 [Drosophila pseudoobscura pseudoobscura] (6e-69)	Full (1 to 241)	Whole adult
Bactericera cockerelli	31763	ref|XP_001850887.1| aquaporin [Culex quinquefasciatus] (4e-59)	Full length (1 to 270)	Whole adult
Bactericera cockerelli	12021	ref|XP_002429480.1| Aquaporin AQPcic, putative [Pediculus humanus corporis] (6a-103)	Full length (1 to 266)	Whole adult
Bactericera cockerelli	39565	gb|EGI59562.1| Aquaporin AQPAe.a [Acromyrmex echinatior] (8e-19)	18-152 (345)	Whole adult
Diaphorina citri	11	ref|XP_003694175.1| aquaporin-like [Apis florea] (1e-54)	Full length (272 AA)	Midgut and testes
Diaphorina citri	12	dbj|BAG72255.1| aquaporin [Coptotermes formosanus] (1e-34)	1 to 115 (246)	Testes
Diaphorina citri	13	gb|EFN76752.1| Aquaporin AQPcic [Harpegnathos saltator] (7e-24)	19 to 127 (278)	Testes
Homalodisca vitripennis	14	dbj|BAG72255.1| aquaporin [Coptotermes formosanus] (9e-62)	Full length (248AA)	Whole body adult and midgut
Homalodisca vitripennis	15	gb|EHJ66754.1| aquaporin [Danaus plexippus] (5e-76)	Full length (265AA)	Whole body adult, instars and midgut

Table 5 Continued

Species	Uni-gene	Best Blastx similarity hit (E value)	Sequence coverage	Tissue used for cDNA production
Homalodisca vitripennis	16	dbj|BAF62091.1| aquaporin [Polypedilum vanderplanki (1e-20)	157-246 (end)	Nymphs
Homalodisca vitripennis	17	ref|XP_003403182.1| aquaporin-like [Bombus terrestris] (3e-10)	209 to end (272)	Midgut
Maconellicoccus hirsutus	18	ref|XP_002429480.1| Aquaporin AQPcic, putative [Pediculus humanus corporis] (5e-26)	beginning to 143 (263AA)	Whole body
Maconellicoccus hirsutus	19	ref|XP_003694175.1| aquaporin-like [Apis florea] (6e-32)	58 to 243 (295)	Whole body
Peregrinus maidis	20	ref|XP_318238.4| AGAP010325-PA [Anopheles gambiae str. PEST] (7e-26)	Full	Gut
Nilaparvata lugens	21	ref|XP_001656932.1| aquaporin [Aedes aegypti] (4e-61)	58-end (264)	Nymphs
Nilaparvata lugens	22	ref|NP_001106228.1| aquaporin [Bombyx mori] (6e-41)	97-259 (end)	Nymphs
Nilaparvata lugens	23	ref|XP_002425393.1|Aquaporin AQPAe.a, putative [Pediculus humanus corporis] (1e-90)	Full length but probably frame shift	Nymphs
Rhodnius prolixus	24	emb|CBY77924.1|aquaporin [Blattella germanica]	67 to 250 (277)	Nymph CNS
Rhodnius prolixus	25	dbj|BAI60044.1| aquaporin 1 [Anopheles gambiae] (7e-36)	143 to 256 (259)	Gut or fat body

Very few of the ESTs came from tissue-specific libraries. Based on the tissue of origin of some of the ESTs, aquaporin genes appeared to be expressed in digestive tract, salivary glands and testes. Interestingly, ESTs with similarity to *A. pisum* LOC100168499 (ApAQP2) were identified from *M. persicae* and *A. pisum* salivary gland libraries.

2.4 Discussion

Expression Sequence Tags are important resources that can be used for gene discovery and they are of great help to study species with no published genome. However, because of their nature ESTs can only be used as an estimation of the number of genes that a particular organism has. For instance, EST are obtained from mRNA, therefore, only genes being expressed in the tissues used to produce the cDNA library can be identified. Genes expressed in different tissues, developmental stages, or biotic or abiotic conditions cannot be identified using this type of resource. Similarly, because ESTs might only represent a portion of the encoded gene, several ESTs might in fact represent a single gene (as is the case with *A. pisum* unigenes 3 and 4). Finally, because a same gene can encode more than one protein if splicing variants exist, several cDNAs can again represent a single gene. The results from the transcriptomic analyses and the analysis of *A. pisum* genome indicate that hemipterans might encode, on average, less than 5 aquaporin genes. This is lower than the 6-8 genes identified in dipteran species, and could possibly indicate that hemipterans have a different number of functional aquaporin genes.

Aphids are the most studied hemipterans, for which several transcriptomic resources were created, even if those resources are not as developed as for other insect species. It is therefore not surprising that the majority of the candidate genes were found within this group. However, next-generation sequencing allows for in-depth transcriptomic sequencing, and the advances in bioinformatics have open the doors for the application of these technologies to non-model species for which no genomic or transcriptomic resources are available. As an example, the unique *B. cockerelli* dataset,

obtained using Illumina, allowed the identification of 4 candidate aquaporin genes. BIB-like aquaporins were only found in *A. pisum* and *A. gossypii*, though since very few candidate aquaporins are full sequence, it is not possible to be certain that some of the other candidates are not BIB-like aquaporins. Similarly, the sequencing of the analyzed datasets was not deep enough to allow the identification of lowly expressed genes.

Several of the identified candidates have already been characterized. For instance ApAQP1 matches *A. pisum* unigene 1 (Shakesby et al. 2009) and ApAQP2 matches *A. pisum* unigene 2 (Wallace et al. 2012). However, many of the candidate aquaporins match putative aquaporins identified in genome sequence analyses. Since in most cases, obtained sequences were not complete, it is not possible to assign the candidates to potential classes based on phylogenetic analyses (DRIP, PRIP, or BIB).

The majority of the aquaporin ESTs were obtained from whole body insect. Therefore, it is not possible to speculate about the expression profile of the identified genes. However, the analysis of tissue-specific libraries used to produce the cDNAs can give an idea of where the particular transcripts are expressed. Hence, in aphids, homologs of ApAQP2 might be expressed in the salivary glands. The expression of ApAQP2 in the salivary glands was not tested when the *A. pisum* gene was characterized (Wallace et al. 2012).

3. ANALYSIS OF PSYLLID AQUAPORIN CANDIDATES

3.1 Introduction

Psyllids are phloem feeding insect vectors of economically important bacterial pathogens causing huanglongbing in citrus and zebra chip in potato (Halbert & Manjunath 2004b; Munyaneza et al. 2007a; Munyaneza et al. 2007b). These two diseases arrived in the US in the early 2000 (Halbert & Manjunath 2004a; Crosslin et al. 2010) and their control has become a priority of the US agriculture. In the last years, important advances on the knowledge of the two main vectors, the potato psyllid, *Bactericera cockerelli*, and the Asian citrus psyllid, *Diaphorina citri*, have been reported. However, important gaps on the knowledge of these insects basic biology still need to be filled.

As other phloem feeding insects, psyllids have established an obligatory symbiosis with bacteria (*Carsonella ruddii*) which supplement the insects with essential amino acids (Buchner 1965; Nakabachi et al. 2006). On retribution, the insect provides the bacteria with a stable environment and sugars. The model system to study phloem-feeding insect- primary endosymbiont is aphid-*Buchnera aphidicola*. Recent advances on the field have shown that both organisms share the biosynthetic pathway for several essential amino acids (Wilson et al. 2010). However, transport of compounds between the two organisms is still poorly understood. Interestingly, an aquaporin has been shown to be a candidate transporter for sugars in the bacteriocyte (Wallace et al. 2012), the organ that houses the primary endosymbiont.
Aquaporin genes are highly conserved (Campbell et al. 2008). The proteins contain conserved regions including the hydrophobic alpha helixes and the NPA regions located in loops B and E (Gomes et al. 2009). Because of the high sequence conservation, it is possible to datamine genomic databases to identify candidate aquaporins as shown in chapter II. Datamining of hemipteran transcriptomic resources identified candidate aquaporins in aphids, psyllids, cicadas, mealybugs and planthoppers. In order to start characterizing the relationship between psyllids and their

associated bacteria, *C. ruddii* and the bacterial pathogens "*Candidatus* Liberibacter asiaticus" and "*Candidatus* Liberibacter solanacearum", psyllid candidate aquaporins from *D. citri* and *B. cockerelli* were cloned and sequenced.

3.2 Materials and Methods

Primer design

The four putative aquaporin candidates identified in *B. cockerelli* and the three *D. citri* transcriptomic resources (*B. cockerelli* unigenes 7752, 31763, 12021 and 39565, and *D. citri* unigenes 11, 12 and 13) were used as templates to design unique primers in order to amplify the full length cDNA. Primers were designed using Primer3 (http://frodo.wi.mit.edu/). Candidate primers were further selected based on the compatibility scores and the absence of sequence matches with ESTs from hemipteran species using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). The primers used are provided in Table 6.

Psyllid rearing

Batericera cockerelli colonies were maintained on tomato plants (*Solanum lycopersicum*) var "Moneymaker" in 14'' X 14'' X 24" insect cages (BioQuip, Rancho Dominguez, CA, USA) on a 16:8 light:dark cycle at ambient conditions. *Diaphorina citri* individuals were obtained from Dr. Mamoudou Sétamou at the Texas A&M-Kingsville Citrus Center in Weslaco, Texas. Individuals were kept on orange jasmine plants (*Murraya paniculata*) or on orange rootstock (*Citrus sinensis*) on a 16:8 light: dark cycle at ambient conditions.

Table 6: List of Primers Used for Conventional PCR or RACE-PCR.

Unigene	Type of PCR	Primer sequence
Diaphorina citri unigene 11	Amplify full length (conventional PCR)	F Primer CACCATCTTCCAATCAACCG R Primer GTAGCCTGATAAATCGCTACCTTG
Diaphorina citri unigene12	Amplify full length (conventional PCR)	F Primer GGTCAGGCAGCAGTTCTAGC R Primer TGTAGCTATGCTCCCCGAAG
Diaphorina citri unigene12	Amplify 3' end by RACE-PCR	F Primer CAATTGGTCACGTGAGTGGATGTC
Diaphorina citri unigene13	Amplify 3' end by RACE-PCR	F Primer TCAGGACCGTTGCACATTTA
Bactericera cockerelli unigene31763	Amplify full length (conventional PCR)	F Primer CTTTCACCATGCCGATTGTA R Primer TCTCATGCACATGTCCCCTA
Bactericera cockerelli unigene12021	Amplify full length (conventional PCR)	F Primer AAGTTCGTGTTTTTCTGACGGATA R Primer AAGGACTCTCAAGCCAAGTC
Bactericera cockerelli unigene7752	Amplify full length (conventional PCR)	F Primer GTCACCATGCCATCGCTATC R Primer CGGGTGCACCACTTCAATTG
Bactericera cockerelli unigene39	Amplify full length (conventional PCR)	F Primer AAACAAGAGTATCGCCAAGAGC R Primer GCTTCCTCTCAAAAGCGTACTT

The primers were designed to amplify the full length sequence of the candidate aquaporins or to obtain the full length sequence by RACE-PCR. F stand for Forward primer and R for Reverse primer.

