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The Role of Aquaporins in Water Balance in *Cheilanthes lanosa* (Adiantaceae) Gametophytes

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ABSTRACT.—Aquaporins are transmembrane proteins that move water specifically and bidirectionally in response to internal cell signaling. With aquaporins, plant cells can control how, where, and when water moves across membranes. Thus, plants are in strong control of their environmental responses. Therefore, it seems likely that aquaporins would have a key role in water balance in xerophytic ferns, particularly in the gametophyte stage. To investigate the role of aquaporins in desiccation avoidance in xerophytic ferns gametophytes, *Cheilanthes lanosa* gametophytes were poisoned with micromolar mercury solutions, which block aquaporin channels, exposed to several osmolytes, and quantified the efflux of water from the cells was quantified. Results strongly suggest that aquaporins may very well play a role in water balance, but also pose some questions concerning the ability of the protonemal stage to fully manage water flow.

KEY WORDS.—aquaporin, *Cheilanthes lanosa*, fern, flux, gametophyte, mercury, NaCl, osmotic potential, sucrose, water

Water and osmoregulation has been a challenge since the beginning of cellular life. The first protocells would have had to constantly control the solutes and water content of the cytoplasm to maintain life. Therefore, it seems plausible that all bacterial, protozoan, animal, fungal, and plant cells would have and should still contain highly conserved mechanisms to move water in and out of cells (Chrispeels and Agre, 1994; Chrispeels and Maurel, 1994; Maurel, 1997).

Water-specific channels for water balance, aquaporins, exist in every kingdom and species. And, although common sense calls for the existence of such a practical mechanism, discovery of these channels was difficult. Indirect evidence demonstrated the rationale for such a simple mechanism at least twenty years before their discovery. Philip (1958) reviewed the data on water movement across lipid bilayers, and averred that the actual rate of water movement into cells is much more rapid than what should occur across a lipid bilayer. Yet, all future botanists were taught that water movement occurs through osmosis across a plant plasma membrane. Finally, in 1984, aquaporins were concurrently discovered in bovine eyes (Gorin *et al.*, 1984) and erythrocytes (Macey, 1984; Agre *et al.*, 1987). Very quickly, aquaporins were

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identified in rat kidney tubules, fungi, bacteria, and of course, plants (reviewed in Agre *et al.*, 1995; Chrispeels and Agre, 1994).

One impediment to the discovery of aquaporins was specificity. Any water-specific channel would have to exclude ions, which are smaller than water molecules. Confusion over potential structure helped delay the search for a water control mechanism. However, following the discovery of aquaporins, the elucidation of the structure revealed how an organism could effectively move water and exclude ions. The proteins are a constructed from six membrane regions that form a spiral internal channel (Fotiadis *et al.*, 2001). Water, which is angled, flows through the spiraled channels but smaller molecules, such as sodium and potassium, cannot pass through the center (Fotiadis *et al.*, 2001). Aquaporins are controlled by a phosphorylation switch (Johnson and Chrispeels, 1991; Johansson *et al.*, 1996, 1998), and thus, cells are in control of water movement. The channels are also bidirectional; water may flow out of cells or into cells (Steudle, 1992; Tyerman *et al.*, 1999). For plants, this is a perfect control mechanism. Cytoplasm solute control and aquaporin gating combine to allow a functional cytoplasmic environment within a range of conditions (Kaldenhoff *et al.*, 1993, 1995; Maurel *et al.*, 1995; Maurel, 1997).

Studies on aquaporins have resolved many complicated questions regarding plant functions. Aquaporins exist in xylem and phloem and help prevent cavitation (Voicu *et al.*, 2009). Aquaporins exist in root cortical and endodermal cells and help generate or resolve pressure of water flow (Vandeleur *et al.*, 2005; Steudle and Peterson, 1998). Aquaporins in guard cell tonoplasts respond to changes in blue light and abscisic acid and allow water flow into and out of the vacuole (Kaldenhoff *et al.*, 1993). Aquaporins are also involved in water movement and management during water stress (Suga *et al.*, 2002).

Based on the above findings, we hypothesized that aquaporins may play a key role in the survival of fern gametophytes in xeric environments as mechanisms for water management. To test this hypothesis, we chose a common North American xerophytic fern, *Cheilanthes lanosa* (Michx.) D.C. Eat. for this study because it inhabits a variety of substrates (Mickel, 1979), though it is commonly found among other xerophytic species (Mohlenbrock, 1959), and possesses many of the features that other xerophytic ferns possess, such as microphylly (Pickett, 1931; Hevly, 1963; Quirk and Chambers, 1981), trichomes (Quirk and Chambers, 1981), mycorrhizal associations (Palmieri and Swatzell, 2004), cuticle on the gametophyte (Lingle *et al.*, 2004), and the ability to regenerate after desiccation (Diamond *et al.*, 2003). In addition, like many other xerophytic gametophytes, *C. lanosa* gametophytes are apogamous (Steil, 1933, 1939; Hevly, 1963). The gametophytes are also not heavily covered with wax, are one-cell in thickness, and appear generally unprotected from their surroundings. Thus, the *C. lanosa* gametophytes could represent the physiological state of numerous xerophytic ferns. We predicted that if it was possible to identify aquaporins in this fern gametophyte, and if we could poison these proteins with low levels of mercury, which blocks the protein channel (Preston *et al.*, 1993; Kuwahara *et al.*, 1997), we could effectively

control water movement out of desiccating cells. This would suggest at least a potential role for aquaporins in xerophytic gametophyte water management.

Materials and Methods

To test our hypothesis, we examined different developmental stages and in different microclimates for the *Cheilanthes lanosa* gametophyte. Each development stage and microclimate condition will heretofore be designated as simply a "stage."

Plant Collection

Cheilanthes lanosa sporophylls were collected in the fall of 2003 after the first frost from Makanda, Illinois, placed in glass 9 cm Petri dishes, and stored in the dark at 4°C. After several months, sporophylls were crushed using a mortar and pestle. *Cheilanthes lanosa* spores average 40 μm in diameter (Devi *et al.*, 1971) and thus spores were separated from the plant material using a 65 μm brass mesh sieve. Spores were stored at 4°C in the dark.

Culture Conditions

Wet grown (WG) gametophytes and protonemal callus (callus).—Spores were surface sterilized in a 7% (v/v) commercial bleach solution with 0.1% (v/v) Triton X-100 for 10 min. Spores were then rinsed in sterile ddH_2O and sown on a modified tissue culture medium (TCM; 20 mM NH_4NO_3, 20 mM KNO_3, 1.5 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM $MnSO_4 \cdot H_2O$, 30.0 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM $CuSO_4 \cdot 5H_2O$, 3.0 mM $CaCl_2 \cdot 2H_2O$, 5.0 μM KI, 0.1 μM $CoCl_2 \cdot 6H_2O$, 0.8 mM KH_2PO_4, 0.9 mM H_3BO_3, 1.0 μM $Na_2MoO_4 \cdot {}_2H_2O$, 0.1 mM $FeSO_4 \cdot 7H_2O$, 0.1 mM Na_2EDTA, 0.23 μM kinetin, 0.86 μM 2,4-D, 0.4 μM nicotinic acid, 0.3 μM pyridoxine, 1.3 μM thiamine, 0.56 mM myo-inositol, pH 5.7; Smith, 1992) in 9 cm Petri dishes. Spores were incubated at 25°C in 0.175 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of continuous far red light (650–705 nm) for 10 d.

Following germination and protonemal development, the plates were separated. Some plates were left in the far red light to enhance callus growth and the remaining plates were then exposed to continuous white light and the protonema began planar growth into gametophytes.

Dry grown (DG) gametophytes.—Dry grown gametophytes were sown on fine grain white sand (Décor Sand, Activa Products Inc., Marshall, Texas, USA) wetted with 20 mL of TCM and incubated as the WG and callus cultures described above. Thereafter, upon drying, DG gametophytes wetted erratically with ddH_2O.

Antibody Production

To establish the molecular weight of the target protein, ensure specificity of binding of the anti-aquaporin antibody, and to detect the presence of

aquaporin-like proteins in gametophytes, an antibody against maize PIP1 aquaporin was raised using a homologous sequence from previously mapped aquaporins in maize (Chaumont et al., 2000). Rabbit anti-aquaporin antibody was produced by Biosource (Invitrogen; Biosource, Camarillo, California, USA) against a conserved amino terminus sequence of PIP1 maize aquaporin, MEGKEEDVRVGANKFPERQPIGTSAQS as described by Chaumont *et al.* (2000).

