Isolation and Expression of Two Aquaporin-Encoding Genes from the Marine Phanerogam Posidonia oceanica

Pierluigi Maestrini 1, Tommaso Giordani 1, Andrea Lunardi 2, Andrea Cavallini 1 and Lucia Natali 1,3

1 Department of Agricultural Plant Biology, Genetics Section, University of Pisa, Via Matteotti 1/B, I-56124 Pisa, Italy
2 Department of Physiology and Biochemistry, University of Pisa, Via S. Zeno 31, I-56127 Pisa, Italy
3 Corresponding author: E-mail, lnatali@agr.unipi.it; Fax, (+39-50) 576750.

Seagrasses such as Posidonia oceanica (L.) Delile are marine phanerogams, widespread in various seas, where they form large prairies representing dynamic substrates exceeding the area of the sediment surface several times over and allowing settlement of epiphyte organisms. Studying mechanisms involved in water transport in marine plants, we isolated two aquaporin-encoding genes, PoPIP1;1 and PoTIP1;1, showing high similarity to plasma membrane- and tonoplast-intrinsic protein-encoding genes, respectively. PoPIP1;1 is unique in the genome of P. oceanica, while PoTIP1;1 belongs to an aquaporin subfamily of at least four members. PoPIP1;1 and PoTIP1;1 encode functional proteins, as indicated by expression experiments in Xenopus oocytes. Both genes are constitutively expressed in the leaves, with higher levels of transcripts in young than in differentiated leaf tissues. Variations of salt concentration in aquarium determined different PoPIP1;1 and PoTIP1;1 transcript accumulation, indicating the existence of adaptation mechanisms related to gene expression also in marine plants, i.e. adapted to very high salt concentrations. Hyposalinity induced lower levels of PIP1 transcripts, while hypersalinity determined more PIP1 transcripts than normal salinity. TIP1 transcripts increased in response to both hypo- and hypersalinity after 2 days of treatment and went back to control levels after 5 d.

Keywords: Aquaporin — Marine plants — Posidonia oceanica — Salinity — Seagrass.

Introduction

Knowledge of the molecular physiology of seagrasses allows the verification of the influence of an extreme environment on physiological and genetic processes that are known in terrestrial plants. Such knowledge is necessary to establish adequate strategies of marine ecosystem conservation.

Terrestrial plants depend on water supply for their growth and development and constantly absorb and lose water. The diffusion of water can be driven by concentration gradients of osmotically active solutes or by physical pressure, generating an osmotic or hydrostatic force, respectively. Beyond simple diffusion across a lipid bilayer, the existence of proteinaceous water channels, aquaporins, in plant membranes has been established (see Johansson et al. 2000).

Aquaporins belong to a highly conserved group of membrane major intrinsic proteins (MIPs) with molecular masses of 26–30 kDa. The functional unit of aquaporin is a tetramer, with each monomer providing an independent water pore. Each monomer typically contains six membrane-spanning α-helices with the N- and C-termini both located on the cytoplasmic side of the membrane (Tyerman et al. 1999). Structurally, the N- and C-terminal halves of the polypeptide are arranged as a tandem sequence repeat, each containing a hydrophobic loop including a conserved NPA motif (Reizer et al. 1993). According to the so-called ‘hourglass model’, the two halves show obverse symmetry with the NPA loops overlapping in the middle of the lipid bilayer to form a pore that mediates bi-directional water flow (Cheng et al. 1997). Within the pore, four bound waters are localized along three hydrophilic nodes, which punctuate a hydrophobic pore segment, facilitating water-specific transport (Murata et al. 2000, Sui et al. 2001).

Functionally, the MIP superfamily of integral membrane proteins may be divided into two groups: aquaporins, and transporters of glycerol and other small neutral molecules (Johansson et al. 2000). In addition, plant aquaporins may be distinguished into plasma membrane- and tonoplast-intrinsic proteins (PIPs and TIPs). Two other groups occur, NIPs and SIPs, including various aquaporins whose subcellular localization is generally unknown (Johansson et al. 2000). In their turn, TIPs may be distinguished into TIP1s, TIP2s, TIP3s, TIP4s and TIP5s, depending on their sequence and subcellular localization, and PIPs into PIP1s and PIP2s, according to the occurrence of specific arrays of amino acids at the N- and C-termini (Johansson et al. 2001).