RNA extraction

Approximatly 20 *D. citri* or *B. cockerelli* adults were collected by hand in a 1.7 mL tube and either flash frozen and stored at -80 degrees until used, or used immedatly for extraction. RNA was extracted using the Purelink RNA extraction kit (Invitrogen) with the following modifications. Psyllids were ground with 300 µL Purelink RNA extraction lysis buffer and 3 µL β-mercaptaethanol using a sterile-RNAse free plastic pestle. Once the sample appeared homogenous, another 300 µL of lysis buffer and 3 µL of β-mercaptaethanol was added to the mixture as recommended in the manufacturer's

protocol and passed through a 21 gauge needle with a syringe 10-12 times in order to further disrupt tissues. The sample was then centrifued for 2 minutes at 12,000g at room temperature to pellet debris, and the supernatant was collected in a new 1.7 mL Eppendorph tube. Then, 600 µL of 70% ethanol was added to the supernatant and mixed thouroughly before transfering into a Purelink RNA extraction column (Invitrogen). Extraction of the RNA followed as recommended in the Purelink RNA extraction protocol from Invitrogen (from the **Binding, Washing, and Elution** protocol pg. 27 http://tools.invitrogen.com/content/sfs/manuals/purelink_rna_mini_kit_man.pdf). Samples were eluted in 30 µL of RNase free water and quantified using a Nanodrop (NanoVue). RNA integrity was visualized in a 1% agarose gel electrophoresis. DNA contamination was removed using Turbo DNase kit (Ambion) following manufacturer's procedure.

RACE amplification

For aquaporin candidates with incomplete cDNAs (*D. citri* unigenes 12, 13) Rapid Amplification of cDNA Ends (RACE) was performed using the SMARTer RACE cDNA Amplification kit (Clontech) in accordance to the manufacturer's manual using the designed primers and psyllid RNA (Figure 5).

Amplified RACE products were run on a 1% agarose gel, stained with gel star (Lonza) and visualized on an UV transilluminator. Bands of interest (good size) were excised from the gel using a razor blade and purified using the PureLink Quick Gel Extraction Kit (Invitrogen) as per the kit's protocol. Extracted samples were then used as template with the specific aquaporin primer used during RACE and the Nested Universal Primer provided with the SMARTer RACE cDNA amplification kit. Amplifications were performed using GoTag Flexi PCR mix and ran as described in Figure 6. Amplified products were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide. If a single band of correct size was obtained, the PCR product was purified using the PureLink® PCR Purification Kit (Invitrogen), otherwise, selected bands were cut using a razor blade and purified using PureLink™ Quick Gel Extraction Kit (Invitrogen).

Figure 5: PCR Parameters and Conditions for RACE-PCR

PCR setup:	**PCR program:**
6.25 μL GoTag Felxi 2X buffer	1.30 sec 94 ºC
5.0 μl 10X Advantage 2 PCR Buffer	2.30 sec 68 ºC
5.0 μL universal primer (10X)	3.3 min 72 ºC
1.0 μL candiate primer (10 μM)	4.Repeat steps 1-3 for 24 cycles
1.0 μL 50X Advantage 2 Polymerase	
1.0 μL dNTP Mix (10 mM)	
2.5 μL RACE cDNA template	
34.5 μL Molecular Grade H_2O	
50.0 μL reaction	

RT-PCR amplification

To amplify full length aquaporins, purified RNA was used to produce cDNA using the Superscript VILO cDNA synthesis kit (Invitrogen) following manufacturer's protocol. Full length candidate aquaporins were amplified as shown on Figure 6. PCR products were visualized on a 1% agarose gel, stained gel star (Lonza) and visualized on an UV transilluminator.

Figure 6: PCR Parameters and Conditions for Conventional RT-PCR or PCR

PCR setup:	**PCR program:**
6.25 μL GoTag Felxi 2X buffer	1.2:00 min 95ºC
0.5 μL primer F(10 μM)	2.30 sec 95 ºC
0.5 μL primer R(10 μM)	3.30 sec 55 ºC
1.0 μL cDNA or DNA template	4.1:00 min 72 ºC
4.25 μL Molecular Grade H_2O	5.Repeat steps 2-4 for 34 cycles
12.5 μL reaction	6.10:00 min 72 ºC

Cloning and sequencing

Purified PCR products were cloned using the pGEMTeasy kit (Promega) following manufacturer's instructions. Briefly, the PCR products were ligated into the pGEMT vector following the pGEMTeasy protocol for an overnight ligation at 4ºC. After the overnight ligation, plasmids were transformed into 50 µL JM109 strain *E. coli* (Promega) by heat shock as follows: bacteria were removed from storage at -80C and thawed on ice for ten minutes, then 2 µL of the ligation mixture were added and the mix was incubated for 30 mintues on ice. The *E. coli*-vector mixture was then heat shocked at 42ºC for 40 seconds and then immediately placed on ice for five minutes. Gently, 950 µL of SOC broth was then added to the sample and incubated at 37º C for an hour and a half. A portion of the sample was then spread on LB ampicilin (100 µg/mL) plates with X-Gal (80 µg/mL) and incubated at 37º C overnight. Colonies were selected using blue/white selection. Selected colonies were placed in 5 mL LB broth with ampicilin (100 µg/mL) for overnight incubation at 37º C and 200 rpm. Selected plasmids were purified using PureLink™ Quick Plasmid Miniprep Ki t(Invitrogen) following manufacuturer's protocol. Plasmids were submitted for sequencing to Eton Bioscience.

In silico analyses

Obtained sequences analyzed as follows: vector was cleaned using vecscreen BLAST, identity was checked using Blastx. Obtained sequences were aligned with candidate aquaporin sequences to complete cDNAs or to identify splicing variants. Candidate cDNAs were considered full length aquaporin sequences if they had a putative start codon, a stop codon and all aquaporin signatures.

Protein sequence was obtained by *in silico* translation using Expasy translate tool. Candidate aquaporin proteins were aligned with *Drosophila melanogaster*, *Aedes aegypti*, *Acyrthosiphon pisum* and *Bemisia tabaci* aquaporins (accession numbers in Table 7) for phylogenetic analysis. Alignment was performed using ClustalW and phylogenetic linkage of the protein sequences was assessed using the Neighbor Joining method with 1000 replicates and computed using the

Poisson correction method using MEGA 5.0. Aquaporins from the study were also analyzed using InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/ EMBL) for identification of aquaporin domains such as intermembrane regions. To identify signal peptide regions, SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used with default settings.

Table 7: Sequences Included in the Aquaporin Tree

Acession number	**Name of protein**
XP_001648046.1	A. aegypti AQP6
XP_00165169.1	A. aegypti AQP5
XP_00165168.1	A. aegypti AQP4
XP_001649747.1	A. aegypti AQP3
XP_001656932.1	A. aegypti AQP2
XP_001656931.1	A. aegypti AQP1
NP_476837.1	D. melanogaster big brain
NP_611811.3	D. melanogaster CG17664
NP_611812.2	D. melanogaster CG17662
NP_611810.1	D. melanogaster CG5398
NP_725052.1	D. melanogaster CG7777
NP_725051.2	D. melanogaster DRIP
NP_523728.1	D. melanogaster CG12251
NP_611813.1	D. melanogaster CG4019
NP_001139376.1	A. pisum ApAQP1
NP_001232971	A. pisum ACYPI009194 (ApAQP2)
XP_001948407.1	A. pisum PREDICTED: neurogenic protein big brain-like
B5L019	B. tabaci BtAQP 1

3.3 Results and Discussion

Identifying full length sequence

The complete CDS of the 5 candidate aquaporins was successfully amplified using total RNA purified from adult psyllids (the 4 *B. cockerelli* candidates and *D. citri* unigene 11). Table 8 shows the protein length obtained from each candidate unigene by *in silico* translation.

Table 8: Protein Length Obtained from Each Candidate Unigene by *in silico* Translation

Organism	Unigene	Encoded protein
B. cockerelli	7752	279 AA
B. cockerelli	39565	282 AA
B. cockerelli	31763	269 AA
B. cockerelli	12021	266 AA
D. citri	11	300 AA
D. citri	12	249 AA

Surprisingly, 2 amplicons of different size were obtained for *D. citri* unigene 11. Sequencing of both amplicons revealed that they both encode for the same protein. The difference between both amplicons was an insertion of 229 bp in the 5'UTR region (Figure 7). Therefore, both cDNAs encoded for the same protein. This difference in the 5'UTR might affect protein expression. Further analyses are needed to evaluate if these 2 forms are expressed and/or translate at different speeds or localizations.

RACE-PCR for the incomplete *D. citri* candidate unigene12 successfully amplified the full length sequence. The obtained sequence encodes a 249 AA protein. Similarity search using Bastx identified BAG72254.1 aquaporin from *Coptotermes*

formosanus as the best hit. This protein is also 249 AA long. On the other hand, RACE-PCR for *D. citri* candidate unigene 13 revealed that, that unigene probably does not encode an aquaporin, since the identified sequence lacked major hallmarks of aquaporins (NPA boxes and transmembrane regions). Except for *D. citri* unigene 11, a single form was identified for each of the psyllid candidate aquaporins.

Bioinformatic analyses

The six putative aquaporin candidates were analyzed using the full protein coding regions. All proteins showed high sequence similarity to established aquaporins (Figure 8). Each of these sequences showed six intermembrane regions within the protein coding sequence and had two highly defined NPA regions. However, *B. cockerelli* unigenes 7752 and 39565 encoded a NPT and NPS, respectively, instead of the first NPA. Alignment with other aquaporins showed that those same *B. cockerelli* unigenes have a longer C-loop which might suggest an aquaglyceroporin function (Kaufmann et al. 2005a). Furthermore, for none of the sequences, SignalP detected cleavage sites.