ELISA (enzyme-linked immunosorbent assay)

In order to test for the presence of aquaporin-like proteins, an enzyme-linked immunosorbent assay was performed to detect a wide range of aquaporin concentrations in various gametophyte stages. Approximately 20 mg of WG gametophyte material was homogenized in 200 µL of coating buffer (15 mM Na_2HCO_3, 35 mM $NaHCO_3$, pH 9.5) in a mortar and pestle. The sample was then centrifuged at 10,000 rpm for 10 min. The supernatant was retained and centrifuged again at 10,000 rpm for 10 min. The sample was then diluted in 1 ml of coating buffer. The concentration was approximately 3 µg of material for every 50 µL of coating buffer. The antigen-coating buffer solution was then pipetted in 50 µL increments into a 96-well polystyrene microtiter plate. The plate was covered and allowed to incubate overnight at 4°C. The following morning, the antigen solution was removed by inverting the plate and washing three times with Tris-Tween washing buffer (TTW; 10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.2). In all the washing steps, the wells were completely filled (~350 µL). Plates were blocked with 3% (w/v) low-fat milk powder in TTW for 30 min. The wells were then washed three times with TTW. Once the TTW was removed, 100 µL of rabbit anti-aquaporin (1:500; Biosource, Camarillo, California, USA) in TTW, rabbit preimmune serum (1:500; Biosource, Camarillo, California, USA) in TTW, or TTW only (secondary antibody only control) was placed into the wells and allowed to incubate for 60 min. The wells were washed three times with TTW. The TTW was removed and 50 µL of goat anti-rabbit horseradish peroxidase (1:500; Sigma-Aldrich, St. Louis, Missouri, USA) in TTW, or TTW only (primary antibody only control) was added and allowed to incubate for 60 min. The wells were again washed three times with TTW. The chromogenic enzyme reaction was then initiated by addition of freshly prepared solution of 1.5 mg *o*-phenylenediamine and 2 µL of 30% (v/v) H_2O_2 dissolved in 2 mL of citrate-phosphate buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, pH 5.0) at 50 µL per well. H_2O_2 was added immediately prior to use. The enzyme reaction was allowed to incubate for 15 min and the reaction was halted by the addition of 50 µL of 0.5 M H_2SO_4 per well. The plate was then read at 492 nm on a Beckman DU640B spectrophotometer (Beckman Coulter, Fullerton, California, USA).

Immunoblotting

To establish the molecular weight of the target protein and to ensure specificity of binding of the anti-aquaporin antibody, immunoblotting was

performed. Approximately 100 µL of *Cheilanthes lanosa* callus, WG, or DG plant material was ground in homogenization buffer [10 mM KCl, 1 mM $MgCl_2$, 50 mM HEPES, 300 mM sorbitol, 0.1% (w/v) BSA and 1 mM EDTA, pH to 7.2]. Samples were mixed 1:1 2X Laemmli sample buffer [Sambrook et al., 1989; 4% (w/v) SDS, 10% (v/v) mercaptoethanol, 0.002% (w/v) Bromphenol Blue, 20% (v/v) glycerol, 120 mM Tris, pH 6.8], incubated for 10 min at 90° C, centrifuged at 10,000 rpm for 10 min. Total protein concentration was determined using the Bradford Assay (Stoschek, 1990). Samples were loaded onto a 12% (w/v) polyacrylamide gel at 35 µl per well and 20 µg/mL concentration. Samples were electrophoresed at 16 mA constant current for approximately 30 min. A broad range marker (Kaleidoscope; BioRad, Hercules, California, USA) was used for reference. Proteins were then transferred at 45 mA constant current for 20 min onto a nitrocellulose membrane (0.2 µm pore size). Following transfer, the membranes were rinsed three times in TTBS [137 mM NaCl, 2.7 mM KCl, 24.8 mM Tris, 0.2% Tween (v/v)], and blocked in 3% (w/v) bovine serum albumin (BSA) in TTBS. Membranes were then rinsed three times for 5 min each in TTBS. Membranes were then incubated overnight at 4°C in either preimmune serum (BioSource, Camarillo, California, USA; 1:500 in TTBS), rabbit anti-PIP1 aquaporin (BioSource, Camarillo, California, USA; 1:500 in TTBS), or TTBS only (secondary antibody only control). Membranes were again rinsed 3 times for 5 min in TTBS. Following the rinse, membranes were incubated in goat anti-rabbit alkaline phosphatase (1:500 in TTBS; Sigma-Aldrich, St. Louis, Missouri, USA), with the exception of the primary antibody only control, which was incubated in TTBS only, each for 1 h at 25°C. Membranes were rinsed three times for 5 min each in TTBS. The membrane was then colorized with a fresh mixture of 20 mL of alkaline phosphate buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5), 132 µL of nitro blue tetrazolium (NBT) stock, and 66 µL of 5-Bromo-4-chloro-3′-indolyphosphate *P*-Toluidine (BCIP) stock (Sambrook *et al.*, 1989). After agitation for 5 min, the membrane was briefly rinsed with ddH_2O and the reaction was stopped with 20 mM ethylene diamine tetraacetic acid (EDTA).

Osmolality Stress

In order to test for aquaporin function and potential control of function in gametophyte stages, gametophytes were exposed to desiccating levels of osmolytes with and without mercury exposure. Although the preferred test for function of aquaporins is expression of mRNA in *Xenopus* oocytes (which do not express aquaporins), PIP1 mRNA does not express well in oocytes (Preston *et al.*, 1992). Therefore, another standard, the immersion of samples in mercuric chloride at low levels (1 mM; Macey, 1984) was used. Wet grown (WG) gametophytes, callus and dry grown (DG) gametophytes were exposed to 2 separate treatments. One treatment consisted of time increments of NaCl, $CaCl_2$, or sucrose and was used to show gametophyte response to long term increasing environmental stress. Gametophytes were pretreated in 200 µL well slides in 100 µL of ddH_2O or ddH_2O plus 1 mM $HgCl_2$ for 20 min. Gametophyte images were captured digitally. At t_0, and subsequently every 5 min afterward,

50 mM increments of the respective solute were added until the solution in the well slide reached 500 mM osmolality. Images of gametophytes were captured after 1 h. The second treatment was used to show gametophyte response to immediate stress. This treatment began with a 20 min pretreatment in a 200 µL well slide in 100 mM isotonic solute solution (NaCl, $CaCl_2$, or sucrose) or 100 mM isotonic solution plus 1 mM $HgCl_2$. Gametophyte images were captured digitally. At t_0, 400 mM of solute in 100 µL of ddH_2O was added to the well so that the solution in the well reached 500 mM osmolality. Images of the gametophytes were captured after 1 h.

Cell volume was determined using six diameter measurements. Average radii were used to extrapolate the sphere volume and surface area. Only hourly rates of efflux were calculated. This is because diffusion out of these cells was slow enough to allow the necessary resolution to demonstrate loss of function of water channels under mercury poisoning. Well slides were incubated in a moisture chamber in the interim times between increments to prevent water loss from the wells. The efflux of water out of each of 100 cells from each treatment was measured using Fick's Law:

$$\text{Flux} = -\text{PS}[(\text{Osm}_o - \text{Osm}_i)/\text{D}]$$

Where -P = permeability (negative because against the concentration gradient), S = surface area, Osm_o = osmolality outside of cell, Osm_i = osmolality inside of cell, and D = distance (modified from Qui et al., 2000).

Data Capture and Image Analysis

For cell volume and trichome position, gametophyte images were captured digitally on an Olympus SZ40 (Olympus America, Center Valley, Pennsylvania, USA) with SPOT Advanced 3.2 software (Diagnostic Instruments, Sterling Heights, Michigan, USA). Measurements were made using SPOT Advanced 3.2 software. Measurements of 100 cells were conducted for each treatment. To assess the influence of treatment, medium, fern stage and timing, a three-way Analysis of Variance (ANOVA; P = 0.05; n = 3600) with interaction was undertaken using the SAS General Linear Model Procedure (SAS 1999–2000; SAS, Cary, North Carolina, USA). A Tukey's Studentized test was then performed to determine significant differences between media, with a primary focus on the comparison of $HgCl_2$ and ddH_2O pretreatments. To graph individual treatments, box plots were developed using JMP Statistical Discovery Software (SAS Programming, Serial No. GV0KZ9JJ07, © 2007; SAS, Cary, North Carolina, USA) and modified using PaintShopPro 6.02 (Jasc Software, Ottawa, Ontario, Canada).

Results

ELISA and Immunoblotting

To establish the potential presence of an aquaporin-like protein in germinating spores and gametophytes, ELISA was performed on callus and

immunoblotting was performed on DG and WG gametophytes. In ELISA, primary antibody only, secondary antibody only, and the preimmune serum controls were negative (Fig. 1A). The positive control, application of anti-aquaporin antibody applied to radish root homogenate, resulted in a strong cross-reaction of root total protein with the anti-aquaporin antibody. The immunoblotting procedure produced a single band when the anti-aquaporin antibody was applied to the mature gametophyte sample (Fig. 1B). Primary antibody only, secondary antibody only, and preimmune serum controls were negative. The positive control, application of anti-aquaporin antibody applied to radish root homogenate, resulted in a strong cross-reaction of root total protein with the anti-aquaporin antibody.

