

Functional analysis of the aquaporin gene family in *Caenorhabditis elegans*

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Huang CG, Lamitina T, Agre P, Strange K. Functional analysis of the aquaporin gene family in *Caenorhabditis elegans*. *Am J Physiol Cell Physiol* 292: C1867–C1873, 2007. First published January 17, 2007; doi:10.1152/ajpcell.00514.2006.—Aquaporin channels facilitate the transport of water, glycerol, and other small solutes across cell membranes. The physiological roles of many aquaporins remain unclear. To better understand aquaporin function, we characterized the aquaporin gene family in the nematode *Caenorhabditis elegans*. Eight canonical aquaporin-encoding genes (*aqp*) are present in the worm genome. Expression of *aqp-2*, *aqp-3*, *aqp-4*, *aqp-6*, or *aqp-7* in *Xenopus* oocytes increased water permeability five- to sevenfold. Glycerol permeability was increased three to sevenfold by expression of *aqp-1*, *aqp-3*, or *aqp-7*. Green fluorescent protein transcriptional and translational reporters demonstrated that *aqp* genes are expressed in numerous *C. elegans* cell types, including the intestine, excretory cell, and hypodermis, which play important roles in whole animal osmoregulation. To define the role of *C. elegans* aquaporins in osmotic homeostasis, we isolated deletion alleles for four *aqp* genes, *aqp-2*, *aqp-3*, *aqp-4*, and *aqp-8*, which are expressed in osmoregulatory tissues and mediate water transport. Single, double, triple, and quadruple *aqp* mutant animals exhibited normal survival, development, growth, fertility, and movement under normal and hypertonic culture conditions. *aqp-2;aqp-3;aqp-4;aqp-8* quadruple mutants exhibited a slight defect in recovery from hypotonic stress but survived hypotonic stress as well as wild-type animals. These results suggest that *C. elegans* aquaporins are not essential for whole animal osmoregulation and/or that deletion of aquaporin genes activates mechanisms that compensate for loss of water channel function.

water channel; osmoregulation

AQUAPORINS ARE CHANNEL-FORMING proteins that facilitate the transport of water and small solutes such as glycerol and urea (1). Genome sequencing projects have revealed the presence of >450 aquaporin-encoding genes in all kingdoms of life (36). These channels fall into two functionally distinct subgroups that include the water-permeable aquaporins and the glycerol-permeable aquaglyceroporins. Both channel types assemble as a homotetramer. Aquaporin monomers are composed of six transmembrane helices and contain two hemipores (9, 23). A highly conserved Asn-Pro-Ala (NPA) signature sequence is located near the center of each hemipore. Amino acids surrounding the NPA boxes confer water and/or solute permeability to the channel (10).

Animal genomes contain anywhere from seven (*Drosophila melanogaster*) to 13 (*Homo sapiens*) aquaporins, and these genes are expressed in highly tissue-specific patterns (1, 16, 32). However, several aquaporin knockout mutants fail to exhibit

detectable phenotypes in these tissues (30), suggesting that these channels may function redundantly to regulate transmembrane solute and water flux. An essential test of this hypothesis is the analysis of animals harboring mutations in multiple aquaporin genes. To date, only a limited number of aquaporin double knockout mutant mice have been analyzed (27, 28, 33–35).

The nematode *Caenorhabditis elegans* offers numerous experimental advantages for defining basic physiological processes such as water and solute transport and cellular osmoregulation (29). In addition, the aquaglyceroporins substrate glycerol is the primary organic solute used by worms to adapt to and recover from hypertonic stress (19). Therefore, we hypothesized that the aquaporin gene family may play important functional roles in osmotic adaptation in *C. elegans*. The worm genome contains eight canonical aquaporin-encoding genes (*aqp-1*–*8*). Biophysical characterization of heterologously expressed *aqp-2* and *aqp-4* has been carried out by Kuwahara et al. (17, 18). However, nothing is known about the cellular and tissue expression patterns of these channels or about their physiological roles. In this study, we cloned the cDNAs for all eight *C. elegans* *aqp* genes and characterized their water and glycerol permeabilities in *Xenopus* oocytes. We also determined their expression patterns using green fluorescent protein (GFP) translational and transcriptional reporters. Finally, we isolated deletion alleles for four aquaporins that are expressed in osmoregulatory tissues and created single, double, triple, and quadruple aquaporin mutant worms to explore the physiological roles of the channels in whole animal osmotic homeostasis.

MATERIALS AND METHODS

C. elegans strains. Nematodes were cultured using standard methods (4). Worm strains used in this study were wild-type Bristol N2 strain and the *ced-3* loss-of-function mutant strain MT2405.