Figure 7: Alignment of Both cDNA Forms Identified for *D. citri* Unigene 11

```
1     GTAGGTTCTCGTACCTACTACTTGTTGAAACTAATCAATCAAAAAGAAATTTACTACTTACTAGTACACCATCTTCCAATCAACCGGTCC
1     GTAGGTTCTCGTACCTACTACTTGTTGAAACTAATCAATCAAAAAGAAATTTACTACTTACTAGTACACCATCTTCCAATCAACCGGTCC

91    AATTAAAAGCTATTTAGGTACCAACATTTCGTTTTTATTTATTTTTTCAAATCAACCATGATGCTCCCGAATTAAAGAAGACAAAAAAAA
91    AATTAAAAGCTATTTAG-------------------------------------------------------------------------

181   AAATAAAAAAAAAATCCGTTTTTTCGCGAGTGATTTGGCTGAGACGAATAGAAAACAAAATTTCTAATTTTGTATTTTTTATTGTTTAATT
107   ------------------------------------------------------------------------------------------

271   TATTGTTCAACTTGTCAAAAAATATGTTGTAAATATATTTTTACTAAAAATAAAAATAAAATAAAGTCTTATTGAACCAGTGTGAGTGTG
107   ------------------------------------------------------------------TCTTATTGAACCAGTGTGAGTGTG

361   AGAAGAATACCACACGACAGTTTTCCACTTTCTGGGAAGTCGAGTAAAAAATAGCCCAGGAACAATGCCGGTCAAAATTGGAAGCAAAAA
132   AGAAGAATACCACACGACAGTTTTCCACTTTCTGGGAAGTCGAGTAAAAAATAGCCCAGGAACAATGCCGGTCAAAATTGGAAGCAAAAA

451   AAGTTTCAAGAAGACCTTGCTCGGAGATGAACTACAAACCCTCAACGGGGAGAGGTTCTTCGAAATCCTCAAACGTGCCGTTGCGGAAAT
222   AAGTTTCAAGAAGACCTTGCTCGGAGATGAACTACAAACCCTCAACGGGGAGAGGTTCTTCGAAATCCTCAAACGTGCCGTTGCGGAAAT

541   GTTGGGTACCGCCATTCTACTCGGTCTGGGATGTATGAGTACGAAAGACACCACGGGGGGCGCTGCTAGTCATTTGAACATTGTGCTCTC
312   GTTGGGTACCGCCATTCTACTCGGTCTGGGATGTATGAGTACGAAAGACACCACGGGGGGCGCTGCTAGTCATTTGAACATTGTGCTCTC

631   GTTTGCCTTTGCCGTGGCCACGTCTGTTATGATTTTCGGACACATCAGCGGATCTCACATCAATCCTGCCCTCTCATTGGTGGGCGTAGT
402   GTTTGCCTTTGCCGTGGCCACGTCTGTTATGATTTTCGGACACATCAGCGGATCTCACATCAATCCTGCCCTCTCATTGGTGGGCGTAGT

721   CATGGGCAAAATCTCCCTTCAGATATTCGTGGTGTACACCATAGCCCAATGCATAGGAGCAACACTCGGCTATTCCATAGCAAGGTCGCT
492   CATGGGCAAAATCTCCCTTCAGATATTCGTGGTGTACACCATAGCCCAATGCATAGGAGCAACACTCGGCTATTCCATAGCAAGGTCGCT

811   GTTTCCGGCACATTACCTGGGCGAAACGTTCTGTTGCACTCTACCCAATCCTAACGTTGAGCTGTCGCAAGCGTTCTCTGCAGAGTTTTT
582   GTTTCCGGCACATTACCTGGGCGAAACGTTCTGTTGCACTCTACCCAATCCTAACGTTGAGCTGTCGCAAGCGTTCTCTGCAGAGTTTTT

901   GCTGACAGTCATCATTGCCATGGTCTTGTGTGCAGCTTGGGATTACAAATGTTTAGACAAGCACGATTCTTTACCGCTCAAGTTCGGATT
672   GCTGACAGTCATCATTGCCATGGTCTTGTGTGCAGCTTGGGATTACAAATGTTTAGACAAGCACGATTCTTTACCGCTCAAGTTCGGATT

991   TGCTGTCACCGCCTTGGCCATTCCAGGGGCTCAATTCGCCGGCTGCAGTATTAACCCAGCTCGTAGTTTCGGACCGGCTTTGGTGACTGG
762   TGCTGTCACCGCCTTGGCCATTCCAGGGGCTCAATTCGCCGGCTGCAGTATTAACCCAGCTCGTAGTTTCGGACCGGCTTTGGTGACTGG

1081  ACATTGGGAGAACCATTGGGTCTATTGGGTGGCGCCACTATCGGGGGCGTTCGTCGGATCCTTCATCTATCGGGTTCTATTCTACGACAA
852   ACATTGGGAGAACCATTGGGTCTATTGGGTGGCGCCACTATCGGGGGCGTTCGTCGGATCCTTCATCTATCGGGTTCTATTCTACGACAA

1171  TCCGGACAAACATAATACTGTAACGTACGATACGAATGAATCGGTGAAACTTCAAACTGTGGACGAGGCCTAAACCACTCGAGTGAAACA
942   TCCGGACAAACATAATACTGTAACGTACGATACGAATGAATCGGTGAAACTTCAAACTGTGGACGAGGCCTAAACCACTCGAGTGAAACA

1261  TCAAACTCTGGATGAGCCTTAAACACGAACACATGGATGAAACAGTGAGCCTGTGCTGAATGAAGTACTCAGACTACTGTGGAATGTGGA
1032  TCAAACTCTGGATGAGCCTTAAACACGAACACATGGATGAAACAGTGAGCCTGTGCTGAATGAAGTACTCAGACTACTGTGGAATGTGGA

1351  TTTCACCTGTTTTTTCAGCTCCTAGAAGGCGTGTTAAGCTAACAACCTGTTAATTTACATTTGATTTACACAGGCTTAACAGGGCTGTTA
1122  TTTCACCTGTTTTTTCAGCTCCTAGAAGGCGTGTTAAGCTAACAACCTGTTAATTTACATTTGATTTACACAGGCTTAACAGGGCTGTTA

1441  AGCTAACAGGGCTGTTAGGAGCTAAAAAGATGGGTGGTGACTGGTGAAACCCACCCCTAAACTGTCACGTAATTGATCCATTCTTGTCAT
1212  AGCTAACAGGGCTGTTAGGAGCTAAAAAGATGGGTGGTGACTGGTGAAACCCACCCCTAAACTGTCACGTAATTGATCCATTCTTGTCAT

1531  ATATTGTGAAATACCAATGTACCCTACCTATACCTACAGGTAGACAAGTCTAAGTAGGTAGCTAAGTCCCATTATGCCATCAGTTTGCCT
1302  ATATTGTGAAATACCAATGTACCCTACCTATACCTACAGGTAGACAAGTCTAAGTAGGTAGCTAAGTCCCATTATGCCATCAGTTTGCCT

1621  GTTGGTACCTACAATGGGTAACTTATGACCATAACCGATGTTTTAGGTATAGGTATCTATACTAATACTTACGAATAGCCAAATTTGTCC
1392  GTTGGTACCTACAATGGGTAACTTATGACCATAACCGATGTTTTAGGTATAGGTATCTATACTAATACTTACGAATAGCCAAATTTGTCC

1711  GATCAAAACGTACCTAGGTAATATGTAAGTACCAGGTACCTACTCGTAGTTTGTACCTACACAATACCAGGATACAAGGTAGCGATTTAT
1482  GATCAAAACGTACCTAGGTAATATGTAAGTACCAGGTACCTACTCGTAGTTTGTACCTACACAATACCAGGATACAAGGTAGCGATTTAT

1801  CAGGCTACCTAATACCTAATTACCTAATAAAATGAATAAGGTTTTTACTTACCTAACTGACT
1572  CAGGCTACCTAATACCTAATTACCTAATAAAATGAATAAGGTTTTTACTTACCTAACTGACT
```

Alignment of cDNA forms identified for *D. citri* unigene 11. ATG encoding the beginning of the protein sequence and TAA encoding the stop codon are emphasized