Increment Solute Treatments

NaCl and $CaCl_2$.—To measure the response of different stages of gametophytes to hyperpolarizing solutes, which destabilize plasma membranes and often alter membrane protein function, samples were exposed to incremental increases of NaCl and $CaCl_2$. When preincubated in ddH_2O and then exposed to NaCl increments, callus quickly plasmolyzed to the extent that protoplasm fluid was almost entirely extracted and chloroplasts clumped tightly with nuclei and other cell contents (Fig 2A). Callus cells visibly plasmolyzed when exposed to slow increments in NaCl concentrations. Mean of cell volume loss in treatments in which NaCl was added in slow increments was $6.65 \cdot 10^{-4}$ cm^{-3} $\pm$ $10.85 \cdot 10^{-4}$. Following an incubation in $HgCl_2$, callus flux levels significantly dropped (Fig. 2B). Cells appear fully intact up to 500 mM NaCl. In addition, variation in the response was greatly reduced. Mean of cell volume flux in NaCl treatments with a $HgCl_2$ incubation prior to the incremented NaCl treatment was $0.21 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.47 \cdot 10^{-4}$. Plasmolysis also occurred when WG gametophytes were exposed to slow increases in NaCl (Fig. 2C). Mean of cell volume loss in treatments in which NaCl was added in slow increments was $4.86 \cdot 10^{-4}$ cm^{-3} $\pm$ $6.93 \cdot 10^{-4}$. WG flux levels significantly dropped after the introduction of $HgCl_2$ (Fig. 2D). Cells appear uncompromised. Standard deviations and variation was similar for both WG treatments. Mean of cell volume flux in NaCl treatments with $HgCl_2$ incubation prior to the incremented NaCl treatment was $0.55 \cdot 10^{-4}$ cm^{-3} $\pm$ $3.23 \cdot 10^{-4}$. DG gametophytes appeared to maintain cell viability in NaCl increments up to 500 NaCl (Figs. available from author). Some cell volume loss was visible in older cells toward the center of the gametophyte. Mean of cell volume loss following a slow increase in NaCl was $3.96 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.40 \cdot 10^{-4}$. Mean of cell volume loss in NaCl increment treatment following a pre-incubation in $HgCl_2$ was $0.80 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.15 \cdot 10^{-4}$. DG flux levels significantly dropped after the introduction of $HgCl_2$.

These results were mirrored in the $CaCl_2$ increment treatments (Fig. 3). Callus cells visibly plasmolyze when exposed to slow increments in $CaCl_2$ concentrations (Fig 3A). Mean of cell volume loss in treatments in which $CaCl_2$ was added in slow increments was $12.18 \cdot 10^{-4}$ cm^{-3} $\pm$ $6.59 \cdot 10^{-4}$. Flux in callus cell volume levels significantly dropped following pre-incubation in $HgCl_2$ (Fig 3B). Mean of cell

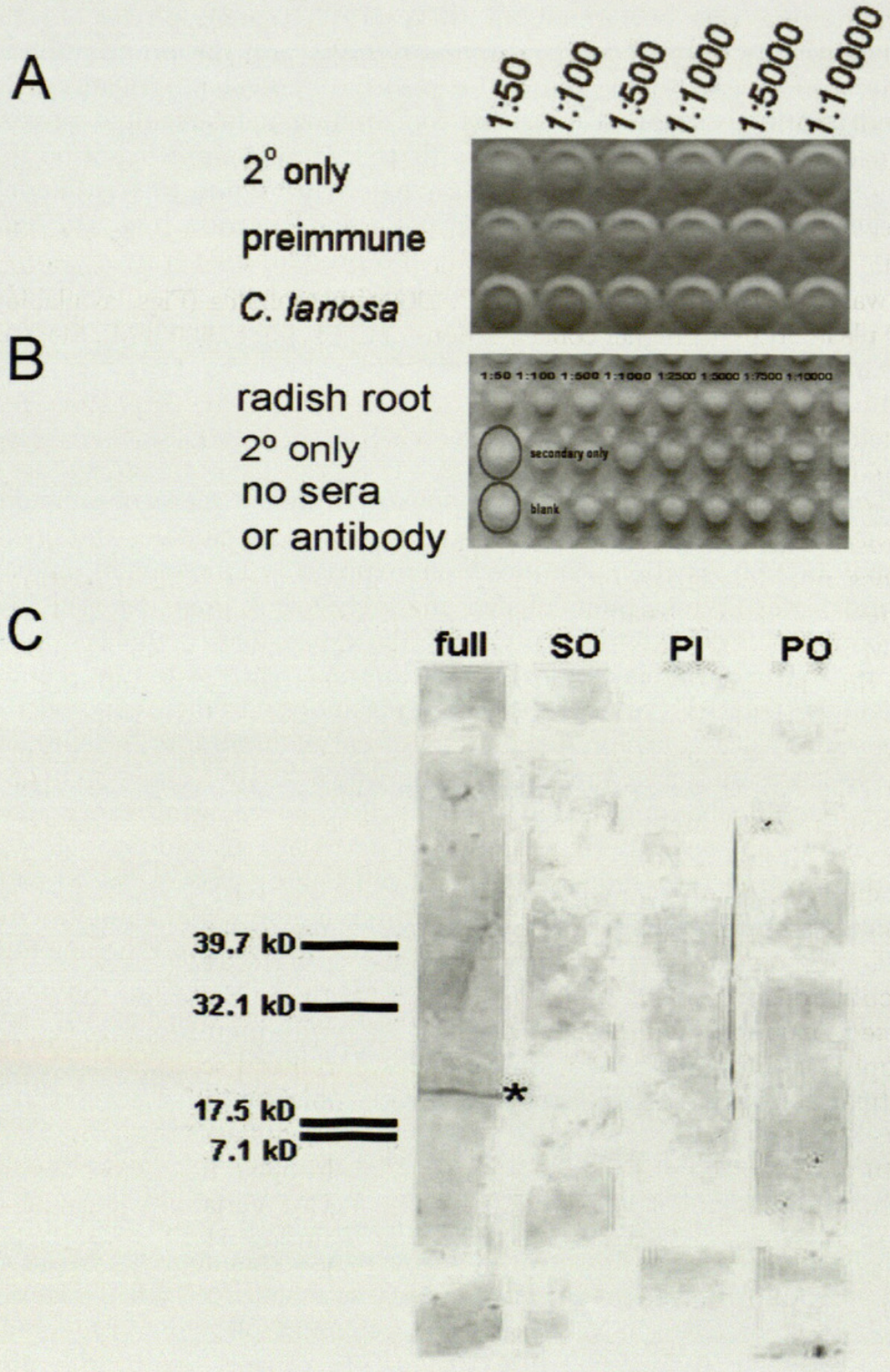

FIG. 1. Immunolabeling of Aquaporin-like Protein. (A) ELISA signal from cross-reaction of anti-aquaporin antibody with callus total protein is yellow. Stronger cross-reaction due to greater concentrations of antibody (antibody dilutions of 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10000) results in more intense coloration and absorbance. Primary antibody only (not shown), secondary antibody only, and preimmune serum controls were negative. The positive control, anti-aquaporin antibody cross-reaction with radish root, is not shown in this image, but is located on the same

volume loss following $CaCl_2$ treatment with a pre-incubation in $HgCl_2$ was $2.20 \cdot 10^{-4}$ cm^{-3} $\pm$ $2.80 \cdot 10^{-4}$. The treatment with pre-incubation in $HgCl_2$ also exhibited a much smaller amount of variation than the treatment without $HgCl_2$. Heavy plasmolysis occurred when WG gametophytes were exposed to slow increments of $CaCl_2$ up to 500 mM (Fig. 3C). Mean of cell volume loss in treatments in which $CaCl_2$ was added in slow increments was $6.95 \cdot 10^{-4}$ cm^{-3} $\pm$ $6.59 \cdot 10^{-4}$. WG flux levels significantly dropped after the introduction of $HgCl_2$ (Fig. 3D). Mean of cell volume loss in $CaCl_2$ increment treatments were preceded by pre-incubation in $HgCl_2$ was $1.95 \cdot 10^{-4}$ cm^{-3} $\pm$ $1.23 \cdot 10^{-4}$. DG gametophytes (Figs. available from author) plasmolyzed at higher concentrations of $CaCl_2$ (up to 500 mM). Mean of cell volume loss after increasing increments of $CaCl_2$ was $5.75 \cdot 10^{-4}$ cm^{-3} $\pm$ $2.38 \cdot 10^{-4}$. DG cell volume flux levels were significantly lower when gametophytes were pre-incubated in $HgCl_2$. There was also a substantial increase in variation in the response with pre-incubation in $HgCl_2$. Mean of cell volume loss in gametophytes pretreated with $HgCl_2$ was $2.65 \cdot 10^{-4}$ cm^{-3} $\pm$ $1.05 \cdot 10^{-4}$.