Alignment and phylogenetic analysis. All *C. elegans* aquaporin predicted sequences were obtained from Wormbase (www.wormbase.org). Sequences were aligned using ClustalX version 1.8. A phylogenetic tree was generated from the alignment using the Phylodendron, which can be accessed from <http://www.es.embnnet.org/Doc/phylogendron/treeprint-form.html>.

cDNA cloning. Basic local alignment search tool (BLAST) homology searches of WormPep and the *C. elegans* genome with human AQP-1 yielded eight homologous predicted open reading frames (ORFs). The *C. elegans* ORFs encoding Ce-AQPs (sequence name followed by the gene name) are F32A5.5b (*aqp-1*), C01G6.1b (*aqp-2*), Y69E1A.7 (*aqp-3*), F40F9.9 (*aqp-4*), C35A5.1 (*aqp-5*), C32C4.2 (*aqp-6*), M02F4.8 (*aqp-7*), and K02G10.7b (*aqp-8*). Primers were designed using the predicted gene models as a template. Aquaporin cDNAs were obtained with a one-step RT-PCR kit (Qiagen, Valencia,

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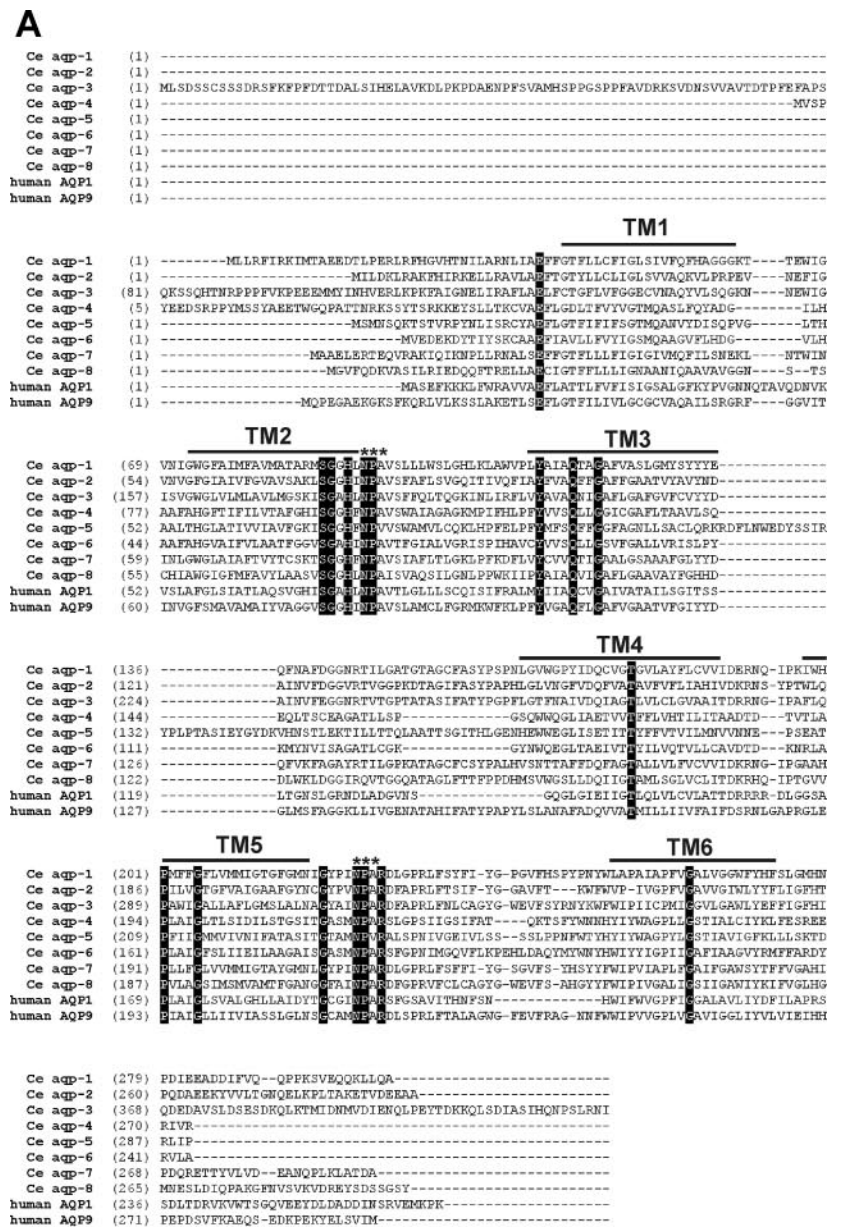
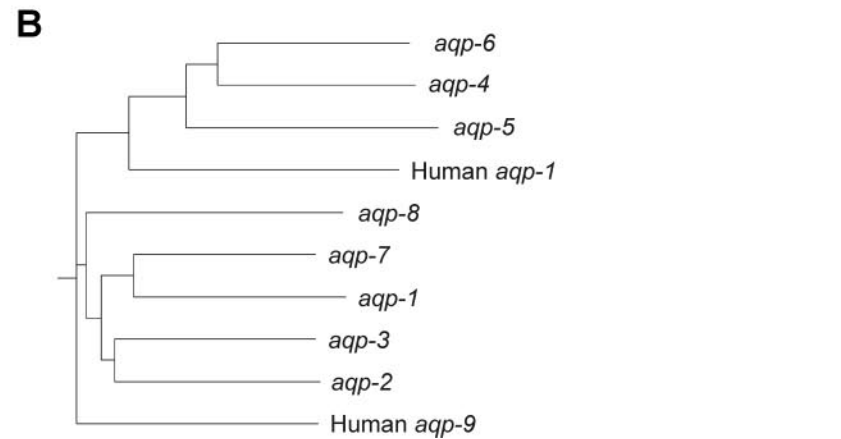