Figure 8: Amino Acid Alignment of Seven *D. melanogaster* and 6 Psyllid Putative AQPs

```
                                                                             ***
D. cit 11       1    EMLGTAILLGLGCMSTKD-TTGGA---ASHLNIVLSFAFAVATSVMIFGHISGSHINPALSLVGVVMGKI
D. cit 12       1    ELIGTFVLVFVGTGSIMWPNDP---NTVDVTKIALTFGFVIATIAQAIGHVSGCHINPAVTIGLFCSGHI
B. coc 12021    1    ELFGNLLLNFFGCLSCVSLLEQPAGTPPNIVLVAFTFGLVIFTSVQALGHVSGGHFNPAVTVGMLATGNV
B. coc 31763    1    EFLGTAVLLGLGCMGCAN-TTGKG---LVHMEVVFSFAFAVATAVMIFGHVSGAHINPAVSLVALVMGKI
B. coc 7752     1    EVLGSAMLMYFGCMSLVSGFSQAP---LPGMQGGLMFGFVVSTIIVIFGHISGAHLNPTVSLSAYLLDMI
B. coc 39565    1    ELLGTSFLMFFGCMSLVSGFAQAP---VSDMQPALVFGFVVSTIIVVFGHISGAHLNPSVTVAALVLRDI
D. mel BIB      1    ECLASFMYVFIVCGAAAGVGVGAS-VSSVLLATALASGLAMATLTQCFLHISGAHINPAVTLALCVVRSI
D. mel CG7777   1    EFLGNLILNFFACGACTQIEDG--------TFKALAFGLAIFMAITIVGHLSGGHVNPAVTAGMLVAGRI
D. mel CG4019   1    ELIGTGILVFLGCMGCVK-TDLFP---NNHLQIVLNFGFAVLIAIQCFGCVSGAHLNPAVTVAAYIYEMV
D. mel CG17664  1    EMIATAMLMFLGCMGSVE-NSVFT---NSDFQSALNFGFVVLICIQCFGCVCGAHLNPAVTLATYVYNMI
D. mel CG17662  1    ELAATAVFVFIACMGCVE-TPLFQ---NSHFRSGLTFGLAILIAIQCFGSVSGAHLNPAITLAAWLYGAI
D. mel CG5398   1    EFSATALLILLGCMGDST-NQGGE---SKFLVASVHYGLTVMVVMHVFGFVSGAHSNPCISISCYLMGYI
D. mel DRIP     1    ELVGTFFLIFVGVGSTTSG---------SVPQIAFTFGLTVATIAQGLGHLSGCHINPAVTLGFLIVGEI

D. cit 11       67   SLQIFVVYTIAQCIGATLGYSIARSLFPAHYL------GETFCCTLPNPNVELSQAFSAEFLLTVIIAMV
D. cit 12       68   SLLKGFFYIIMQCVGAVAGSAVLEAVTPNPCC--------KLGMTGLNPSINATQGLIIEAIITFVLVLT
B. coc 12021    71   SVIRGVFYVVAQCLGAIAGSLILKSLTPVDFQG-------NLGMTTLNKHLTPTQGMGIEFFLGFVLVLV
B. coc 31763    67   SIQLWVVYAIAQCAGAVFGYSITRSLFPAVYL------SDDFCVTLPNPRVELSQAFTAEFILTAIIALV
B. coc 7752     68   PLIELPVYFVSQIVGCLIGVGLLHVVTPQEIL-YPLGGSVGFCVTVPHASLTQAQAFLAEFLSTSLLIFT
B. coc 39565    68   SVLEAVVYVLAQCAGCILGYALLGVATPHEIFSYGVPAGSGQCVTMPHPSLSHPQALLVEFLGTALLVFT
D. mel BIB      70   SPIRAAMYITAQCGGGIAGAALLYGVTVPGYQG------NLQAAISHSAALAAWERFGVEFILTFLVVLC
D. mel CG7777   63   SLIRAFFYVVFQCLGAIAGTAAVKILIDQDYYN-------GLGHTSLAPNITELQGLGIEFFLGLLLVLV
D. mel CG4019   67   TLRMAFAYFAAQMLGAFIGYGLLMVLLPSPTL-TVG---AGLCVTLPHTSVTTGQALGIEFVITSILVIV
D. mel CG17664  67   SLPMALAYFVAQMVGAFIGYGLLKAVLPESAI-YSAENPNGVCLTSLNSTLTPWQGLAVEFLITCVLISV
D. mel CG17662  67   GWIRAIAYFVAQAAGALIGYGLLVAVLPGNSI-KGVDNPSGVCVTILAPGISVLQGVFIEFLITCCLVMV
D. mel CG5398   67   ALEVMMMYVVCQMAGAFLGYFLLMQLLPKELVDKSK---PGICLVQPMDTLSTYQVVIIECLLTAVLVLG
D. mel DRIP     62   SILKAAFYIIVQCVGAIAGAAVIKVALDGVAGG-------DLGVSSFDPSLNCAQAVLIEALITFILVFV

                                                            ***
D. cit 11       131  LCAAWDYKCLDKHDSLPLKFGFAVTALAIPGAQFAGCSINPARSFGPALVTGHWENHWVYWVAPLS
D. cit 12       130  VEAVCDDRRTDIKGSVPVAVGLAITCCHLAAIKFTGASMNPARTLGPAVIGNHWDNIWVYWAGPIL
B. coc 12021    134  IFGVCDGNKPHAKAPAALAIGLTVALGHLAAIDFTGASMNPARTFGSAVVANIWTDHWVYWVGPSL
B. coc 31763    131  LCAAYDQRCLDKHDSLPIKFGFAVVALAVPGAQFAGCSVNPARSFGPALITGVWDNHWIYWVAPLS
B. coc 7752     137  CCGVWDPRNARHGDATPIKFALVIALCSITVGPYTGASMNPARSLAPAVFSQVWTAHWLYWVAPML
B. coc 39565    138  CCGVWDHRNAHQGDSNPVKFALVITLLSITVGPYTGASLNPARSLAPAVVNNVWTNLWIYWVAPPL
D. mel BIB      134  YFVSTDPMKKFMGNS-AASIGCAYSACCFVSMPY----LNPARSLGPSFVLNKWDSHWVYWFGPLV
D. mel CG7777   126  VFGACDPHKPDSRYTAPLAIGMAVTLGHLGTIRYTGASMNPARTVGTAFATDIWASHWVYWVGPVL
D. mel CG4019   133  CCGVWDPRNSKFHDSVGIRFGLAIACLACAAGPFTGGSMNPARSFAPALWNKHFESNWIYWLAPLS
D. mel CG17664  136  CCGVWDPRNATKQDSLPVRFGLAIACLSLTAGQLTGASMNPVRSFAPAIWNGFWDDHWIYWVGPMA
D. mel CG17662  136  ACSVWDPRNAKLQDSVPVRFGLTVSCLILTAGLFTGASMNPTRSLGPAVWNDSWAHHWIYWVGPLV
D. mel CG5398   134  WCSLWDVRNGRFLDSVAIRMGLLVIACSFAGIQLTGASMNPAKTLVPAIFYGSPNSVLMQLTGQIL
D. mel DRIP     125  VKAVSDPGRQDIKGSAPLAVGLAIAAGHLCAIKLSGASMNPARSFGPAVVQGVWTYHWVYWVGPIA
```

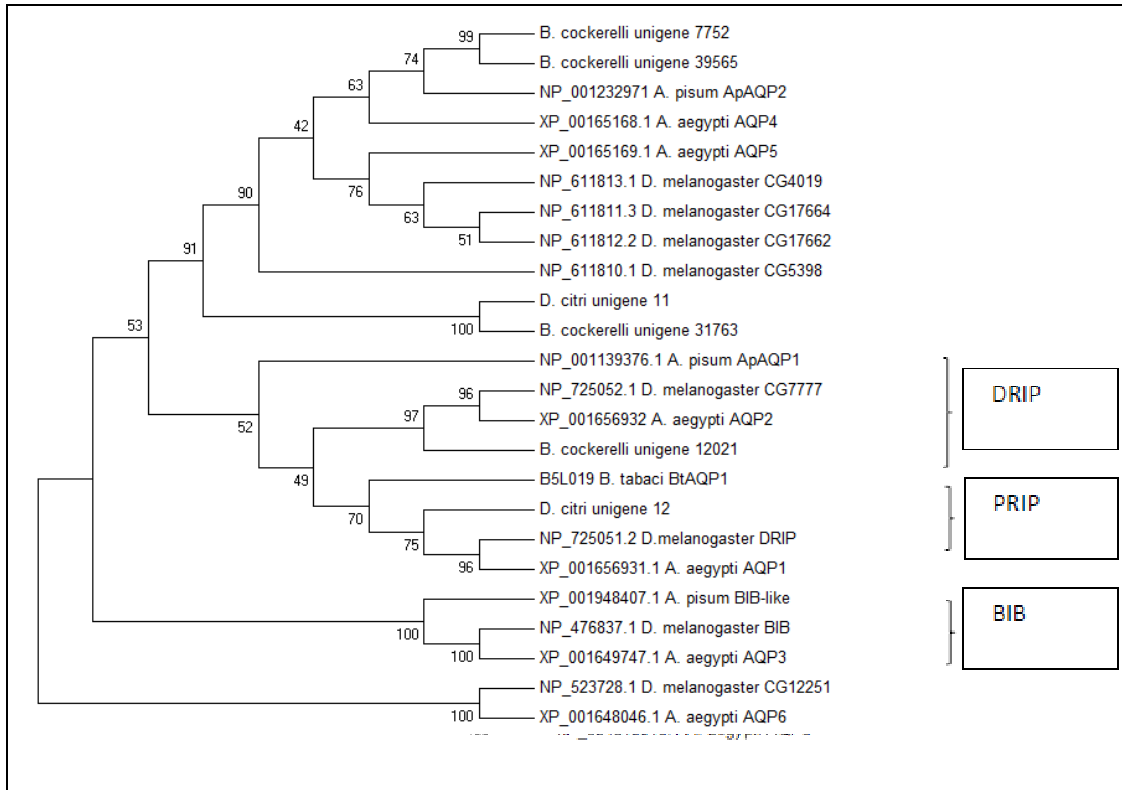
Amino acid alignment of the seven D. melanogaster AQPs and the six psyllid putative AQPs. NPA motifs are shown by ***. C-loop region is boxed. Nh2 and COOH-terminal regions are not included.

Phylogenetic analysis

Sequence similarity between aquaporins has been used to classify aquaporins into subfamilies (Campbell et al. 2008; Gomes et al. 2009). Insect aquaporins have been traditionally subdivided into three subfamilies, the DRIPs, PRIPS, and BIBs based on sequence similarities (Campbell et al. 2008). The three subfamilies can be seen in Figure 9.

Phylogenetic analyses showed that *B. cockerelli* unigenes 7752 and 39565 are similar and cluster with *A. psium* ACYP009194 and *A. aegypti* AaAQP4 and AaAQP5, as well as *D. melanogaster* CG4019, 17664, 17662 and 5398 which have a longer C-loop and might suggest an aquaglyceroporin function (Kaufmann et al. 2005a). Interestingly, *A. psium* ACYP009194 (ApAQP2) has been shown to be expressed in the bacteriocyte and transport polyols (Wallace et al. 2012). Therefore, these two *B. cockerelli* candidates might be involved in a similar function. Unigenes *B. cockerelli* 31763 and *D. citri* 11, clustered together but apart from the other aquaporins. Similarly, the phylogenetic analysis showed that *B. cockerelli* unigene 12021 and *D. citri* unigene 12 clustered with DRIPs and PRIPs. PRIPs and DRIPS are very similar proteins. According to the tree, *B. cockerelli* unigene 12021 would cluster with PRIPs while *D. citri* unigene 12 would cluster with DRIPs. However, in the same tree BtAQP1 would also be a PRIP while this protein has been described as DRIP-like. Finally, none of the psyllid candidate aquaporins belonged to the BIB group, which is composed by *D. melanogaster* BIB protein as well as *A. aegypti* and *A. pisum* BIB-like proteins. Since BIB-like genes have been identified in many insects, including aphids, it is probable that psyllids also encode BIB-like proteins that were not identifiable through the available transcriptomic data. The sequencing of *D. citri* genome is in progress and would allow not only to identify BIB-like aquaporins, but many others.

Figure 9: Evolutionary Relationships of AQPs



The evolutionary history was inferred using the Neighbor-Joining method (Saitou, Nei 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Poisson creection method and are in the units of the number of amino acid substitutions per site. The analysis involved 25 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 187 positions in the final dataset. Evolutionary analysis were conducted in MEGA 5 (Tamura et al, 2011).