Aquaporin activity can be regulated mostly in response to post-translational modifications but also at transcriptional level. Some aquaporins are constitutively expressed (Johansson et al.
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Results

Putative aquaporin-encoding cDNAs of _P. oceanica_ were obtained by a rapid amplification of cDNA ends (RACE) protocol (Frohman et al. 1988). After overlapping of 5’- and 3’-RACE sequence fragments, the two cDNA sequences, _PoPIP1;1_ (accession number AJ314584) and _PoTIP1;1_ (accession number AJ314583), were found to be 1,548 and 1,212 bp long, respectively. The deduced proteins sequences showed that _PoPIP1;1_ has a 5’-UTR of 10 nucleotides and a 3’-UTR of 240 nucleotides, while _PoTIP1;1_ has a 5’-UTR of 79 nucleotides and a 3’-UTR of 261 nucleotides. Analyses performed using the BLASTx program at NCBI showed high similarities to putative plant aquaporins: _PoPIP1;1_ deduced protein is most similar to plasma membrane aquaporin _PIP1;1_ of the hybrid _Vitis berlandieri × Vitis rupestris_ (acc. number AAFT1817, 86% identity) and _PoTIP1;1_ to a tonoplast aquaporin of _Ricinus communis_ (accession number CAE53881, 70% identity).

After establishing cDNA sequence, primers designed on the 5’-end and on the 3’-UTR of _PoPIP1;1_ and _PoTIP1;1_ were used to amplify by PCR the corresponding aquaporin genes from genomic DNA. Comparison of the genomic sequence to that of the cDNA shows that _PoPIP1;1_ is interrupted by two introns, 314 and 95 bp long, in contrast to the corresponding _PIP1s_ of _Arabidopsis_, which are interrupted by three introns (Johanson et al. 2001). The _PoTIP1;1_ gene is interrupted by one 100 bp intron, as in _Arabidopsis_ _TIP1s_. Insertion sites of introns of both _PoPIP1;1_ and _PoTIP1;1_ correspond to those observed in _Arabidopsis_ aquaporin-encoding genes; introns interrupt regions that do not code for transmembrane helices (Johanson et al. 2001).

The hydrophobicity profiles of _PoPIP1;1_ and _PoTIP1;1_ deduced protein sequence, calculated using the program ProtScale, at the Expasy server (Switzerland), according to amino acid scale values by Kyte and Doolittle (1982), showed six
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highly hydrophobic regions (not shown) corresponding to membrane-spanning putative α-helices that are typical of aquaporins. Moreover, two minor peaks are observed, corresponding to the two NPA domains, which form the water pore within the membrane lipid bilayer (Tyerman et al. 1999).

To establish to which aquaporin class PoPIP1;1 and PoTIP1;1 deduced proteins belong, a similarity analysis was performed, using the neighbour-joining method, comparing PoPIP1;1 and PoTIP1;1 deduced proteins with various membrane-intrinsic proteins of plants and animals (Fig. 1). PoPIP1;1 protein is similar to PIP1s and PoTIP1;1 to TIP1s.

In order to establish whether these two genes belong to multigene families, Southern blot experiments were performed using restriction enzymes not cutting within the PoPIP1;1 or PoTIP1;1 gene sequences. Two probes per gene were used, the first, named ‘generic’, corresponding to regions encoding highly conserved domains in PIPs or TIPs, the second, named ‘specific’, corresponding to the 3′-UTRs. Hybridization patterns are shown in Fig. 2. PoPIP1;1 is detected as a unique hybridization band per lane with either the generic or specific probe, suggesting that this is a single-copy gene. PoTIP1;1 corresponds to one band using the specific probe, while in the BamHI and EcoRI digestion products, four bands are detected by the generic probe, suggesting that this particular TIP1 belongs to a multigene family of at least four members. No differences in hybridization pattern were observed washing membranes at low (1× SSC) instead of high (0.3× SSC) stringency (not shown).

To find out whether the PoPIP1;1- and PoTIP1;1-encoded proteins are functional aquaporins, the cDNAs were cloned in a vector for in vitro synthesis of cRNA, and the corresponding cRNAs were injected into Xenopus laevis oocytes. The same experiment was carried out with ZmPIP2;5 cDNA (Chaumont et al. 1999).
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et al. 2000) used as positive control. Three days later, the oocytes were tested in swelling assays by exposing them to a hypotonic solution. If heterologous water channel proteins are present in the plasma membrane, the oocytes swell much more quickly than the water-injected oocytes. In these conditions, oocytes injected with Po\textit{PIP1;1} cRNAs swelled only slightly more rapidly than the water-injected oocyte (Fig. 3).