Fig. 1. Phylogenetic analysis of *Caenorhabditis elegans* aquaporin gene family. *A*: alignment of *C. elegans* aquaporins (Ce-aqp) with human AQP-1 and AQP-9. Transmembrane domains (TM) are predicted from the human AQP-1 structure (31). Amino acid residues conserved in all channels are highlighted. Asterisks indicate location of conserved Asn-Pro-Ala (NPA) boxes. *B*: rooted phylogenetic tree of *C. elegans* aquaporins. Tree was constructed using the neighbor-joining method (see MATERIALS AND METHODS). Human *aqp-1* and *aqp-9* were used as references in the tree.



CA) using mRNA from mixed-stage worms. In all cases, *aqp* cDNA clone sequences matched those predicted in Wormbase.

Oocyte transport studies. Aquaporin cDNAs were inserted into the *Xenopus* expression vector pXβG-ev1, which includes an in-frame NH₂-terminal myc tag. Capped cRNAs were synthesized in vitro from linearized plasmids (26). Defolliculated *Xenopus* oocytes were injected with 10 ng of cRNA dissolved in 50 nl of diethyl pyrocarbonate-treated water. Injected oocytes were incubated for 3 days at 18°C in 200 mosmol/kgH₂O Barth's solution containing (in mM) 88 NaCl, 1 KCl, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 2.4 NaHCO₃, and 10 HEPES, pH 7.4. Osmotic water permeability (P_f) was determined by measuring the rate of oocyte swelling induced by transferring oocytes to 67 mosM Barth's solution (Barth's solution diluted 1:3 with H₂O) as previously described (26). Glycerol permeability (P_s) was measured by placing oocytes in Barth's solution diluted 1:1 with 200 mM glycerol. The rate of glycerol-induced cell swelling was used to calculate P_s as described previously (6). The expression and localization of each channel to the oocyte plasma membrane was confirmed using an anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and immunofluorescence microscopy (data not shown).

Construction of transgenes and transgenic worms. Ce-AQP transcriptional and translational GFP reporters were created by PCR amplification and cloning of putative promoter regions into pPD95.75 using standard procedures (courtesy of A. Fire, Departments of Genetics and Pathology, Stanford University School of Medicine, Stanford, CA). We defined the promoter region of the *aqp* genes as the sequence between the start codon and the stop codon of the next upstream gene. When the promoter region was less than 3 kb, all promoter sequence was included in the fusion construct. Otherwise, at least 3 kb of upstream sequence was used. The size of the upstream promoter sequence used for each gene is approximately as follows: *aqp-1*, 1.5 kb; *aqp-2*, 5 kb; *aqp-3*, 2.8 kb; *aqp-4*, 2.2 kb; *aqp-5*, 3 kb; *aqp-6*, 3.7 kb; *aqp-7*, 4.5 kb; and *aqp-8*, 2.5 kb. Transgenic worms were generated by DNA microinjection with rol-6 as a cotransformation marker (22). GFP expression patterns were determined by confocal microscopy using a Zeiss LSM510 laser scanning microscope.

Isolation of deletion alleles. Ce-AQP deletion alleles were generated using a method described previously (13). Mutant worms were outcrossed four times to the wild-type N2 strain before phenotypic analyses were performed. All of the deletion mutations cause frame shifts and give rise to early stop codons. In the unlikely event that any of these mRNAs are translated, they would give rise to proteins missing at least four transmembrane domains. Therefore, these mutations are almost certainly null mutations. The following double, triple, and quadruple mutants were generated by crossing and PCR genotyping: *aqp-2;aqp-3*, *aqp-2;aqp-4*, *aqp-2;aqp-8*, *aqp-3;aqp-4*, *aqp-3;aqp-8*, *aqp-4;aqp-8*, *aqp-2;aqp-3;aqp-8*, *aqp-2;aqp-3;aqp-4*, and *aqp-2;aqp-3;aqp-4;aqp-8*.