Sucrose.—Sucrose was used to test gametophyte response to a pure osmolyte that does not depolarize or hyperpolarize membranes (Fig. 4). Callus cells were desiccated in a treatment of slow sucrose increments up to a total of 500 mM sucrose. Mean of cell volume loss was $9.45 \cdot 10^{-4}$ cm^{3} $\pm$ $8.86 \cdot 10^{-4}$. Callus flux levels significantly dropped in treatments that included a pre-incubation in $HgCl_2$ (Fig. 4B). Mean of callus cell volume loss was $0.09 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.51 \cdot 10^{-4}$. In addition, variation was greatly reduced when protonemal callus was pre-incubated in $HgCl_2$. WG gametophytes in a treatment in which sucrose is increased in increments up to 500 mM exhibited some cell volume loss in older cells (Fig. 4C). Mean of cell loss in sucrose increment treatment was $9.62 \cdot 10^{-4}$ cm^{-3} $\pm$ $1.64 \cdot 10^{-4}$. WG flux levels significantly dropped in sucrose increment treatments that were pre-incubated in $HgCl_2$ (Fig. 4D). Mean of cell volume loss in these treatments with $HgCl_2$ pre-incubation was $0.48 \cdot 10^{-4}$ cm^{-3} $\pm$ $3.20 \cdot 10^{-4}$. Variation in response decreased markedly when gametophytes were pre-incubated in $HgCl_2$. DG gametophytes (Figs. available from author) experienced some cell volume loss in treatments involving increments in sucrose molarity (up to 500 mM). Mean of DG cell volume loss in sucrose increment treatments was $10.80 \cdot 10^{-4}$ cm^{-3} $\pm$ $54.15 \cdot 10^{-4}$. DG cell volume loss significantly dropped in sucrose increment treatments that included a pre-incubation in $HgCl_2$. Variation in gametophyte response was also less in treatments that included $HgCl_2$ pre-incubation. Mean of cell volume loss in treatments with $HgCl_2$ pre-incubation was $0.95 \cdot 10^{-4}$ cm^{-3} $\pm$ $2.45 \cdot 10^{-4}$.

←

plate. (B) ELISA Positive Radish Root Control. Each immunoblot and ELISA contained a radish root control. PIP1 aquaporins express in germinating seeds and new plant roots and freshly germinated radish root reliably produced a strong signal. (C) Immunoblotting procedures resulted in a single band of total protein that cross-reacted with anti-aquaporin antibody. *= approximately 22.4 kD. Secondary only (SO), preimmune serum (PI), and primary only (PO) controls did not result in any cross reaction with the primary or secondary antibodies alone, or with the pre-inoculation serum.

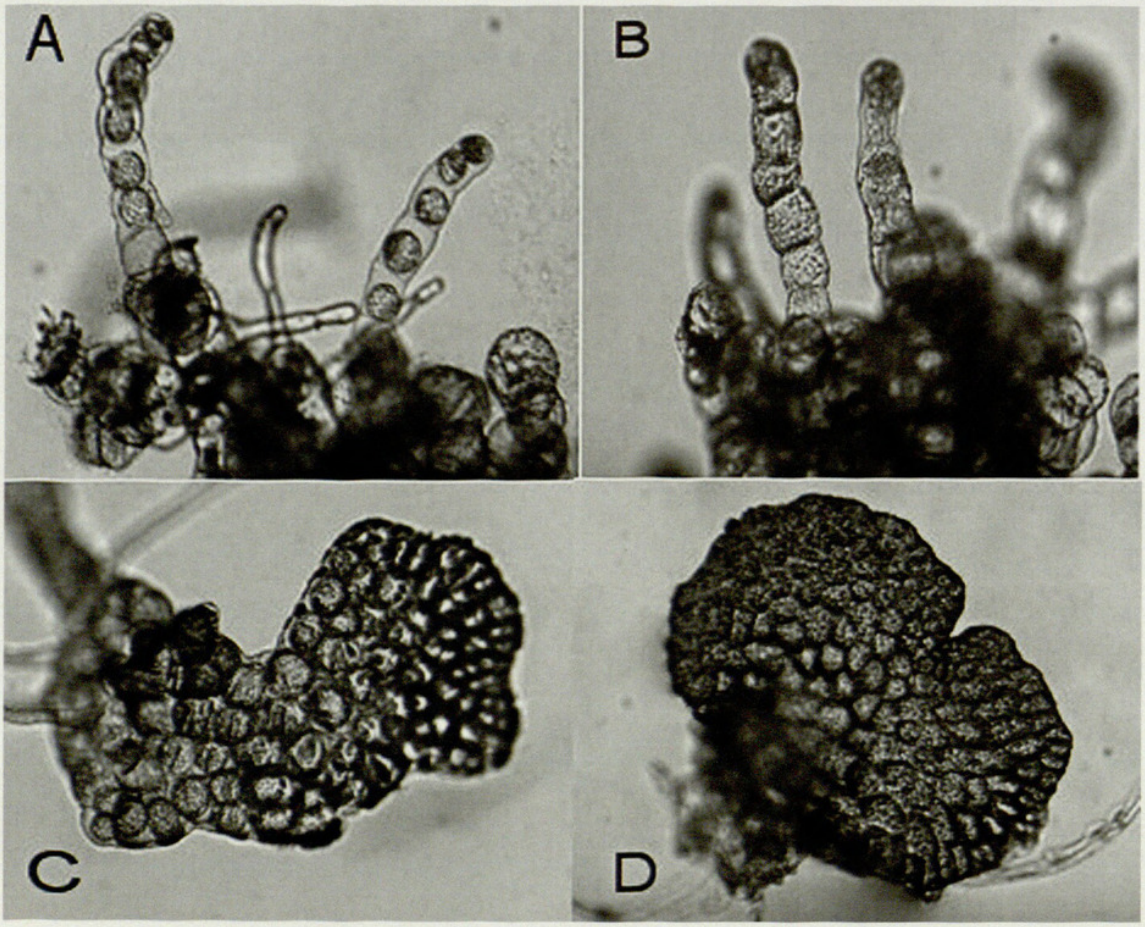

FIG. 2. Flux in Cell Volume of Gametophytes exposed to NaCl Increments. (A) Callus cells visibly plasmolyze when exposed to slow increments in NaCl concentrations. Mean of cell volume loss in treatments in which NaCl was added in slow increments = $6.65 \cdot 10^{-4}$ cm^{-3} ± $10.85 \cdot 10^{-4}$. (B) Following an incubation in $HgCl_2$, callus flux levels significantly dropped. Cells appear fully intact up to 500 mM NaCl. In addition, variation in the response was greatly reduced. Mean of cell volume flux in NaCl treatments with a $HgCl_2$ incubation prior to the incremented NaCl treatment was $0.21 \cdot 10^{-4}$ cm^{-3} ± $0.47 \cdot 10^{-4}$. (C) Plasmolysis occurred when WG gametophytes were exposed to slow increases in NaCl. Mean of cell volume loss in treatments in which NaCl was added in slow increments = $4.86 \cdot 10^{-4}$ cm^{-3} ± $6.93 \cdot 10^{-4}$. (D) WG flux levels significantly dropped after the introduction of HgCl2. Cells appear uncompromised. Standard deviations and variation was similar for both WG treatments. Mean of cell volume flux in NaCl treatments with $HgCl_2$ incubation prior to the incremented NaCl treatment = $0.55 \cdot 10^{-4}$ cm^{-3} ± $3.23 \cdot 10^{-4}$.

Immediate Immersion Treatments

$NaCl_2$ and $CaCl_2$.—To test for differences in flux that could be based on diffusion or aquaporin deactivation, stages were introduced to the same solute treatments but the timing of introduction was changed to immediate immersion in 500 mM solute. Following $HgCl_2$ pretreatment, all stages exhibited a significant reduction in flux. Callus cells lost cell volume very quickly when immersed in 500 mM NaCl (Fig. 5A). Mean of cell volume loss was $6.30 \cdot 10^{-4}$ cm^{-3} ± $3.82 \cdot 10^{-4}$. Callus pre-incubated in $HgCl_2$ before immersion remains intact (Fig. 5B). Cell volume was significantly different than those cells treated only with NaCl. Mean cell volume loss in treatment