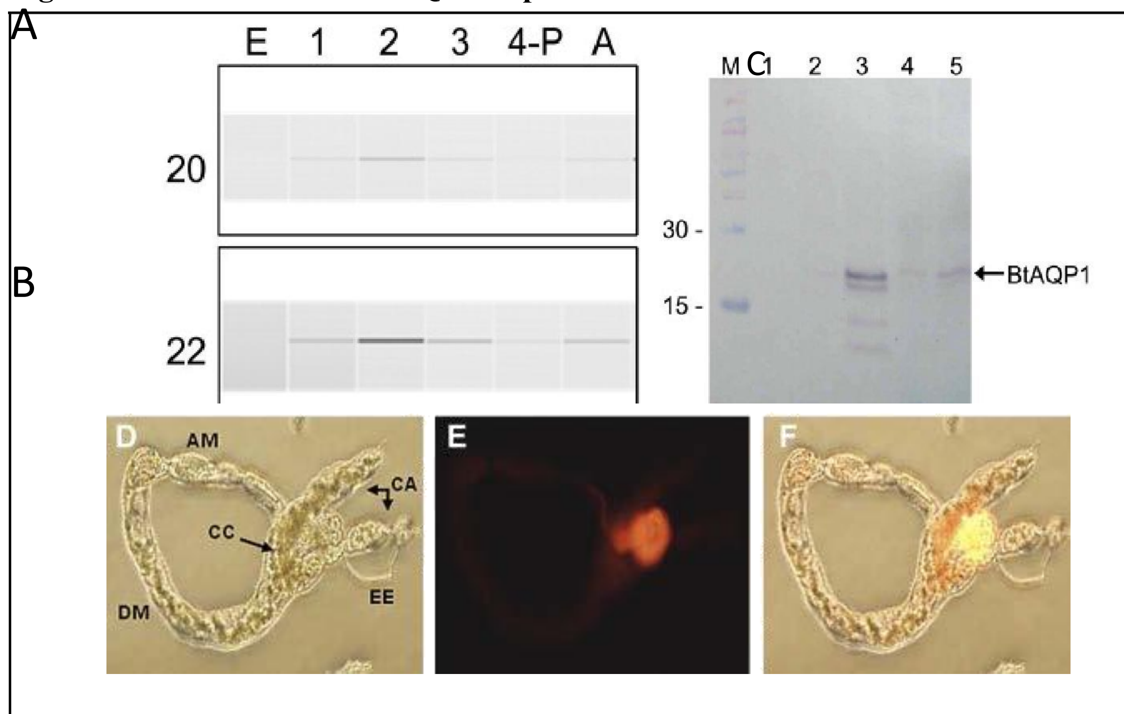
4. EXPRESSION ANALYSES OF *D. citri, B. cockerelli* AQUAPORINS

4.1 Introduction

Aquaporins from phloem feeding hemipterans have only been studied in *Acyrthosiphon pisum* and *Bemisia tabaci* (Shakesby et al. 2009; Mathew et al. 2011*;* Wallace et al. 2012*).* For *B. tabaci*, aquaporin BtAQP1, a DRIP-like aquaporin, protein expression was localized in the gut tissue of adult whiteflies, more specifically in the filter chamber (Figure10 C-F). Gene expression analyses showed a pick in 2nd instar nymphs, and no expression within eggs (see figure10 A,B). This protein is hypothesized to be involved in overcoming the osmotic pressure barrier posed by phloem feeding. Expression profile obtained from different life-stages and tissues supports this function.

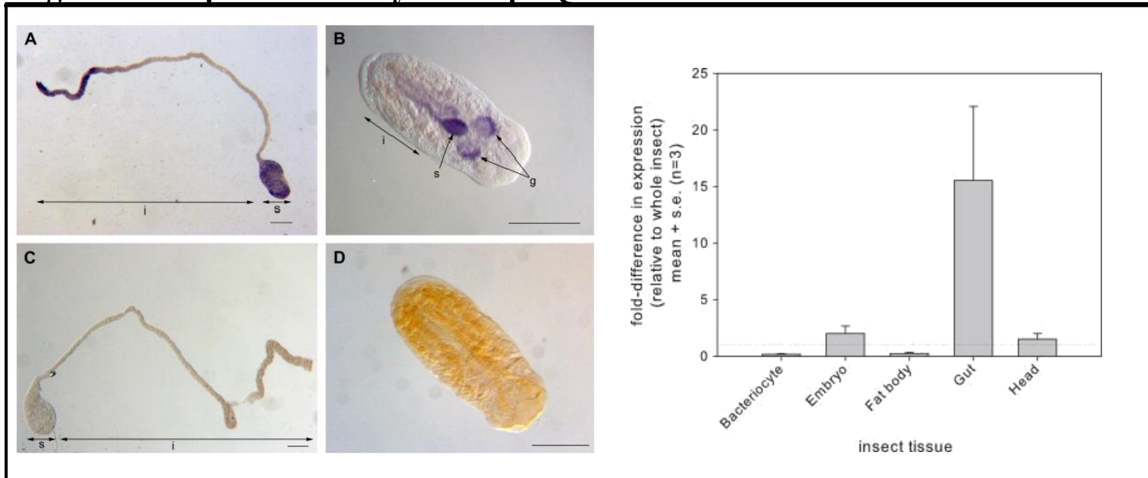
Similarly, expression of the DRIP-like *A. pisum* aquaporin 1, ApAQP1, was found in the gut where the hindgut and midgut come in close contact with each other (Figure11 A-E) (Shakesby et al. 2009). However, for ApAQP2, an aquaglyceroporin, a different expression pattern was found. In adults, this gene appeared downregulated within gut tissue but upregulated within the bacteriocyte and fat body tissue (Figure 12) (Wallace et al. 2012).

Figure 10: *Bemisia tabaci* BtAQP1 Expression



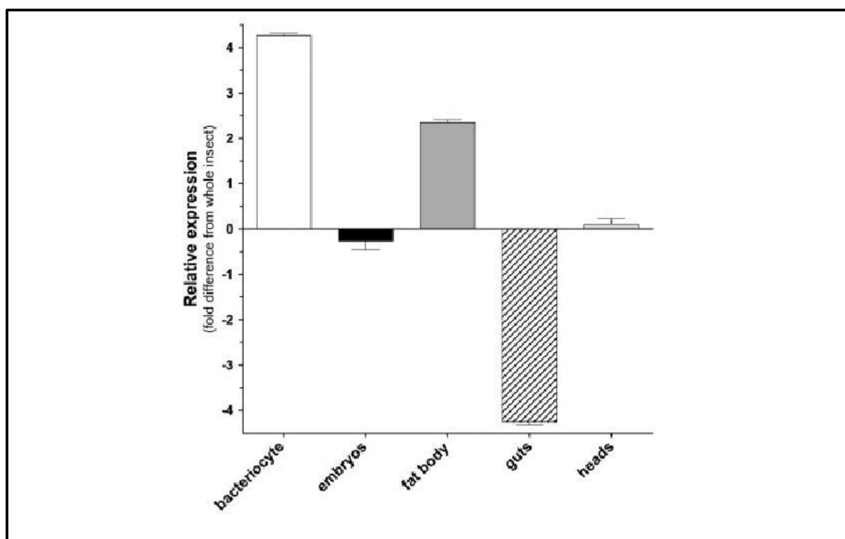
A,B) Expression of *BtAQP1* in the egg (E), 1st instart (1), 2nd instar (2), 3rd instart (3), 4th instar and Pupae (4-P), and the adults (A) using RT-PCR analyzed using a Angilent 2100 Bioanalyzer for cycles 20 and 22. C) The expression of BtAQP1 in leg tissue (lane 1), heads (lane 2), intact gut tracts (lane 3), whole body minus gut (lane 4), and whole adult homogenates (lane 5) separate on a SDS-PAGE gel and analyzed by immunoblotting with anti-BtAQP1. D,E, F) BtAQP1 immunolocalization using anti-BtAQP1. Samples show the ventral side of the gut with external esophagus (EE), descending midgut (DM), ascending midgut (AM), connecting chamber (CC), filter chamber (FC), ileum (IL) and hindgut and the caeca (CA) all shown in D. E and F show immunoflorescence of the Anti-BtAQP1 on the sample (Mathew et al. 2011).

Figure 11: Expression Analysis of ApAQP1



Expression analysis of ApAQP1 (Shakesby et al. 2009) A-D) In situ localization of the ApAQP1 sequence within the adult gut (A,C) and the embryo (B,D) using the antisense DIG -Labled RNA (A, B) and Sense DIG-Labeled RNA (C,D). E) Expression of *ApAQP1* in different tissues normalized against *GAPDH*, *βTUB* and *RPL32*.

Figure 12: Expression Analysis of ApAQP2



The relative expression analysis of ApAQP2 (Wallace et al. 2012). ApAQP2 expression quantitative analysis normalized to *RPL32* gene expression

Analysis of hemipteran ESTs identified several putative aquaporin candidates. The tissues used to produce the cDNA libraries from which putative aquaporins were identified included body parts such as head, or organs such as testes, guts and salivary glands. Moreover, candidate aquaporins were identified in different life stages (nymphs and adults).

In order to further characterize the putative aquaporins identified in psyllids, we performed expression analyses from different life-stages and from dissected tissues of 2 *B. cockerelli* and 2 *D. citri* candidate aquaporins (*B. cockerelli* unigenes 21012 and 31763 and *D. citri* unigenes 11 and 12). For one of the candidates, *B. cockerelli* unigene 31763, we further characterized the expression by performing *in situ* hybridizations.

4.2 Materials and Methods

Insect collection

Bactericera cockerelli were maintained as described in Chapter III. Insects were collected at different life stages (Figures 13 and 14). Samples were separated in four life stages: eggs, young nymphs (L2-3), old nymphs (L4-5), adult males and adult females. Eggs were retrieved by using a razor blade to carefully slice the stalk of the egg and a pair of tweezers was used to move the egg to a 1.7 mL Eppendorf tube with Trizol and ground with a plastic pipette until homogenous. The resulting mixture was stored at -80ºC until RNA extraction could be completed.