Hypotonic stress assays. To assess the effect *aqp* gene deletion of hypotonic stress resistance, we monitored worm motility. Worms were grown on high-salt (365 mM NaCl) nematode growth medium (NGM) for at least 1 wk. Low-salt (1 mM NaCl) 6-cm NGM plates were prepared by spreading one-third of the plate with 50 μl of OP50 bacteria. L4 and young adult worms were manually transferred from high-salt plates to the side of low-salt plates lacking bacteria. The number of worms that reached the bacterial food source was counted every 10 min for the first 3 h and every hour after that. Mobility assays were performed similarly, except that the low-salt plates did not contain food. Worm motility was defined as spontaneous movement of at least one full body length.

Statistical analysis. Data are presented as means ± SE. Statistical significance was determined using either one-way analysis of variance or Student's two-tailed *t*-test for unpaired means.

RESULTS AND DISCUSSION

Phylogenetic analysis of putative *C. elegans* aquaporins. A BLAST search using the human AQP-1 sequence revealed the presence of eight putative aquaporin-encoding genes in the *C. elegans* genome. The genes encode proteins predicted to be 244–421 amino acids long and share 20–35% identity with human AQP-1. Phylogenetically, *aqp-4*, *aqp-5*, and *aqp-6* belong to the water-specific aquaporin subfamily, whereas *aqp-1*, *aqp-2*, *aqp-3*, *aqp-7*, and *aqp-8* belong to the aquaglyceroporin subfamily (Fig. 1). All *C. elegans* aquaporins have six predicted transmembrane helices and two NPA boxes that are the hallmarks of this gene family (37). *aqp-5* and *aqp-8* are the most distant member of each subgroup. *aqp-5* contains a valine instead of an alanine in the third position of both NPA boxes, which is the most common substitution found in over 450 analyzed aquaporin-encoding genes (36). *aqp-5* also exhibits an extended extracellular region between the second and third transmembrane domains. *aqp-3* possesses an extended NH₂-terminal cytoplasmic domain of 76 amino acids.

Water and glycerol permeability of *C. elegans* aquaporins. To characterize their transport properties, the cDNAs for all eight *C. elegans* aquaporins were cloned and expressed in *Xenopus* oocytes. Expression of *aqp-2*, *aqp-3*, *aqp-4*, *aqp-6*, or *aqp-7* induced a significant ($P < 0.01$) five- to sevenfold increase in oocyte water permeability (Fig. 2A). Water trans-

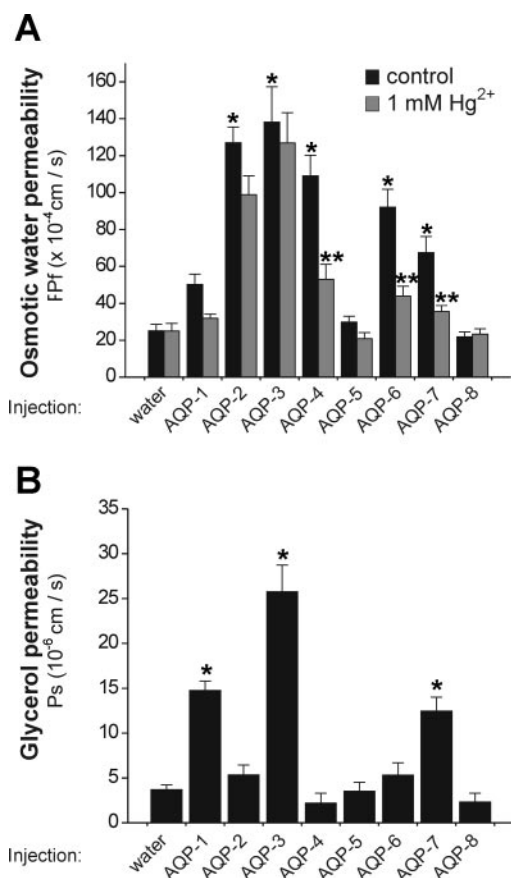


Fig. 2. Transport properties of *C. elegans* aquaporins. Water (A) and glycerol (B) permeabilities of *Xenopus* oocytes injected with water or *C. elegans* aquaporin-encoding cRNAs. P_f , water permeability; P_s , glycerol permeability. * $P < 0.05$ compared with water-injected oocytes. ** $P < 0.05$ compared with control water permeability in the absence of 1 mM HgCl₂. Values are means ± SE ($n = 6-11$).

port through AQP-4, AQP-6, and AQP-7 was inhibited significantly ($P < 0.04$) by 1 mM mercury, whereas AQP-2 and AQP-3 were mercury insensitive (Fig. 2A).

AQP-1, AQP-3, and AQP-7 showed significant ($P < 0.01$) glycerol permeability (Fig. 2B). The low water permeability of AQP-1 and AQP-7 (Fig. 2A) is similar to that of other members of the aquaglyceroporin subfamily (6). Expression of *aqp-5* or *aqp-8* had no effect on oocyte glycerol, water (Fig. 2), or urea (data not shown) permeability despite abundant surface expres-

sion that was detected using immunofluorescence (data not shown).