To analyse Po\textit{PIP1;1} and Po\textit{TIP1;1} transcript levels in vivo, Northern blots and hybridization with specific probes were performed using RNA isolated from different portions of leaves collected in the sea and immediately frozen in liquid nitrogen (Fig. 4). Densitometric analysis of hybridization bands indicates a decreasing gradient in the transcript accumulation of both Po\textit{PIP1;1} and Po\textit{TIP1;1}, from the meristem region to the leaf subapex, containing fully differentiated (i.e. elongated) cells.

Northern blot analysis was also performed on \textit{Posidonia} leaves from shoots cultivated in aquaria for different time periods (2 and 5 d) in different saline concentrations, ranging from 30\% to 45\%. The salinity of seawater in which the \textit{Posidonia} plants used in our experiments lived (40\%) was used as a con-
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Fig. 5 shows that $PoPIP1;1$ transcripts increased (compared with the control) after 5 d of 45‰ salinity treatment, while, in low salinity conditions, the transcripts decreased more rapidly with 30‰ than with 35‰ salt concentration. In contrast, transcript levels of $PoTIP1;1$ increased after 2 d treatment with all salt concentrations; but after 5 d treatment, transcript levels similar to that of control plants were observed with all concentrations (Fig. 5).

Discussion

Characterization of two $P. oceanica$ aquaporin-encoding genes

Aquaporins are key elements of water transport in terrestrial plants, favouring water transfer within the symplast, driven by leaf transpiration. In marine plants, the roots serve essentially as anchorage, and water supply to photosynthetic epidermal and mesophyll cells is obtained mainly by diffusion, as indicated also by the reduced functionality of xylem vessels in the leaf (Tomlinson 1979).

Because of the importance of diffusion through cell membranes for water conductance, studying aquaporins and their genes in marine phanerogams such as $P. oceanica$ can give further information on mechanisms of water transport, especially considering the highly saline environment in which Posidonia lives.

$P. oceanica$ DNA sequences isolated in our experiments show very high similarity to plant membrane-intrinsic protein-encoding genes, whose main features (NPA boxes, six main putative $\alpha$-helices) can be found in the deduced proteins. Both genes are expressed, hence presumably active. These are the first putative aquaporin-encoding genes to be isolated from a marine plant. Putative aquaporin-encoding genes have been isolated from a halophyte (Aster tripolium), but this plant can tolerate up to 9‰ salinity (Uno et al. 1998), compared with 40‰ salinity, which is the salinity at which $P. oceanica$ normally lives.

Analysis of $PoPIP1;1$ and $PoTIP1;1$ deduced proteins shows that they are typical class 1 PIP and class 1 TIP, respectively (Fig. 1). TIP1 aquaporins are generally highly expressed in plants (Chaumont et al. 1998). For example, Higuchi et al. (1998) demonstrated that more than 25% of radish tonoplast proteins are class 1 TIPs. Heterologous expression of $PoTIP1;1$ in Xenopus oocytes showed a high, rapid increase in volume, similar to that of the positive control ZmPIP2;5, indicating the presence of a facilitated water transport pathway (Fig. 3).

Concerning the function of tonoplast aquaporins in Posidonia, it is known that mesophyll tissues of seagrasses usually contain parenchyma cells with one large vacuole, especially in differentiated portions of the leaf (Kuo and McComb 1980). Unfortunately, only a few studies have contributed to the molecular characterization of seagrass vacuoles and their function (Pak et al. 1995, Carpaneto et al. 1997). Plant cells must have turgor pressure, i.e. osmotic pressure is higher inside the cell than outside. In seagrasses, which tolerate very high Na⁺ concentrations, this would imply very high salt concentrations within the cell, especially in the vacuole, in which high salinity is more compatible with metabolism than in the cytoplasm (Tyerman 1980). Strong Na⁺ accumulation in the vacuoles of leaf parenchyma cells is probably related to the activity of Na⁺-permeable ion channels (Carpaneto et al. 1997). In this respect, it is conceivable that the occurrence of tonoplast aquaporins contributes to adjust the osmotic gradient in seagrasses, as it does in terrestrial plants.

$PoPIP1;1$, in contrast to $PoTIP1;1$, showed a very slow (though significant) rate of swelling (Fig. 4) when expressed in $X. laevis$ oocytes. It is to be noted that proteins belonging to the plant PIP1 subfamily often show no or very limited water channel activity in $X. laevis$ oocytes or in Saccharomyces cerevisiae (Chaumont et al. 2000, Suga and Maeshima 2004). The reason for this is still unknown; however, this class of aquaporin-encoding genes is transcriptionally active in all plant species studied. Hypotheses could be made that such aquaporins do not function as water channels on their own, suggesting that they are transporters of gas or solutes not yet identified (for example CO₂), or that they need to be chemically modified by components not present in $X. laevis$ oocytes, or that strong activation of these aquaporins occurs only after subunit–subunit interaction in plant cells (Chaumont et al. 2000, Fetter et al. 2004, Suga and Maeshima 2004).