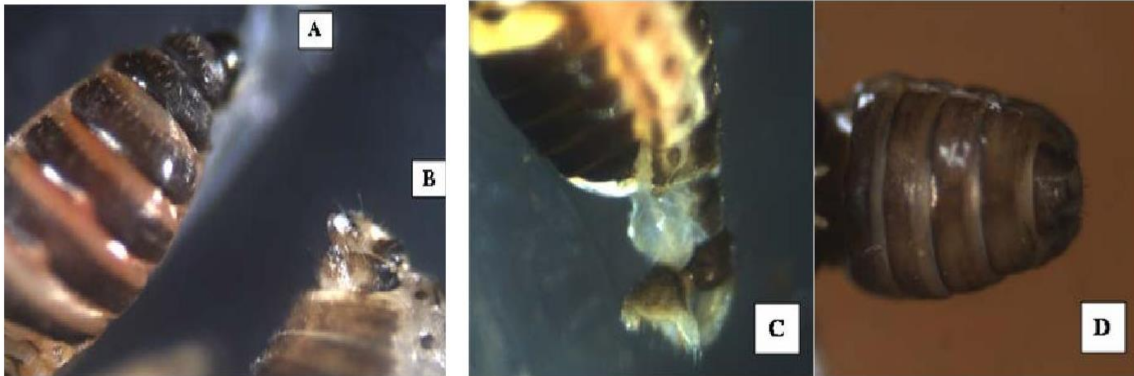
For collecting nymph individuals at stages L2-L3 or L4-L5, insects were classified using their morphological characteristic such as size and development of wing pads (Figure 13). Twenty individuals were collected for each group using a wetted paintbrush and placed into a 1.7 mL Eppendorf tube with Trizol and ground with a plastic pipette until the mixture was homogenous. The resulting sample was then stored at -80ºC until RNA extraction could be completed.

Adult *B. cockerelli* were sexed according to their external morphology (Figure 14). Twenty individuals of each sex were placed into a 1.7 mL Eppendorf tube with Trizol and ground with a plastic pipette until the mixture was homogenous. The mixture was then stored at - 80ºC until RNA extraction could be completed. Similarly, *D. citri* nymphs (Figure 13) and adults were collected from the colonies maintained as described in Chapter III. For *D. citri*, only adult head and guts were tested.

Figure 13: Nymphal Stages of Psyllids

Shown above are the five nymphal stages of *D. citri* nymphs. The nymphal life stages from left to right: L1, L2, L3, L4, L5. These life stages are very similar to *B. cockerelli* nymphs.

Figure 14: ***Bactericera cockerelli*** **Adult Forms**



A- Male and B- female abdomens of *B. cockerelli* adults, in comparison to each other. D shows the female abdomen with the five apparent abdominal segments and the rounded anal regions and C portrays the male penis and 6th segment that visually differentiates the genders (Abdullah 2008).

Dissection for RT-PCR analyses

Twenty adult *B. cockerelli* or *D. citri* were picked at random from the laboratory reared colonies (see Chapter III for rearing conditions) and placed in 1.7 mL Eppendorf tubes on ice to mollify them until dissection. Insects were then placed in RNase free 0.9% (w/v) NaCl on a RNase Zap (Invitrogen) treated dissecting slide and dissected using a pair of tweezers and a lance treated with RNase Zap as well. By swift decapitation and rupturing the abdomen of both *D. citri* and *B. cockerelli* heads and guts were separated. The rupture was caused by tearing a hole between the second or third abdominal segment. The gut would emerge from the tear and once teased out the anal region of the insect was removed. The bacteriocyte was dissected by carefully removing tissues from the ruptured abdomen until the bacteriocyte could be clearly seen and extracted. All tissues (head, guts, bacteriocytes) were removed from the dissection slide using a 200 µL pipette and placed in 200 µL Trizol and stored at -80ºC until RNA extraction could be completed.

Semiquantitative RT-PCR

RNA extraction and cDNA synthesis were done as previously described (Chapter III, RNA extraction and RT-PCR amplification). Semiquantitative RT-PCRs were performed on adult heads, guts, bacteriocytes and whole bodies. RT-PCRs were performed on eggs and nymphal stages (L2-3, L4-5) and males and female individuals.

PCR primers were designed to amplify sequences within each of the target aquaporin candidates (see Table 9). For constitutive reference gene amplification, ferritin primers were also designed to amplify sequences within both *B. cockerelli* and *D. citri*. For *B. cockerelli*, 28S rRNA was also used as a control.

Table 9: Primers to Test Expression of Candidate Psyllid Aquaporin Genes by RT-PCR

Unigene	**Primer sequence**
B. cockerelli unigene 12021	FWD: TCGCTCTGGGACACTTAG REV: GCCTGGTTTTACAAAGAG
B. cockerelli unigene 31763	FWD: CGATCAAAAGGCTTCAAAG REV: ATGCACATGTCCCCTAAG
D. citri unigene 11	FWD: CATCAGCGGATCTCACATCAA REV: ATACTGCAGCCGGCGAATT
D. citri unigene 12	FWD: GTCTGTTTTGCTCCGGTCAT REV: TACAGCTGATCCAGCCACTG
Ferritin primers (these primers amplify ferritin from both psyllid species)	FWD: GATCGCGATGTGGTAGCTCT REV: GGGACTCGTTCACATCCTTC
28S primers (for *B. cockerelli* only)	FWD: CGCAGACTGGTTCGGGATAC REV: GCGAGGACTCAGTTTCGTGTC

PCR reactions were performed using GoTagFlexi Colorless master mix (Figure 15). Five µL aliquots of the PCR reaction were removed at 20, 25, 30 and 35 cycles. For PCR, cDNA was quantified using a nanodrop (NanoVue) and amplification was normalized against ferritin expression. Each sample was then run on a 1% agarose gel stained with 1X GelStar. Three independent replicates were performed.

Figure 15: PCR Parameters and Conditions for Semi-Quantitative RT-PCR

PCR setup:	**PCR program:**
12.5µL GoTag Felxi 2X buffer	1.2:00 min 95ºC
1.0µL primer F(10 µM)	2.30 sec 95 ºC
1.0µL primer R(10 µM)	3.30 sec 60 ºC
1.0µL cDNA template	4.1:00 min 72 ºC
10.5 µL Molecular Grade H_2O	5.Repeat steps 2-4 for 34 cycles
25 µL reaction	6.10:00 min 72 ºC

Semi-quantitative analysis of expression

Samples were analyzed using ImageJ software using the "Single band" function of the program. To do this, images were converted into 8 bit and calibrated using the "Uncalibrated OD" function and global calibration. The band area was then selected and individual bands were manually identified and their intensity measured. Measured intensity of these bands was then compared to define the cycles during which the PCR reaction was within the exponential phase. Bands within this range were then compared to the ferritin (control) bands in the same range to produce a ratio in order to calculate the band intensity. JMP software was used to calculate the standard deviation of each sample, as well as the P-value of each sample in relation to the others in its grouping.

In Situ probe production

Bactericera cockerelli unigene 31763 transcript cloned in pGEMT vector (obtained in Chapter III) was used to produce the probes. Plasmid was purified using PureLink Quick Plasmid Miniprep Kit (Invitrogen) and linearized by specific restriction enzymes (SacII and SalI) during 8 h at 37°C, then the degree of linearization was examined on a 1% agarose gel. After a complete digestion, linearized plasmids were cleaned by ethanol precipitation by incubating the digestion product for at least 2h at -20°C with 0.3 M sodium acetate (pH 5.2) and 75% ethanol. After centrifugation at 12,000 rpm for 30 min at 4°C, linearized plasmids were resuspended in water. *In vitro* transcription was performed with 1 µg of linearized plasmids using the *in vitro* transcription using DIG RNA labeling kit (SP6 T7) (Roche) following the

manufacturer's instructions.

In situ hybridization

In situ hybridizations of dissected tissues were performed as described in Price et al. for aphid gut sucrase expression but with modifications (Price et al. 2007). Insect guts, bacteriocytes, ovaries and testes were dissected from adult *B. cockerelli* as previously described. Dissections were performed in 1x Phosphate-buffered Saline (PBS), then fixed in 3.8% formaldehyde in 1x PBS at room temperature during 2 h. The tissues were washed once for 5 min with 1x Phosphate-buffered saline, 0.1% (v/v) Tween 20 (PBST) and dehydrated with 100% methanol at -20°C until further processing. The tissues were rehydrated through a graded series of methanol/PBST, washed 3 times for 5 min in PBST. The hybridization was carried out overnight at 60 °C with 2.0 ng/µL sense or antisense DIG-dUTP labeled RNA probes in hybridization solution (50% (v/v) formamide, 5× SSC, 1 mg/ml total yeast RNA, 100 mg/ml heparin, 0.1% (v/v) Tween 20). After hybridization, nonspecific probes were washed off at 60°C with the following steps: 2x SSC, 1 h; and 0.2x SSC twice, 1 h. After, the tissues were washed gently twice with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, 0.1% (v/v) Tween 20 pH 7.5) at room temperature for 10 min, and blocked in 1x Blocking Reagent (Roche) for 2.5 h at room temperature. The tissues were then incubated with anti-DIG AP fragments antibody (Roche) at 1:2000 in 1x blocking solution with gently shaking (50 rpm) overnight at 4 °C. The antibody was detected after four washes for 20 min at room temperature in maleic acid buffer and the color developing was performed using BM Purple alkaline phosphatase substrate (Roche) plus 5mM levimasole to block or avoid the endogenous alkaline phosphatase activity.