Localization of aquaporin gene expression. To determine the expression pattern of *C. elegans* aquaporins, we generated transgenic worms expressing transcriptional or translational GFP reporters for each of the eight *aqp* genes. It should be noted that GFP reporters likely recapitulate the normal pattern of aquaporin expression. However, definitive localization of the channels will require immunolocalization methods using specific antibodies.

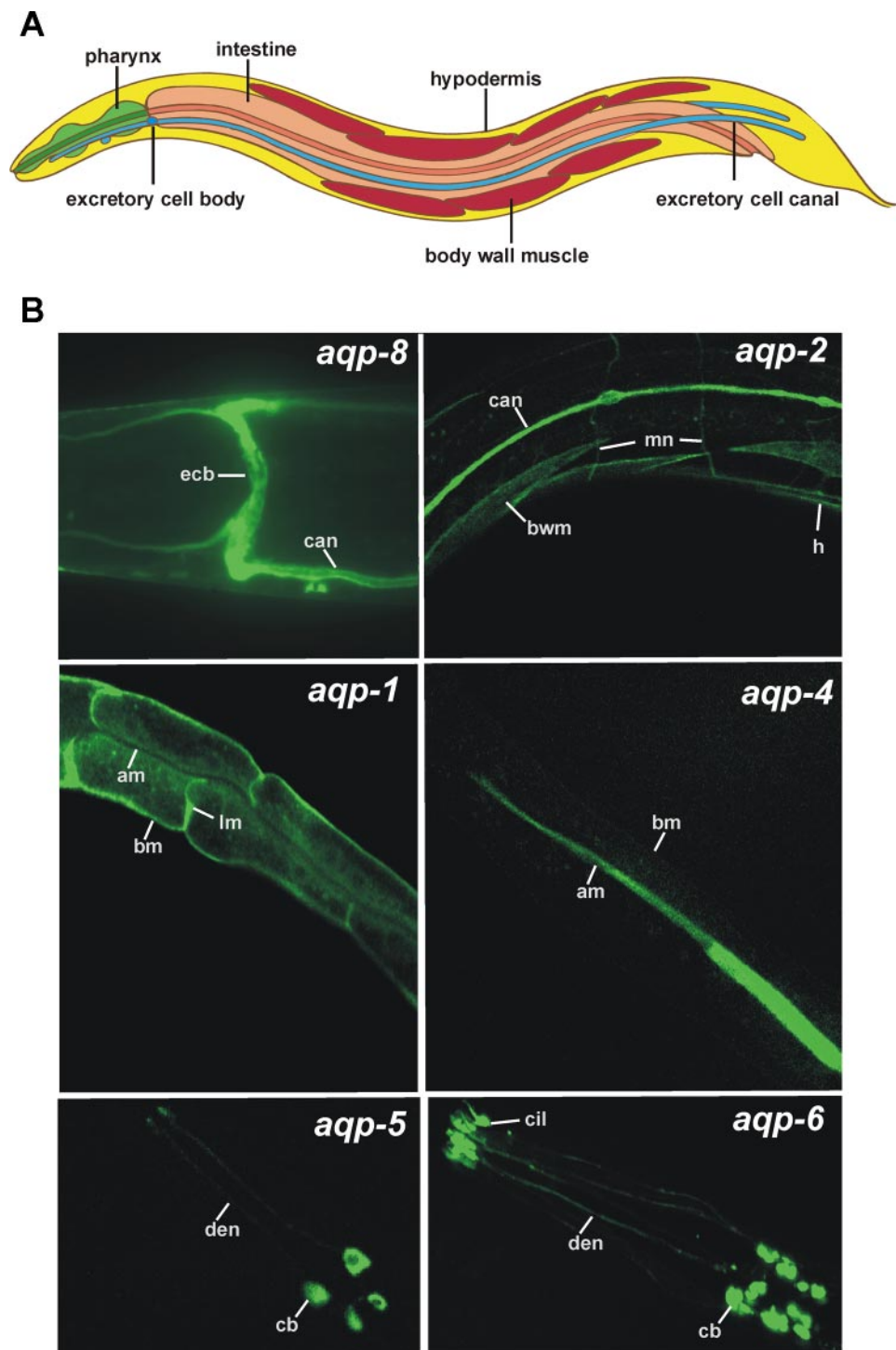


Fig. 3. Expression pattern of *C. elegans* aquaporins. *A*: diagrams of worm anatomy. *B*: representative confocal images of transgenic animals expressing the *C. elegans* aquaporin proteins COOH-terminally fused to green fluorescent protein (GFP). For all images, anterior is to the *left* and posterior is to the *right*. Complete expression pattern for all 8 *C. elegans* aquaporins is summarized in Table 1. ecb, excretory cell body; can, excretory cell canal; bwm, body wall muscle; mn, motor neuron; hyp, hypodermis; am, apical membrane of intestine; bm, basal membrane of intestine; lm, lateral membrane of intestine; cb, cell body; den, dendrite; cil, ciliated dendritic tip.