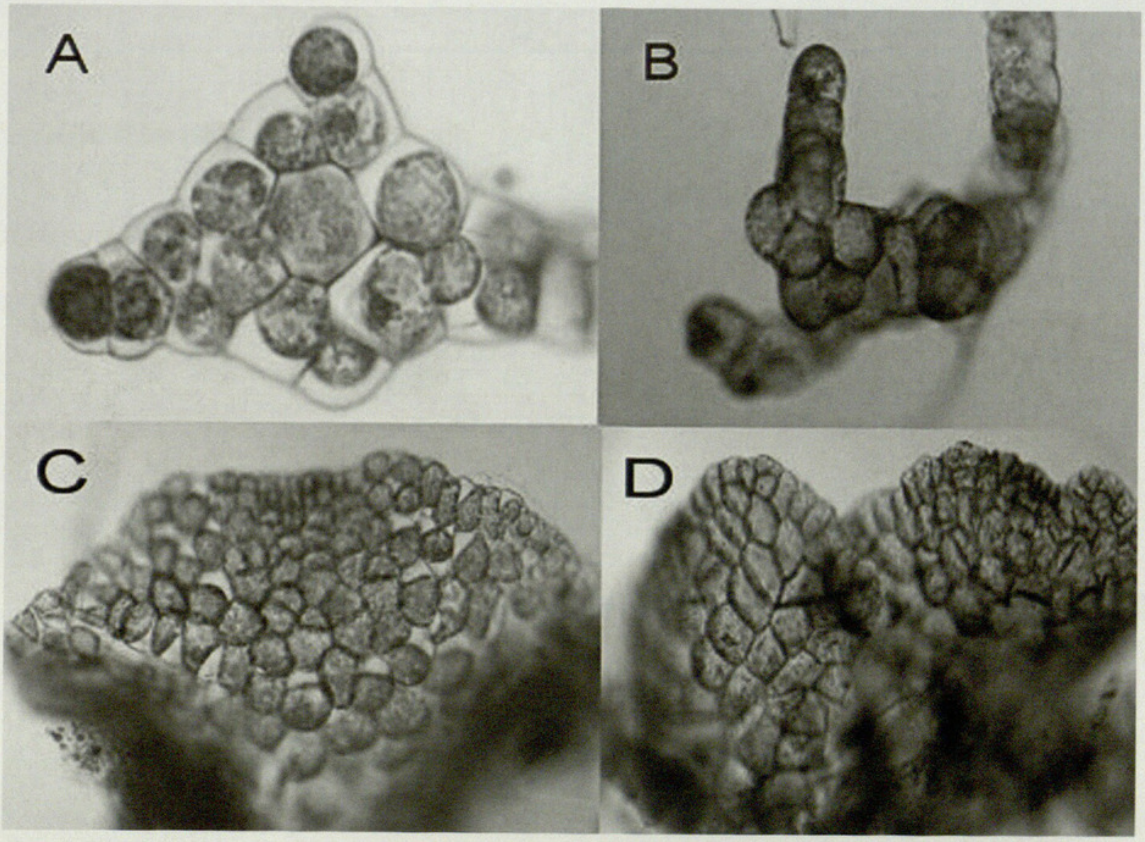

FIG. 3. Flux in Cell Volume of Gametophytes exposed to $CaCl_2$ Increments. (A) Callus cells visibly plasmolyze when exposed to slow increments in $CaCl_2$ concentrations. Mean of cell volume loss in treatments in which $CaCl_2$ was added in slow increments = $12.18 \cdot 10^{-4}$ $cm^{-3} \pm 6.59 \cdot 10^{-4}$. (B) Flux in callus cell volume levels significantly dropped following pre-incubation in $HgCl_2$. Mean of cell volume loss following $CaCl_2$ treatment with a pre-incubation in $HgCl_2$ = $2.20 \cdot 10^{-4}$ $cm^{-3} \pm 2.80 \cdot 10^{-4}$. The treatment with pre-incubation in $HgCl_2$ also exhibited a much smaller amount of variation than the treatment without $HgCl_2$. (C) Heavy plasmolysis occurred when WG gametophytes were exposed to slow increments of $CaCl_2$ up to 500 mM. Mean of cell volume loss in treatments in which $CaCl_2$ was added in slow increments = $6.95 \cdot 10^{-4}$ $cm^{-3} \pm 6.59 \cdot 10^{-4}$. (D) WG flux levels significantly dropped after the introduction of $HgCl_2$. Mean of cell volume loss in $CaCl_2$ increment treatments were preceded by pre-incubation in $HgCl_2$ = $1.95 \cdot 10^{-4}$ $cm^{-3} \pm 1.23 \cdot 10^{-4}$.

with pre-incubation in $HgCl_2$ was $0.26 \cdot 10^{-4}$ $cm^{-3} \pm 14.29 \cdot 10^{-4}$. Variation in responses was also reduced when callus was exposed to $HgCl_2$ pre-incubation. WG gametophytes also plasmolyzed immediately in 500 mM NaCl (Fig. 5C). Mean of cell volume flux in NaCl immersion was $5.08 \cdot 10^{-4}$ $cm^{-3} \pm 0.72 \cdot 10^{-4}$. Conversely, change in WG cell volume was minimal following treatment that included pre-incubation in $HgCl_2$ (Fig. 5D). Mean of cell volume flux in treatments that included $HgCl_2$ pretreatment was $1.85 \cdot 10^{-4}$ $cm^{-3} \pm 0.37 \cdot 10^{-4}$. Variation in flux amounts also significantly decreased in the $HgCl_2$ pre-incubation treatment. DG gametophytes (Figs. available from author) were visibly affected by immediate immersion in 500 mM NaCl. Mean of cell volume loss in NaCl immediate immersion was $3.10 \cdot 10^{-4}$ $cm^{-3} \pm 1.00 \cdot 10^{-4}$. However, when DG gametophytes were pre-incubated in $HgCl_2$ prior to the NaCl immersion, there was a decrease in cell volume loss. Mean of cell volume

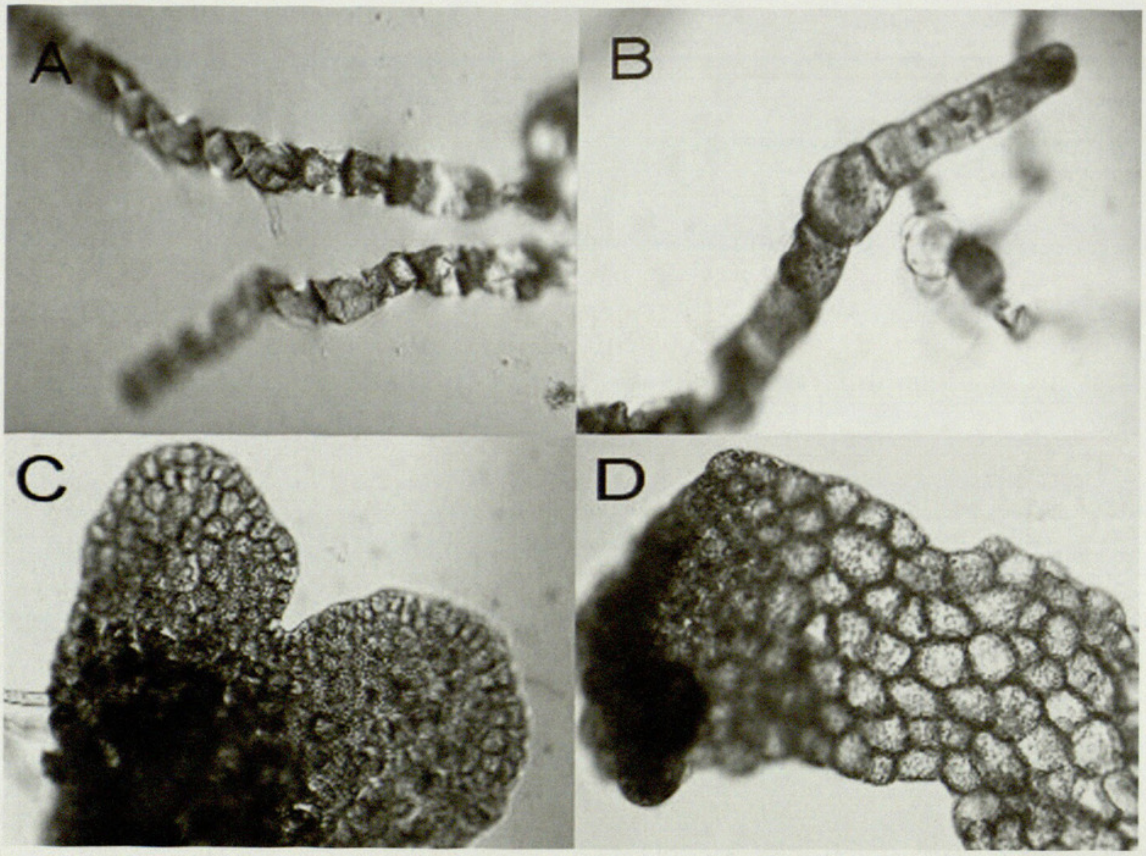

FIG. 4. Gametophytes and Sucrose Increments. (A) Callus cells were desiccated in a treatment of slow sucrose increments up to a total of 500 mM sucrose. Mean of cell volume loss = $9.45 \cdot 10^{-4}$ cm^{-3} ± $8.86 \cdot 10^{-4}$. (B) Callus flux levels significantly dropped in treatments that included a pre-incubation in $HgCl_2$. Mean of callus cell volume loss = $0.09 \cdot 10^{-4}$ cm^{-3} ± $0.51 \cdot 10^{-4}$. In addition, variation was greatly reduced when protonemal callus was pre-incubated in $HgCl_2$. (C) WG gametophytes in a treatment in which sucrose is increased in increments up to 500 mM exhibited some cell volume loss in older cells. Mean of cell loss in sucrose increment treatment = $9.62 \cdot 10^{-4}$ cm^{-3} ± $1.64 \cdot 10^{-4}$. (D) WG flux levels significantly dropped in sucrose increment treatments that were pre-incubated in $HgCl_2$. Mean of cell volume loss in these treatments with $HgCl_2$ pre-incubation = $0.48 \cdot 10^{-4}$ cm^{-3} ± $3.20 \cdot 10^{-4}$. Variation in response decreased markedly when gametophytes were pre-incubated in $HgCl_2$.

loss in $HgCl_2$ pretreated gametophytes was $0.80 \cdot 10_{-4}$ cm^{-3} ± $1.55 \cdot 10^{-4}$. There was also a substantial increase in variation in the response with pre-incubation in $HgCl_2$.