4.3 Results and Discussion

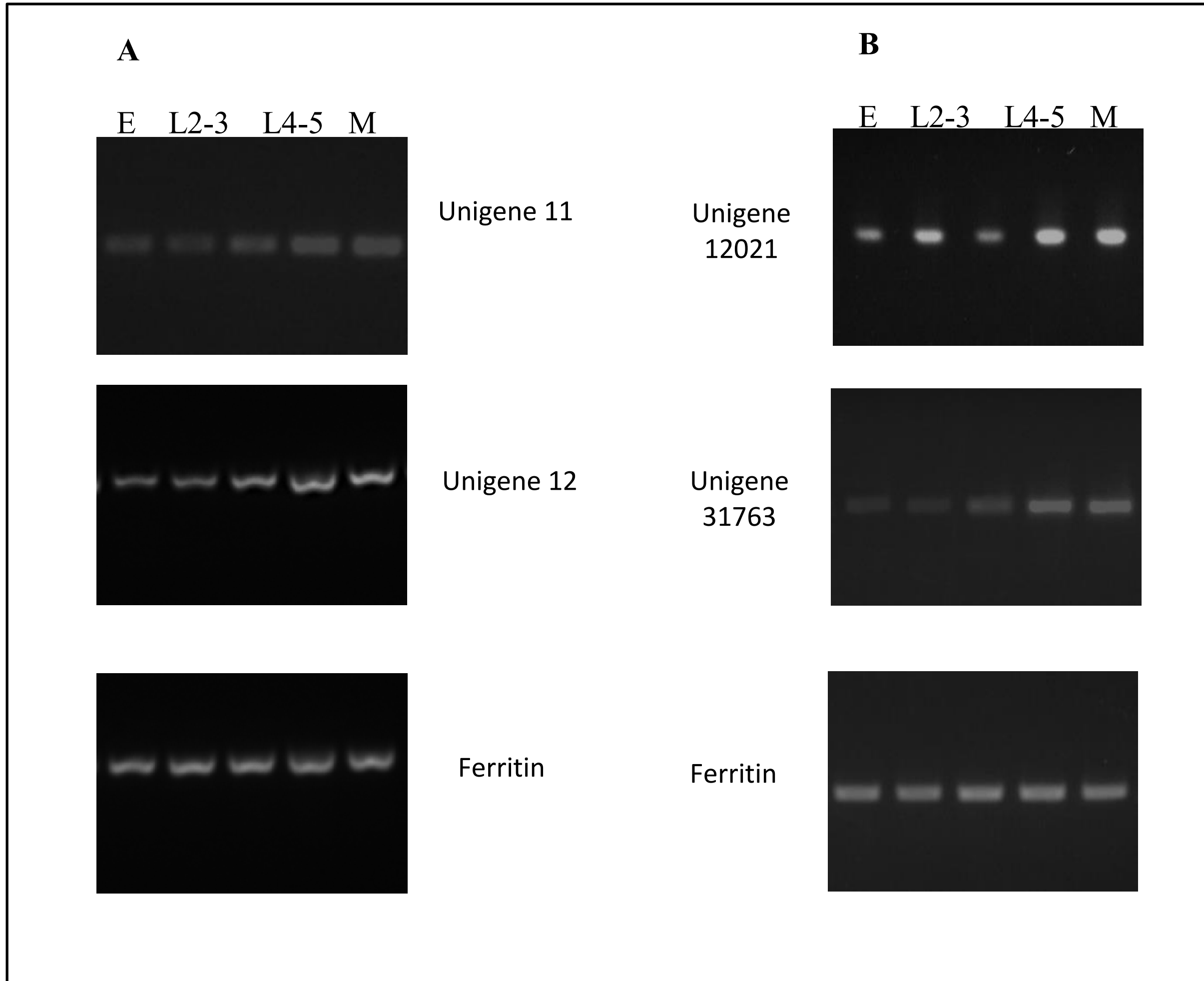
Aquaporin expression at different life-stages

Analysis of RT-PCRs showed that the four tested psyllid candidate aquaporins *B. cockerelli* unigenes 12021 and 31763 and *D. citri* unigenes 11 and 12 were expressed in all life stages (eggs, young nymphs, old nymphs, adult females and adult males). No particular expression pick was seen during the different psyllid life stages (Figure 16).

Aquaporin expression in different tissues

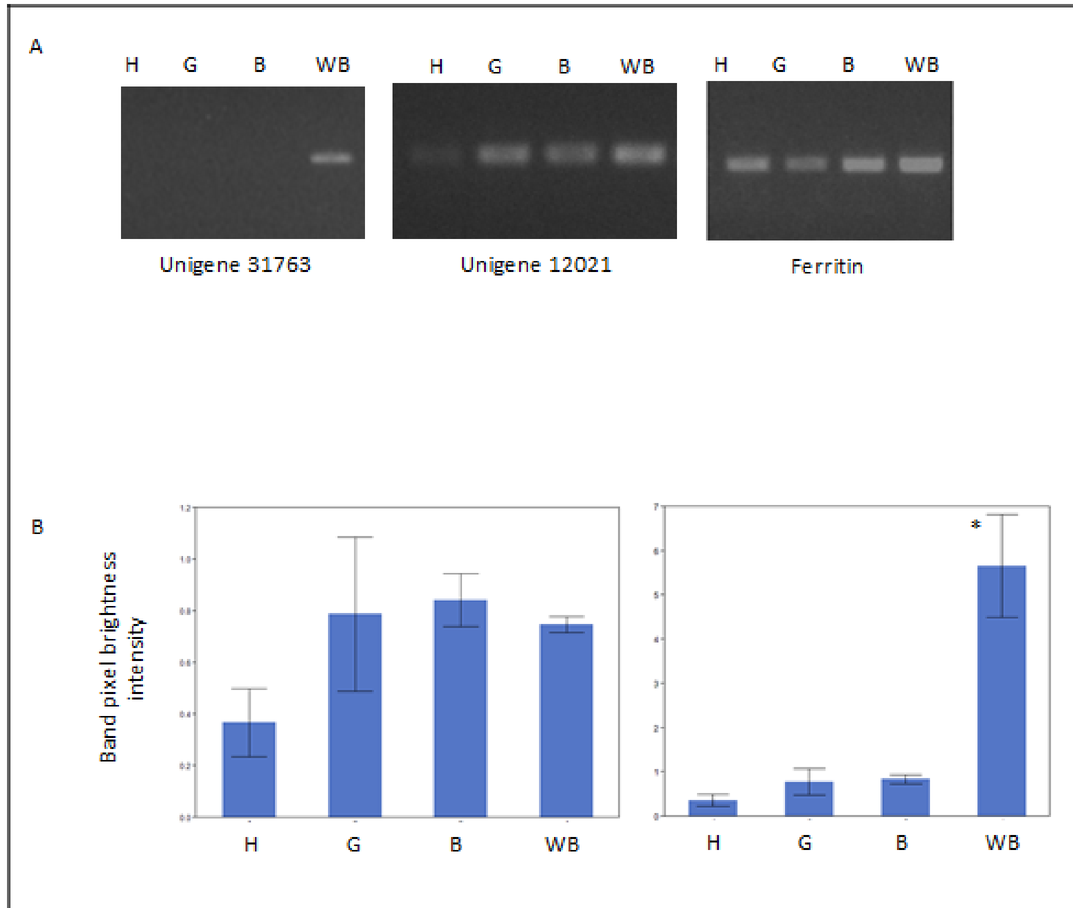
Expression of *B.cockerelli* unigene 12021 was observed in all tested tissues (head, gut, bacteriocyte). No differences in expression level among those tissues were observed (Figure 17). For *B. cockerelli* unigene 31763 low expression level was found in gut, head and bacteriocytes (Figure 17). *Diaphorina citri* aquaporin candidates were found expressed in the 3 tissues (head, gut and whole body see Figure 18). No differences of expression were found among those tissues. However, *D. citri* unigene 12 seems to be highly expressed in the gut (Figure 18).

Figure 16: Expression *of B. cockerelli* Unigenes 12021 and 31763 and *D. citri* Unigenes 11 and 12 in Psyllid Lifestages



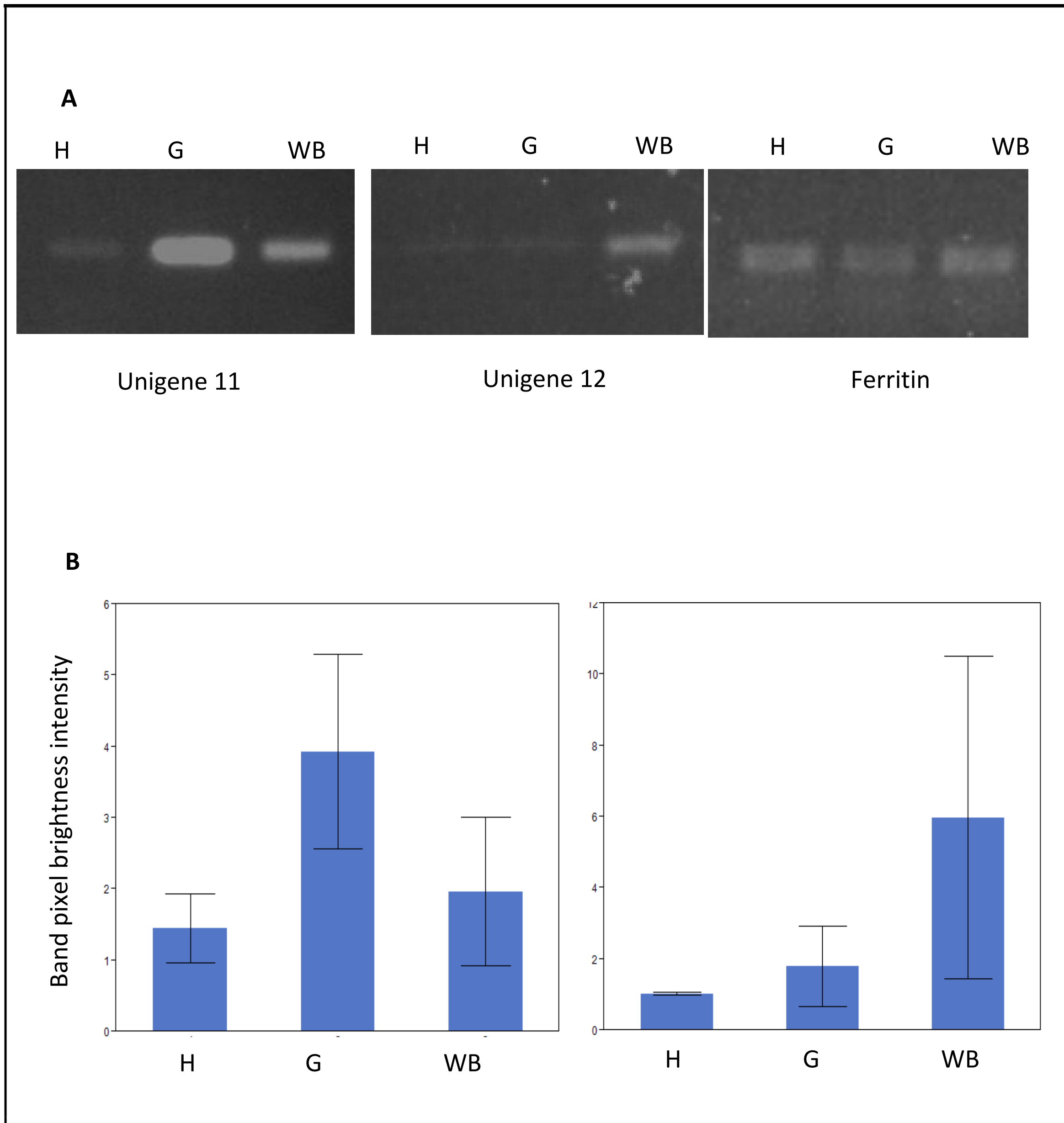
A: Images from *B. cockerelli* unigenes 12021, 31763, and *B. cockerelli* Ferritin RT-PCR.
B: Images from D. citri unigenes 11, 12 and *D. citri* Ferritin RT-PCR. E=egg, L2-3= young nymphs, L4-5= old nymphs, M=male, F= female.