Table 1. Summary of *Caenorhabditis elegans* aquaporin expression patterns

Gene/Sequence Name	Permeability	Expression
<i>aqp-1</i> /F32A5.5	Glycerol	Pharynx, intestine (basolateral membrane)
<i>aqp-2</i> /C01G6.1	Water	Excretory cell, muscle, hypodermis
<i>aqp-3</i> /Y69E1A.7	Water, glycerol	Intestine, excretory cell, seminal vesicle/vas deferens
<i>aqp-4</i> /F40F9.9	Water	Intestine (apical membrane)
<i>aqp-5</i> /C35A5.1	Unknown	I1 neurons
<i>aqp-6</i> /C32C4.2	Water	IL1 neurons
<i>aqp-7</i> /M02F4.8	Water, glycerol	Muscle
<i>aqp-8</i> /K02G10.7	Unknown	Excretory cell

Gene/sequence names are as shown in Wormbase (www.wormbase.org). Statistically significant permeabilities are as shown in Fig. 2 ("unknown" indicates no significant permeability to water or glycerol). Expression indicates cell/tissue localization of aquaporin::green fluorescent protein (*aqp*::GFP) transcriptional and translational transgenes. *aqp-3* transcriptional reporters were expressed in the intestine and excretory cell, as well as male seminal vesicle and vas deferens, whereas *aqp-3* translational GFP reporters were expressed only in the male seminal vesicle and vas deferens. This difference in expression may reflect tissue-specific differences in the processing of the AQP-3::GFP fusion protein. Expression of *aqp-3* transcriptional reporters in the intestine and excretory cell was upregulated in starved worms (data not shown), consistent with previous microarray data (38).

Figure 3 shows examples of *aqp-GFP* gene expression patterns. The results are summarized in Table 1. As expected, many *C. elegans* aquaporins are expressed in cells that play significant roles in water and solute transport. For example, *aqp-2*, *aqp-3*, and *aqp-8* are expressed in the excretory cell, which is a single polarized cell that forms canals analogous to mammalian renal tubules (5, 24). Disruption of excretory cell function by laser ablation causes animals to swell with fluid and die (25), indicating that this cell functions in fluid secretion and excretion.

aqp-1 and *aqp-4* were expressed predominantly in the intestine, which functions in nutrient and probably water absorption. Interestingly, the water-permeable AQP-4 was localized only to the apical membrane of the intestine, whereas the glycerol-permeable AQP-1 was expressed on the basolateral membrane (Fig. 3B). *C. elegans* adapts to chronic hypertonic stress by accumulating the organic osmolyte glycerol (19). The intestine is a major site of osmotically induced glycerol production (20). Expression of AQP-1 on the intestinal basolateral

membrane may promote rapid efflux of glycerol into the pseudocoelomic fluid, which bathes all non-glycerol-producing cells.

In addition to the excretory cell, *aqp-2* was expressed in muscle, motor neurons, and hypodermis. The hypodermis, like the excretory cell, plays an important role in the maintenance of whole animal fluid balance (14). Expression of *aqp-3* in the male seminal vesicle and vas deferens may reflect a requirement for aquaporins in generation of seminal fluids as has been proposed for mammals (7).

The remaining *C. elegans* aquaporins were expressed in diverse cell types. *aqp-7* was expressed in a punctuate pattern in muscle cells. Similar patterns of gene expression in *C. elegans* muscle have been observed for proteins that localize to focal adhesions, which are sites of cell-extracellular matrix interactions that regulate intracellular signaling pathways (12, 21). Both *aqp-5* and *aqp-6* were specifically expressed in specialized head neurons that failed to stain with the vital dye DiI, suggesting they are not amphid or IL2 sensory neurons (data not shown). Based on cell morphology, anatomy, and lineage analysis using *ced-3* mutants in which IL1, I1, and I2 sister cells fail to undergo programmed cell death (8), we conclude that the four *aqp-5*-expressing neurons likely correspond to I1 and I2 pharyngeal interneurons. These neurons function to modulate the rate of pharyngeal pumping in the absence of food (2). The six *aqp-6*-expressing neurons are likely IL1 sensory neurons that function in mechanosensation and food foraging behaviors (11). Expression of AQP-6::GFP was highly enriched in the cilia and cell bodies of these neurons (Fig. 3). Other than mammalian AQP-9, *C. elegans* AQP-2, AQP-5, and AQP-6 are the only aquaporins known to be expressed in neurons (3).