$CaCl_2$ immediate immersion (Fig. 6) results were only slightly different than NaCl results. Callus plasmolyzed heavily upon immediate immersion in 500 mM $CaCl_2$ (Fig. 6A). Mean of callus cell volume loss in $CaCl_2$ was $6.73 \cdot 10^{-4}$ cm^3 ± $2.98 \cdot 10^{-4}$. However, callus gametophytes that were pretreated in $HgCl_2$ before immersion in $CaCl_2$ exhibited little volume flux (Fig. 6B). Mean of callus cell volume loss in treatments with $HgCl_2$ preincubation was $0.55 \cdot 10^{-4}$ cm^3 ± $0.50 \cdot 10^{-4}$. Variation in the responses also decreased in those cells preincubated in $HgCl_2$. WG gametophyte cells also plasmolyzed heavily in $CaCl_2$ immediate immersion treatment (Fig. 6C). Mean

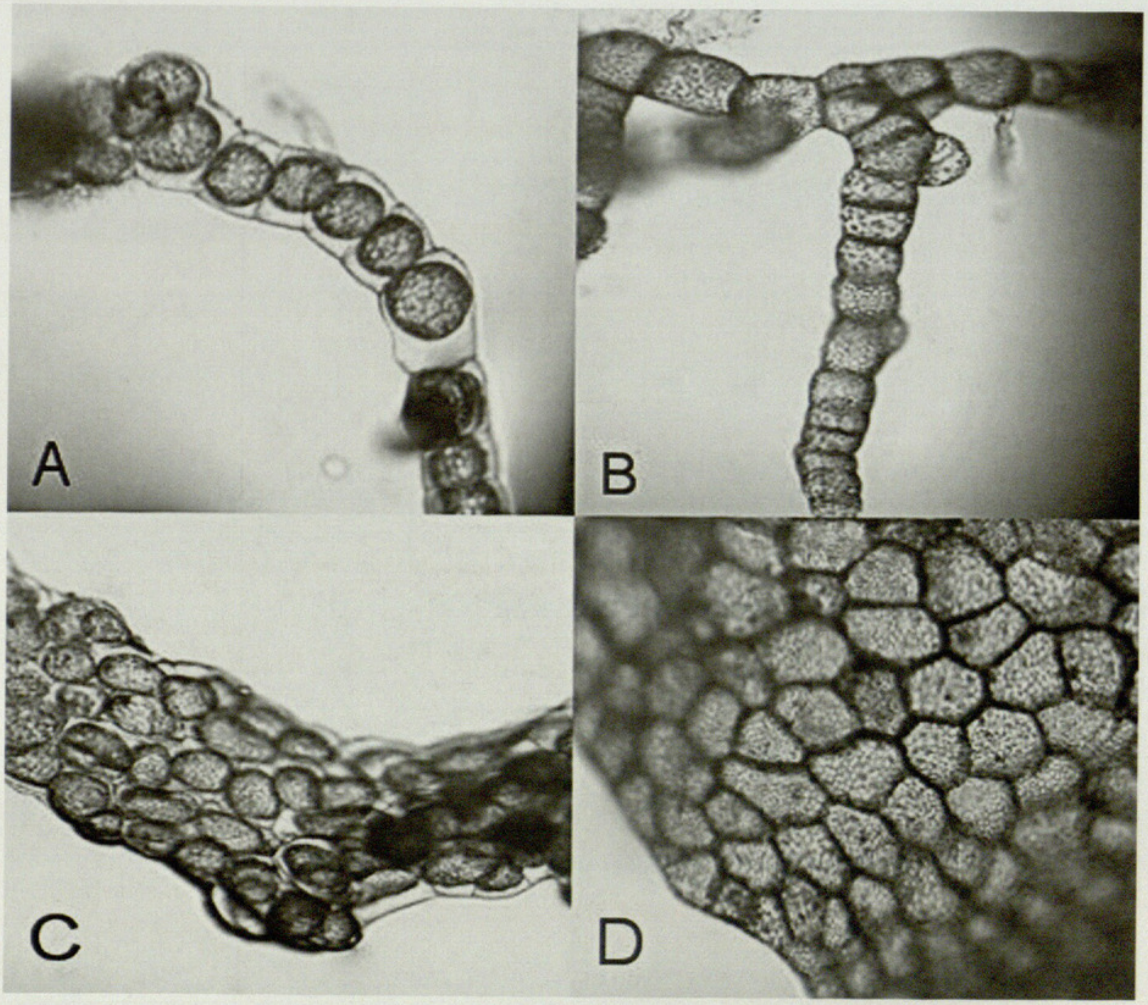

FIG. 5. Gametophytes and NaCl Immediate Immersion. (A) Callus cells lost cell volume very quickly when immersed in 500 mM NaCl. Mean of cell volume loss = $6.30 \cdot 10^{-4}$ $cm^{-3} \pm 3.82 \cdot 10^{-4}$. (B) Callus pre-incubated in $HgCl_2$ before immersion remains intact. Cell volume was significantly different than those cells treated only with NaCl. Mean cell volume loss in treatment with pre-incubation in $HgCl_2 = 0.26 \cdot 10^{-4}$ $cm^{-3} \pm 14.29 \cdot 10^{-4}$. Callus cell volume loss significantly dropped after the introduction of $HgCl_2$. Variation in responses was also reduced when callus was exposed to $HgCl_2$ pre-incubation. (C) WG gametophytes also plasmolyzed immediately in 500 mM NaCl. Mean of cell volume flux in NaCl immersion = $5.08 \cdot 10^{-4}$ $cm^{-3} \pm 0.72 \cdot 10^{-4}$. (D) Conversely, change in WG cell volume was minimal following treatment that included pre-incubation in $HgCl_2$. Mean of cell volume flux in treatments that included $HgCl_2$ pretreatment = $1.85 \cdot 10^{-4}$ $cm^{-3} \pm 0.37 \cdot 10^{-4}$. Variation in flux amounts also significantly decreased in the $HgCl_2$ pre-incubation treatment.

of cell volume loss in $CaCl_2$ immediate immersion was $6.77 \cdot 10^{-4}$ $cm^3 \pm 2.76 \cdot 10^{-4}$. WG gametophytes that were incubated in $HgCl_2$ prior to immediate immersion in $CaCl_2$ showed no visible volume loss (Fig. 6D). Mean of measured volume loss in this treatment was $0.44 \cdot 10^{-4}$ $cm^{-3} \pm 0.08 \cdot 10^{-4}$. DG gametophytes (Figs. available from author) also underwent volume loss following immediate immersion in 500 $CaCl_2$. Mean of DG volume loss was $3.04 \cdot 10^{-4}$ $cm^3 \pm 1.14 \cdot 10^{-4}$. Mean of DG treatments that included pretreatment