Aquaporin-encoding genes are usually grouped in gene families. For example, in Arabidopsis, 35 aquaporin loci have been reported (Johanson et al. 2001) and other members have been discovered after genome sequencing. To analyse the aquaporin gene family in $P. oceanica$ we performed Southern experiments using generic probes, i.e. corresponding to highly conserved protein-encoding regions, or specific probes, i.e. corresponding to the 3′-UTR, a region commonly considered gene specific (see Chaumont et al. 1998). Actually, either $PoPIP1;1$ or $PoTIP1;1$ specific probes produce only one band (Fig. 2). The $PoPIP1;1$ generic probe produces the same band as the specific probe, indicating that this PIP1 gene is present as a single copy in the Posidonia genome (Fig. 2A), although the occurrence of other PIP genes can be supposed. $PoTIP1;1$ is detected in four bands with the generic probe (Fig. 2B), indicating the occurrence of at least four genes highly similar to $TIP1;1$ in the genome. On the whole, our experiments show that only a small number of aquaporin-encoding genes are recognized by our sequences. Since the number of aquaporin-encoding genes is usually much higher, it is conceivable that other gene subfamilies occur, with differences at DNA sequence level.

Expression of aquaporin genes in vivo and in response to changes in salinity

It is known that aquaporin genes in terrestrial plants are in many cases transcribed at higher levels in meristem cells and young tissues than in differentiated cells and mature tissues (Maurel et al. 2002). Chaumont et al. (1998) related transcrip-
tion of a maize TIP-encoding gene in differentiating tissues to vacuole biogenesis and enlargement during cell elongation. Malz and Sauter (1999) have, however, shown that transcription of some rice aquaporin genes is not controlled by growth. We have characterized aquaporin-encoding genes of marine *P. oceanica* by analysing the steady-state levels of transcripts in vivo and after various treatments in aquaria.

In our experiments, the younger the leaf portion, the higher is the *Po*PIP1;1 and *Po*TIP1;1 transcript accumulation (Fig. 4). The diverse behaviour of aquaporin-encoding genes at different developmental stages can be related to the abundance of such genes in plant genomes: it is conceivable that different aquaporins can or cannot be expressed in different tissues and/or environmental conditions, achieving a fine regulation of hydraulic conductivity in the plant.

Hydraulic conductivity in terrestrial plants can be modulated by external solute concentration: for example, variation in hydraulic conductivity has been observed during diurnal cycle, in drought and salt stress, at cold temperatures, and during nutrient or oxygen deficiency (see Mathieu et al. 1995, Carvajal et al. 1996). At cellular level, the main role in determining osmotic gradient is related to ion channels (Carpaneto et al. 2004); however, adjustment of such gradient can be related also to water transport through aquaporins, whose efficiency can be modulated either by post-translational modifications or by modulation of aquaporin gene transcription and translation (Maurel et al. 1997).

Actually, salt concentration affects aquaporin gene regulation (see Maurel et al. 2002 for a review). Especially interesting are the results reported by Uno et al. (1998) on putative aquaporin-encoding gene transcription in leaves of the halophyte *A. tripolium*: they observed that leaf treatments with 200–500 mM NaCl (corresponding to 11.6–29% salinity) increase transcript levels of three out of six aquaporin-encoding genes within 5 h.

We have studied *P. oceanica* aquaporin-encoding transcripts after modifying salinity conditions in the aquarium. Our data indicate that the aquaporin-encoding transcript level is affected by salinity variations: hyposalinity induces lower levels of *Po*PIP1;1 transcripts (after 2 d at 30‰ and after 5 d at 35‰ salt concentrations), while hypersalinity determines more PIP1 transcripts than normal salinity (Fig. 5). Admitting that *Po*PIP1;1 is involved (even though at low efficiency, Fig. 3) in water transport, such changes in transcript levels should determine variations in water flux inside and outside the cells.

Concerning *Po*TIP1;1 transcripts, they increased in response to either hypo- or hypersalinity after 2 d of treatment and went back to control levels after 5 d (Fig. 5), i.e. the response of this TIP-gene is transient.