Phenotypic analysis of aquaporin deletion mutations. To better understand the physiological roles of *C. elegans* aquaporins, we generated deletion alleles for *aqp-2*, *aqp-3*, *aqp-4*, and *aqp-8*. All alleles contained deletions in one or more critical transmembrane helices and pore-lining NPA domains (Fig. 4A). Since water channel structure and function requires intact NPA and transmembrane domains (15), all deletion mutations are likely null alleles. We focused our analyses on *aqp-2*, *aqp-3*, *aqp-4*, and *aqp-8* because they have the high water permeabilities (Fig. 2) or are expressed in osmoregulatory tissues (Fig. 3). In addition, microarray analyses demon-

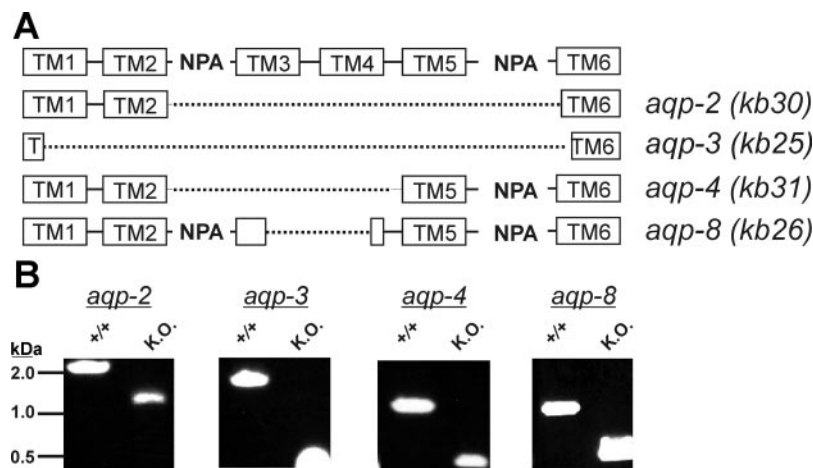


Fig. 4. Isolation of *aqp-2; aqp-3; aqp-4; aqp-8* quadruple mutant animals. A: diagram showing domain structure of a canonical aquaporin and location of deletion mutations (dotted lines) in *aqp-2*, *aqp-3*, *aqp-4*, and *aqp-8*. Allele designations are indicated in parentheses. B: PCR genotyping of wild type (+/+) and *aqp-2; aqp-3; aqp-4; aqp-8* quadruple deletion mutant (K.O.) worms. Primers were designed to flank the deletion sites. Primers located within the deleted region failed to produce a PCR product in *aqp* deletion mutants (data not shown).

strated that *aqp-8* mRNA levels increase approximately eight-fold during hypertonic stress (Lamitina T and Strange K, unpublished observations), which suggests that the channel may be important for osmoadaptation. Crosses were performed between worms carrying single deletion alleles to generate animals lacking all excretory cell-expressed channels (i.e., *aqp-2;aqp-3;aqp-8* triple mutant) or all epithelium-expressed water-permeable channels (i.e., *aqp-2;aqp-3;aqp-4* triple mutant). Furthermore, *aqp* triple mutants were crossed together to generate animals lacking both sets of channels (i.e., *aqp-2;aqp-3;aqp-4;aqp-8* quadruple mutant; Fig. 4B).

aqp-2, *aqp-3*, *aqp-4*, and *aqp-8* homozygous mutants were analyzed for phenotypes individually and in double, triple, and quadruple homozygous mutant combinations. No significant differences in development, life span, fertility, brood size, or motility were detected (data not shown). RNA interference (RNAi)-mediated knockdown of each of the remaining aquaporin genes in *aqp-2;aqp-3;aqp-4;aqp-8* quadruple mutant animals also failed to give rise any obvious defects. For all nonneuronally expressed *aqps*, we observed a striking reduction in expression of GFP-tagged channels, indicating that RNAi was effective in silencing *aqp* gene expression (data not shown).

Single, double, and triple mutants also showed no altered osmotic stress resistance (data not shown). However, *aqp-2;aqp-3;aqp-4;aqp-8* quadruple mutants exhibited an increased sensitivity to hypotonic stress. Worms chronically acclimated to high-salt growth medium swell (19), become temporarily paralyzed, and show decreased motility when returned to low-salt medium (Lamitina T and Strange K, unpublished observations). As shown in Fig. 5A, *aqp-2;aqp-3;aqp-4;aqp-8* quadruple mutant worms moved toward food more slowly than wild-type animals when exposed to hypotonic conditions. The times for 50% of the wild-type and *aqp-2;aqp-3;aqp-4;aqp-8* quadruple mutant worms to reach food under hypotonic conditions were 113 ± 12 and 253 ± 30 min ($n = 6$; $P < 0.005$), respectively. *aqp-2;aqp-3;aqp-4;aqp-8* mutants also became paralyzed more rapidly in response to hypotonicity and remained paralyzed longer (Fig. 5B) compared with control animals. The time required for 50% of the *aqp-2;aqp-3;aqp-4;aqp-8* mutants to recover from paralysis was 24 ± 1 min ($n = 5$), which was significantly ($P < 0.0003$) different from that observed in wild-type worms (recovery time = 15 ± 1 min; $n = 5$). These results suggest that *aqp-2*, *aqp-3*, *aqp-4*, and *aqp-8* play functionally redundant roles in water and/or organic solute transport required for osmotic homeostasis. Because we used NaCl to adjust the osmolality of the growth agar, it is conceivable that these aquaporins may also play a role in ionic homeostasis.