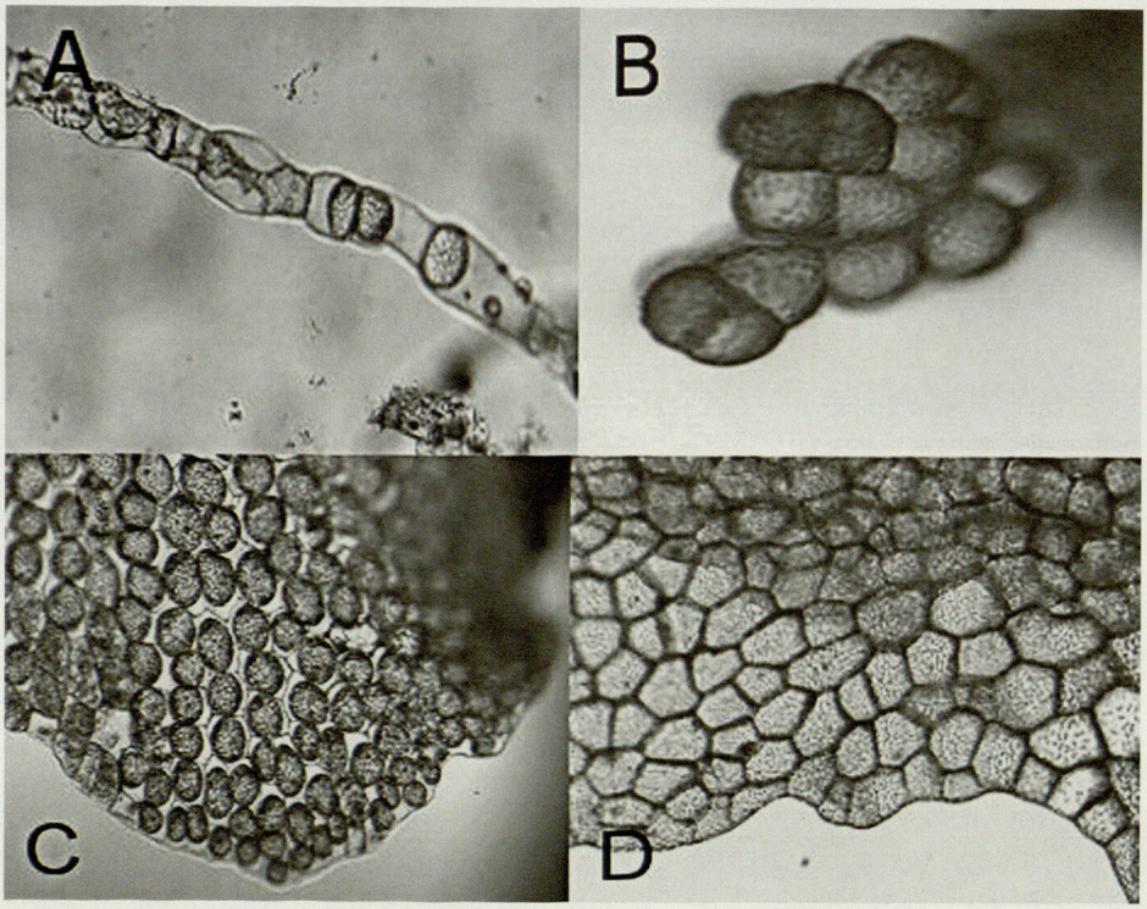

FIG. 6. Gametophytes in $CaCl_2$ Immediate Immersion (A) Callus plasmolyzed heavily upon immediate immersion in 500 mM $CaCl_2$. Mean of callus cell volume loss in $CaCl_2 = 6.73 \cdot 10^{-4}$ cm^{-3} $\pm$ $2.98 \cdot 10^{-4}$. (B) Callus gametophytes that were pretreated in $HgCl_2$ before immersion in $CaCl_2$ exhibited little volume flux. Mean of callus cell volume loss in treatments with $HgCl_2$ preincubation = $0.55 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.50 \cdot 10^{-4}$. Variation in the responses also decreased in those cells preincubated in $HgCl_2$. (C) WG gametophyte cells also plasmolyzed heavily in $CaCl_2$ immediate immersion treatment. Mean of cell volume loss in $CaCl_2$ immediate immersion = $6.77 \cdot 10^{-4}$ cm^{-3} $\pm$ $2.76 \cdot 10^{-4}$. (D) WG gametophytes that were incubated in $HgCl_2$ prior to immediate immersion in $CaCl_2$ showed no visible volume loss. Mean of measured volume loss in this treatment = $0.44 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.08 \cdot 10^{-4}$.

in $HgCl_2$ was $0.49 \cdot 10^{-4}$ cm^3 $\pm$ $0.16 \cdot 10^{-4}$. There was also much less variation in the response with respect to volume loss. Overall, callus was the most vulnerable to desiccation from NaCl and $CaCl_2$ introduction. DG gametophytes were the least affected and visually seemed impervious to NaCl and $CaCl_2$ perturbation.

Sucrose.—Sucrose introduction following pretreatment with ddH_2O resulted in a mode of very little or no water loss in callus (Fig. 7) and WG stages (Fig. 7). Mean of callus cell volume loss was $0.24 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.98 \cdot 10^{-4}$ (Fig. 7A). Callus cells that were preincubated in $HgCl_2$ experienced a gain in cell volume (Fig. 7B). Mean change in cell volume gain was $0.10 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.48 \cdot 10^{-4}$. Variation in cell volume was less in treatments that included a

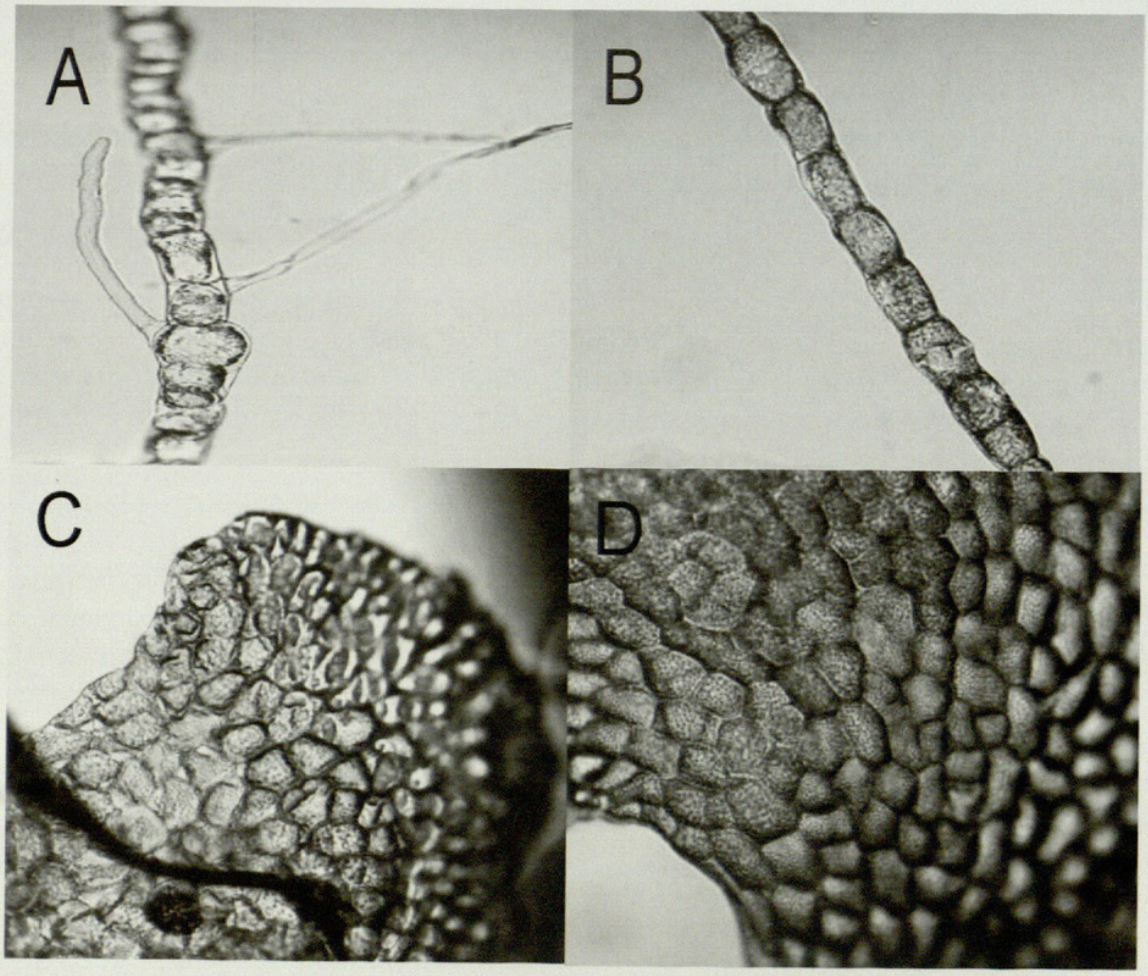

FIG. 7. Gametophytes in Sucrose Immediate Immersion (A) Callus cell moderately plasmolyzed upon immediate immersion in 500 mM sucrose. Mean of callus cell volume loss = $0.24 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.98 \cdot 10^{-4}$. (B) Callus cells immersed in 500 mM sucrose took on cell volume during treatment that included a preincubation in $HgCl_2$. Mean change in cell volume = $0.10 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.48 \cdot 10^{-4}$. Variation in cell volume decreased in treatment that included a preincubation in $HgCl_2$. (C) WG gametophyte cells statistically lost no cell volume in 500 mM sucrose, but that result was highly variable. Mean of cell loss when immediately immersed in sucrose = $0.91 \cdot 10^{-4}$ cm^{-3} $\pm$ $3.00 \cdot 10^{-4}$. (D) WG gametophytes that were pretreated with $HgCl_2$ also showed very little cell volume loss and the variation was quite small in comparison to the treatment with sucrose alone. Mean volume loss in this treatment = $0.93 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.24 \cdot 10^{-4}$.

preincubation in $HgCl_2$ than those preincubated in ddH_2O. WG gametophyte cells statistically lost no cell volume in 500 mM sucrose (Fig. 7C), but that result was highly variable. Mean of WG cell loss when immediately immersed in sucrose was $0.91 \cdot 10^{-4}$ cm^{-3} $\pm$ $3.00 \cdot 10^{-4}$. WG gametophytes that were pretreated with $HgCl_2$ also showed very little cell volume loss and the variation was quite small in comparison to the treatment with sucrose alone (Fig. 7D). Mean volume loss in this treatment was $0.93 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.24 \cdot 10^{-4}$. Likewise, when WG gametophytes were pretreated with $HgCl_2$, cells showed very little cell volume loss and the variation was quite small in comparison to the treatment with sucrose alone. Mean volume loss in this treatment was $0.93 \cdot 10^{-4}$ cm^{-3} $\pm$ $0.24 \cdot 10^{-4}$. The

response to 500 mM sucrose in DG gametophyte cells (Figs. available from author) was similar to that of the WG gametophytes. There was some cell volume loss and the results were highly variable. Mean DG cell volume loss was $0.55 \cdot 10^{-4}$ $cm^{-3} \pm 5.04 \cdot 10^{-4}$. When DG gametophyte cells were preincubated with $HgCl_2$, volume loss dropped dramatically with much less variation. Mean of cell volume loss in this treatment was $0.24 \cdot 10^{-4}$ $cm^{-3} \pm 0.76 \cdot 10^{-4}$.