Figure 17: Expression Analyses of *B. cockerelli* Unigene 12021 and 31763



Analyses in dissected tissues head (H), gut (G), bacteriocyte (B) and whole body (WB) sampels as compared to the consultative expression of ferritin within these tissue groups. A) RT-PCR picture results B) and C) RT-PCR analyses using ImageJ software and normalized against Ferritin expression for unigenes 12021 and 31763 respectively.

Figure 18: Expression Analyses of *D. citri* Unigenes 11 and 12



Analyses in dissected tissues head (H), gut (G), and whole body (WB) samples as compared to the consultative expression of ferritin within these tissue groups. A) RT-PCR picture results, B) and C) RT-PCR analyses using ImageJ software and normalized against Ferritin expression for unigenes 11 and 12 respectively.

Bactericera cockerelli unigene 31763 expression analysis by in situ hybridization

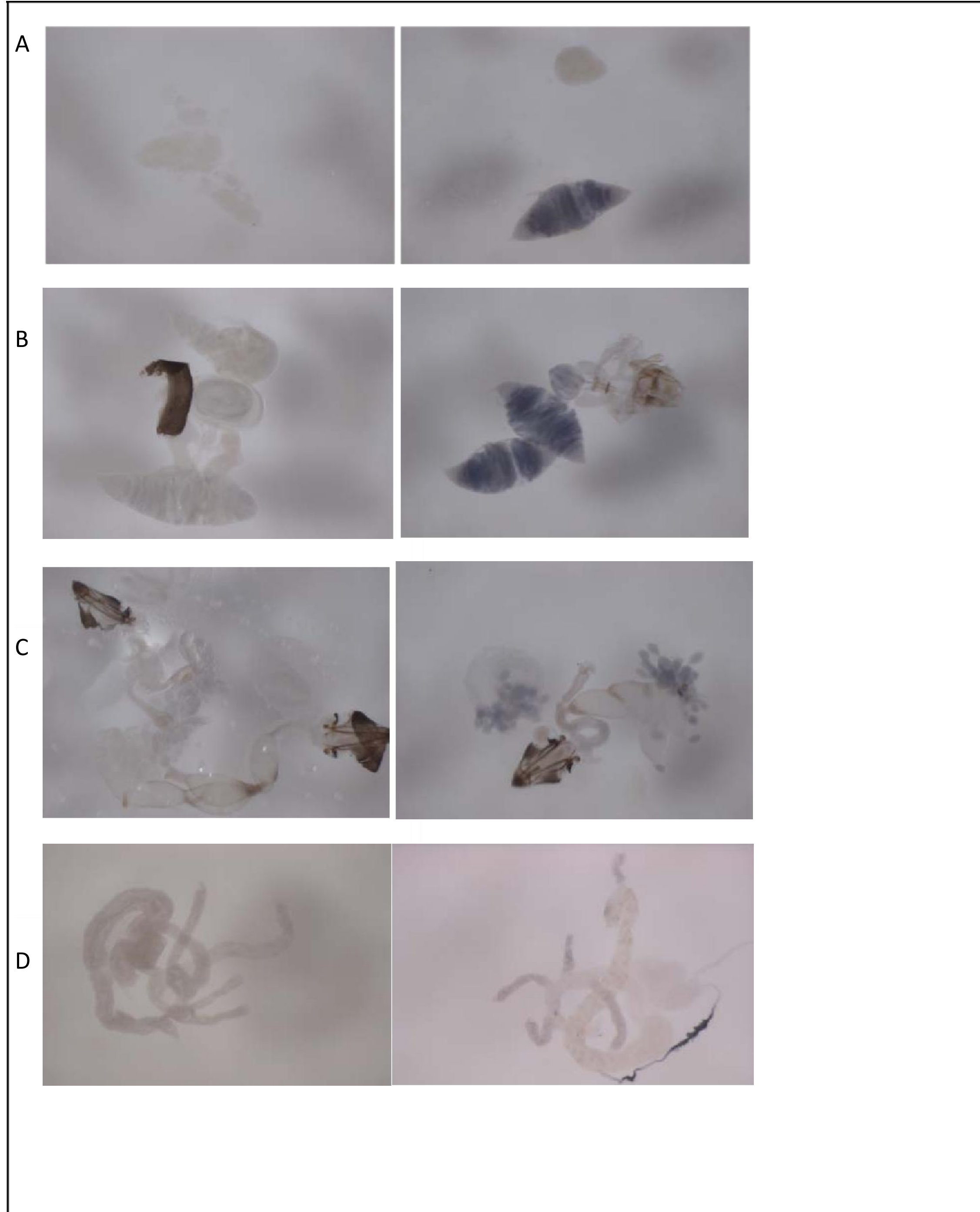
Bactericera cockerelli unigene 31763 was chosen to further investigate transcript expression using *in situ* hybridization. This candidate was chosen because it is similar to *D. citri* unigene 11 and because expression analysis using RT-PCR appeared to show low expression in the tested tissues (head, gut and bacteriocyte).

As shown in Figure 19, expression of this candidate was observed in the reproductive tissues (testis and ovaries), but no signal was observed from guts or bacteriocytes. This result is interesting for several reasons:

- *D. citri* unigene 11 was identified in libraries made from the gut and from testis. It is possible that some tissue contamination might have occurred during library construction, that this gene is expressed at extremely low levels in the gut or simply, that differences exist between the two psyllid species with respect of this gene.
- This unigene and *D. citri* 11 clustered together in the phylogenetic tree, but they appear to share some degree of similarity with the cluster of putative aquaglyceroproteins that are expressed in the bacteriocyte (data from Wallace et al. 2012 and data unpublished from our laboratory). Therefore, our results show, that *B. cockerelli* 31763 is not expressed in the bacteriocyte as the other *B. cockerelli* candidate aquaporins.

According to our expression pattern, it appears that this candidate aquaporin might be involved in reproduction. However, other functions cannot be excluded since the gene was found also expressed in nymphal stages.

Figure 19: *In situ* Hybridization of *B. cockerelli* with Unigene 31763 Probe



Sense probe incubations on the left and antisense probe incubations on the right: A) Bacteriocyte (the antisense shows a part of a testis on the bottom middle of the image) B) Male reproductive organs, C) Female reproductive organs, D) Gut dissection

5. CONCLUSIONS

Prior to this work, only three Hemiptera aquaporins had been identified and characterized. These aquaporins, established within the pea aphid and whitefly, identified aquaporins as potentially having important functions in water retention within the gut of the insect as well as the potential for functioning within the fat body/bacteriocyte (Shakesby et al. 2009, Mathew et al. 2011, Wallace et al. 2012). Our study has identified within the NCBI database an upwards of 25 novel aquaporin candidates within hemiptera species, greatly increasing the available knowledge of aquaporins in these insects. By obtaining these sequences, potential aquaporins in the varied other species of hemiptera could be isolated with greater ease, thereby furthering the phloem feeding hemipteran aquaporin information we have.

Of the aquaporin candidates identified, we were able to isolate the full sequences of two aquaporins candidates from *Diaphorina citri* and four aquaporin candidates *Bactericera cockerelli.* These hemiptera have had no previous aquaporins identified nor a genomic database available for searching, and thus the technique used to isolate these sequences could prove to be useful in obtaining other expressed genes of interest before an annotated genomes become available. From the full coding sequences of these candidate aquaporins, potential aquaglycerol function was identified in two of the *B. cockerelli* aquaporin candidates, Unigene 7752 and 39565, using C loop region homology to *D. melanogaster* aquaporins. Furthermore, phonetic relationships of these candidates to other aquaporin candidates was accessed, giving more credence to the possibility that unigene 7752 and 39565 may have similar function. *D citri* unigene 12 and *B. cockerelli* unigene 12021 were identified with the Aquaporins traditionally found in relation to the gut in phonetic studies, however in expression analysis neither were found in to be significantly expressed in any one tissue type.

However, *D citri* unigene 11 and *B. cockerelli* unigene 31763 both showed phonetic similarities to each other in Neighbor-Joining, and in studying expression unigene 31763 showed significant differences in expression within the whole body. This

indidates that this gene may be expressed within a tissue other than the head, gut, or bacteriocyte, which was confirmed though *in situ* analyses showing increased expression within the sexual organs of male and female *B. cockerelli*.

Further study and assessment

The methods in which the sequences were initially obtained, using EST information and a pipeline to acess candidates for similarities and other important information, appears to be a very adequate means of identifying gene candidates when no genome is available, yet EST or other sequence information can be obtained. This method theoredically needs to assume that the genes searched have a high sequence similarity to other known genes in similar species, as well as expression within the insect in the stages/tissues where ESTs were collected. Any gene having these two factors can be searched in the same manner as these aquaporin candidates and will potentially identify candidates.

Our attempts at localizing several of these aquaporins within the psyllids appeared to show little differentiation in expression within lifestages/tissues. This could be potentially due to a lack of sensitivity in the RT-PCR methods used, and further studies should attempt to increase sensitivity. This includes using better aging methods, such as days from ovapostion/hatching for the early nymphal lifestages or using similarly aged adults (e.g. adults that are 2 days old, versus the random selection of ages in this study). Another area of importance for increasing sensitivity is in the dissections. Improved dissection techniques could potentially lead to more isolated samples, as the techniques used in this study potentially caused some unavoidable contamination of tissue groups. By improving dissection, better analysis can be done to try and identify areas of aquaporin expression.

Full characterization of these aquaporins candidates needs to be completed before any of these candidates can be properly considered aquaporins. This involves characterizing the function of the encoded protein, which has been done with other phloem feeding Hemiptera using zenpus oocyte expression (Mathew et al. 2011). This method would be ideal to characterize the water transport rate of these aquaporins as

well as identify if they function as aquaglyceroporins. This will aid us in identifying if our structural assessments using sequence information is correct, and potentially will help forming predictive models of how other aquaporin candidates may function.

This study has identified several new aquaporin candidates, increasing our knowledge of insect aquaporins in insects with highly unique osmoregulatory challenges. These insects, *D. citri* and *B. cockerelli*, are also known agricultural pests, and our work here may lead to advances in understanding important regulatory functions within these insects and how we may manipulate them for insect control and management. Of the candidates studied, unigene 31763 showed unique expression within reproductive organs that is unlike what has previously been found in phloem feeding Hemiptera, thus not only opening a potentially new area of function for aquaporins in these insect, but perhaps a way to manipulate production.

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