In conclusion, our studies provide the first functional characterization of the aquaporin gene family in *C. elegans*. Remarkably, knockdown of aquaporin channel expression by RNAi (Huang CG, Lamitina T, and Strange K, unpublished observations) or deletion mutagenesis has no overt effect on worm physiology. The relatively modest phenotype observed in hypotonically stressed *aqp-2;aqp-3;aqp-4;aqp-8* quadruple mutants (Fig. 4) suggests that aquaporins expressed in osmoregulatory tissues are not essential for osmotic homeostasis. However, this interpretation should be considered with caution. *C. elegans* is a soil-dwelling nematode. The availability of water in the soil microenvironment as well as the osmotic concentration of soil fluids are likely to vary dramatically both

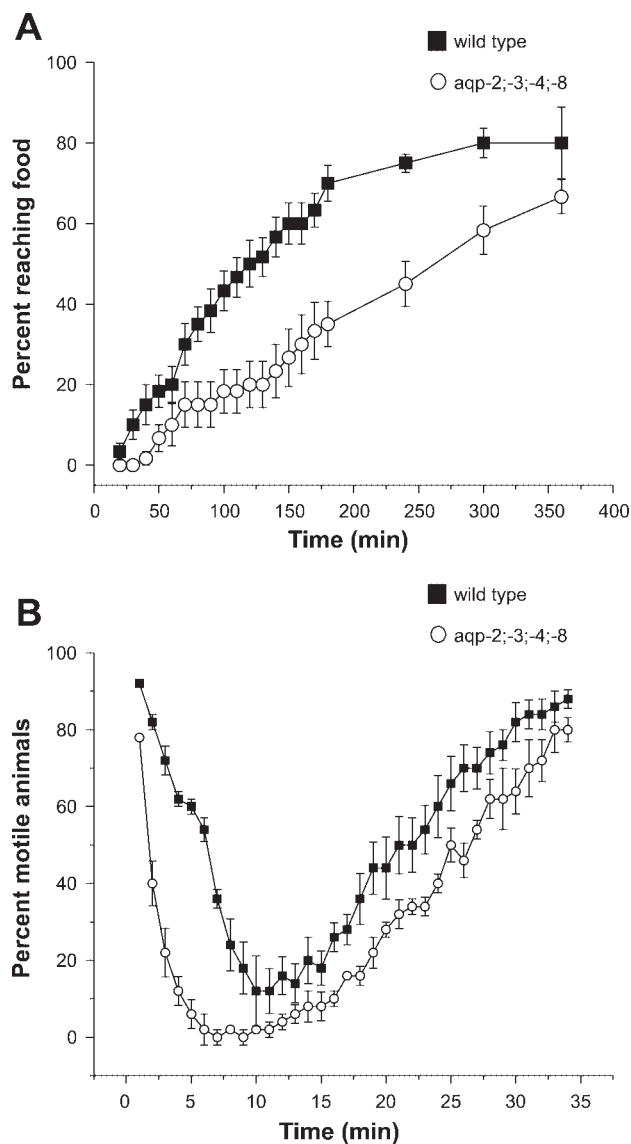


Fig. 5. Hypotonic stress resistance in *aqp-2;aqp-3;aqp-4;aqp-8* quadruple mutant worms. Worms chronically acclimated to high-salt growth medium swell, become temporarily paralyzed, and show decreased motility when returned to low-salt medium (Lamitina T and Strange K, unpublished observations). A: movement toward bacterial food source in hypotonically stressed wild type and *aqp-2;aqp-3;aqp-4;aqp-8* mutants. B: hypotonic stress induced paralysis in wild type and *aqp-2;aqp-3;aqp-4;aqp-8* mutant worms. Values are means \pm SE ($n = 5-6$).

in time and space. The osmotically unstable soil microenvironment may place worms under very different types of osmotic stress than those induced by laboratory culture. Thus it is conceivable that *C. elegans* aquaporins may provide a mechanism for the fine-tuning and constant adjustment of water and solute transport in the face of continual fluctuations in water availability and external osmolality. In the future, it will be important to study the function of aquaporins and other putative osmoregulatory proteins under conditions that more closely mimic those seen in *C. elegans*'s native environment.

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