Discussion

Aquaporin-like proteins were present in all stages of gametophyte growth and play a role in water balance. Overall, $HgCl_2$ pretreatment inhibited water loss in all stages and even resulted in water uptake in some treatments of thinly walled protonemal cells.

Presence of an Aquaporin-like Protein

Results from ELISA controls, the preimmune serum, primary antibody only control, and secondary antibody only control, were negative. Preimmune serum, which is serum from rabbits prior to inoculation with the polypeptide that correlates with the N-terminus of aquaporins (Chaumont *et al.*, 2000), did not show any aquaporins from plants. The primary antibody bound to a single band only when followed by the secondary antibody and neither antibody bound alone. In addition, the antibody bound strongly (not shown) in ELISA and immunoblotting treatments to radish root homogenate (total protein fraction) as a positive control for the procedure. Germinating radish seeds were used as a positive control since PIP1 aquaporins are found primarily in germinating seeds and young shoots (Chaumont *et al.*, 2000). Cross reaction of anti-PIP1 antibodies and radish antigen produced a strong signal. In all gametophyte samples, signal increased linearly with the concentration of the antibody. Significant levels of signal (2–3 times control) were detected in all stages. Immunoblotting resulted in no detectable bands with the preimmune serum control, primary antibody only control, and secondary antibody only control. Only one band in the range of 28 kD, (Agre *et al.*, 1987) was observed, thereby excluding cross-reactivity with other proteins. Taken together, these data suggest that the antibody bound to only one antigen and the procedure was reasonably free of background signal. They also suggest that a PIP1 aquaporin-like protein is found in all stages of gametophyte growth.

Mercury and Aquaporin Function

PIP1 aquaporin-like protein presence and function in *Cheilanthes lanosa* gametophytes is partially responsible for water balance. Because the primary risk for gametophytes in an arid environment is desiccation (Boullard, 1979; Raven *et al.*, 2003), a test of efflux of water from three types of gametophytes was performed: the first, presumably most vulnerable stage, the protonemata [maintained on agar as callus under red light (Raghavan, 1980; Raghavan *et al.*,1989)], the more mature gametophyte [maintained on agar and prompted to mature by blue light (Raghavan, 1980; Raghavan *et al.*,1989), and mature

gametophytes grown in drier conditions [on sand, erratically watered (Diamond and Swatzell, 2003)]. These were exposed to different types of solutes. NaCl and $CaCl_2$ were used to hyperpolarize plasma membranes and sucrose was used as an osmolyte (Lodish *et al.*, 2008). In all solutes, the gametophytes were introduced to up to 500 mM of solutes, either in one large increment or though gradual increments in molarity. Mercury pretreatments in all solutes were adequate to slow or stop water efflux for up to 1 h, even in 500 mM NaCl. Mercury is a direct aquaporin poison (Maggio and Joly, 1995; Heymann *et al.*, 1998). It binds to a cysteine within the water channel and blocks water movement (Preston *et al.*, 1993; Zhang *et al.*, 1993).

Although greater concentrations of $HgCl_2$ could block water movement more aggressively, mercury can also affect other physiological functions in plants (Macey, 1984). The preferred method to show aquaporin function involves the introduction of potential aquaporin mRNA into *Xenopus* oocytes. These oocytes are among the few types of cells that do not express aquaporins. Subsequent production of aquaporins in oocytes prompts water influx and cell disruption (Preston *et al.*, 1992). However, PIP1 aquaporins do not express well in *Xenopus* oocytes (Chaumont *et al.*, 2000). Therefore, a minimum of mercury was used, 1 mM $HgCl_2$ (Carvajal et. al., 1996; Maggio and Joly, 1995; Kaldenhoff et al., 1995), which effectively blocked water flow for the duration of the test. Results suggest that aquaporins are responsible for the majority of water flow across *C. lanosa* plasma membranes, since without $HgCl_2$ in the same respective salt or sucrose solutions, cells immediately desiccate.

Cellular Control of Aquaporin-like Protein Function

Mercury treatment, however, can only show the basic function of aquaporins in gametophytes. Beyond basic function, control mechanisms can alter the way aquaporins function (Luu and Maurel, 2005). Changes in plant cell response to environmental perturbation suggest changes in control methods or activity (Luu and Maurel, 2005). For this reason, gametophytes were challenged with desiccation risk in two different ways. In the first set of tests, gametophytes were exposed to slowly increasing osmolalities. Cells were isotonic at 100 mM of NaCl. For perspective, this concentration would completely plasmolyze a tomato plant cell (Maggio and Joly, 1995). In ddH_2O, callus cells were rounded with substantial turgor. As the solute concentration increased, however, to what would be considered by most plant cells to be a devastating concentration, cells often lost water. Mercury pretreatment was able to block this loss, suggesting flow through aquaporin-like proteins. Most cells were able to avoid desiccation until they reached approximately 350 mM solute concentration. For example, after ddH_2O pretreatment, most callus cells were completely plasmolyzed by the time they reached 500 mM of solute and almost entirely desiccated, with roughly only enough volume for organelles. Desiccation occurred in the final 10–15 min as the environment solution increased beyond 350 mM of solute. However, preincubation in $HgCl_2$ apparently poisoned the major route of water loss. This suggests the presence

of proteins that are aquaporin-like in nature and function but does not test cellular control.

In the second set of tests, the immediate immersion tests, gametophytes were exposed to immediate and violent increases in solutes (Figs. 5–7). Gametophyte response to hyperpolarizing NaCl and $CaCl_2$ (Figs. 5, 6) were appropriately dramatic. Gametophytes desiccated. Due to the hyperpolarizing nature of the solutes, there should have been no control over an aquaporin-like protein (Luu and Maurel, 2005; Qui *et al.*, 2000). The channels would have opened and cells would have predictably desiccated just as the results showed. However, when presented with a true osmolyte, sucrose, the gametophytes with intact and controllable aquaporin-like proteins should have been able to alter aquaporin-like protein function and shut off water efflux (Luu and Maurel, 2005; Chaumont *et al.*, 2000). Predictably, with immediate immersion in sucrose (Fig. 7), gametophytes maintained water balance. Variation may be due to differences in the ability to defer diffusion or differences in development and physiology induced by their respective microenvironments. Therefore, the immediate immersion tests were able to demonstrate control of aquaporin-like protein function. [Higher plants control aquaporin function through phosphorylation (Maurel, 1997). Blue light also stimulates PIP function and inhibits TIP, which suggests signal transduction and cellular control (Ma *et al.*, 2001).

One exception to the differences between increments and immediate immersion experiments was the treatment of the DG gametophytes with sucrose. The treatment means were generally the same (0.0005) in preincubation with ddH_2O. However, the immediate immersion treatment is revealing. In the immediate immersion treatment, preincubation with ddH_2O, there is a huge variation in response. Yet, with preincubation of $HgCl_2$, there is little or no efflux. Clearly, the one difference between these two treatments is the flow of water across the membrane. The rapid influx of water may destabilize the cell and force the cell to rapidly adjust internally (Fitter and Hay, 1987; Taiz and Zeiger, 2006). The large amount of variation possibly suggests the occurrence of numerous signal transduction events or vacuolar adjustments that would characterize a stress response (Fitter and Hay, 1987; Taiz and Zeiger, 2006).

Conclusion

In the first stage of the fern life cycle, protonemal cells express aquaporins, but have thin cell walls and no cuticle. These cells are vulnerable to desiccation. They are also at risk in hypotonic situations. The one thing that protonema can control is aquaporin function. When perturbed, protonema can prevent immediate desiccation by disrupting water flow. However, the much slower osmotic diffusion would eventually result in desiccation. It is not surprising, then, that the protonemal stage thrives in sedimentary rock outcrops, which provide a small and continuous amount of water in the optimal amounts for the gametophytes (Dooley and Swatzell, 2002). WG gametophytes are somewhat

better at desiccation resistance in the face of sucrose, but membrane hyperpolarization in NaCl and $CaCl_2$ still promote rapid desiccation. Admittedly, a Petri dish and agar medium is atypical habitat. The effects on development and function may not be meaningful by themselves. What WG growth does show in conjunction with the DG, more typical growth, is that natural development confers resistance to desiccation. Under long term stress, regardless of the nature of the season, the mature gametophyte in its natural habitat is undeniably impervious to desiccation. This may be the fate of the *C. lanosa* gametophyte generation. The fern's ability to survive desiccation appears to be limited by that brief but vulnerable protonemal stage and by its very need for aquaporins to manage water balance and uptake. Its mechanism for water uptake can become the pathway of its water loss.

Acknowledgments

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Supplemental data website

http://cstl-csm.semo.edu/swatzell/AFJ/diamond2011.htm

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