It can be supposed that, in leaves of this marine plant, at control salinity conditions (40%), water can enter leaf cells according to an osmotic gradient and is distributed to photosynthetic cells. If salinity changes, plasmalemma and tonoplast aquaporins can be involved in adapting the water permeability of the cell. Tonoplast and plasmalemma aquaporins could play different roles: hyposalinity should be accompanied by a reduction in the functionality of plasma membrane water channels to reduce water entering the cells; while tonoplast water channels should be more efficient to favour water transport in the vacuole and buffer hypo-osmolarity in the cytosol consequent to the increased water influx.

On the other hand, salt concentration increase should reduce but not revert the osmotic gradient. In this case, increasing PIP efficiency would be necessary to ensure sufficient water influx into the cells. Tonoplast water channels should also be more functional to buffer possible hypersalinity in the cytosol ensuring high water efflux from the vacuole. Actually, it is known that water transport through protein channels is passive and aquaporins work in both senses (Barone et al. 1997).

The expression patterns of *Po*PIP1;1 and *Po*TIP1;1 after salinity changes fully match this possible adaptation mechanism, suggesting that regulation of aquaporin gene transcription plays an important role in modulating the functionality of water transport in *P. oceanica*.

On the whole, if *Posidonia* aquaporins are probably not involved in water uptake by roots as they are in terrestrial plants, however, aquaporin-encoding genes are active and probably functional in water transport. In fact, our expression data show that they are transcribed constitutively, but variations in salt concentration can determine different steady-state levels of *Po*PIP1;1 and *Po*TIP1;1 transcripts. This indicates that adaptation mechanisms related to aquaporin gene expression occur also in marine plants, i.e. apparently adapted to very high salt concentrations. Since it is reported that water transport through aquaporins in terrestrial plants is modulated mainly by post-translational changes such as phosphorylation (Maurel et al. 1997) or proteolytic maturation from a larger precursor (Inoue et al. 1995), experiments are in progress to analyse post-translational modifications of *P. oceanica* aquaporins.

### Materials and Methods

#### Plant materials

*P. oceanica* (L.) Delile shoots were collected by SCUBA diving between 2 and 5 m depth in the *P. oceanica* meadow off Antignano, Leighorn, Italy. Excess sediment was removed by washing gently in seawater, before placing the plants in plastic bags filled with seawater. Some plants were frozen in liquid nitrogen immediately after explantation for RNA analyses and metal determination.

Other shoots were acclimated to laboratory conditions (closed circuit aquarium, constantly aerated; 16.5 ± 0.5°C; light/dark cycle: 16/8 h; six shoots per aquarium containing 12 liters of natural sea water, collected in the same place) for 24 h before starting the treatments.

#### Salinity treatments

Shoots of *P. oceanica* were acclimated to laboratory conditions as described above for 24 h, then cultivated under the same conditions for 2 or 5 d in aquarium in the same seawater (normal salinity, 40‰) or in seawater supplemented with commercial aquarium salts up to
**Table 1** List of primers used for amplification of putative aquaporin-encoding cDNAs and genes

<table>
<thead>
<tr>
<th>Primer code</th>
<th>Sequence</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIP+</td>
<td>5′-CATCTCCGGCGGCACATCAAC-3′</td>
<td>PIP 3′-RACE</td>
</tr>
<tr>
<td>PIP−</td>
<td>5′-GGGCCAACCAGAGATCCAAT-3′</td>
<td>PIP 5′-RACE</td>
</tr>
<tr>
<td>TIP+</td>
<td>5′-CATCTCGGCGGCAGTBYGA-3′</td>
<td>TIP 3′-RACE</td>
</tr>
<tr>
<td>TIP−</td>
<td>5′-GGGCCAACCAGAGATCCAAT-3′</td>
<td>TIP 5′-RACE</td>
</tr>
<tr>
<td>AP</td>
<td>5′-GGGCCAACCAGAGATCCAAT-3′</td>
<td>3′-RACE adapter primer (Gibco)</td>
</tr>
<tr>
<td>AUAP</td>
<td>5′-GGGCCAACCAGAGATCCAAT-3′</td>
<td>5′-RACE adapter primer (Gibco)</td>
</tr>
<tr>
<td>GenePIP+</td>
<td>5′-AAAGGACAGATGGAGGGCAAG-3′</td>
<td>PCR on genomic DNA</td>
</tr>
<tr>
<td>GenePIP−</td>
<td>5′-CTGTAAGAGACTCGAGCAATT-3′</td>
<td>PCR on genomic DNA</td>
</tr>
<tr>
<td>GeneTIP+</td>
<td>5′-GGGCATAGAAGAGATGGTAGAGAACA-3′</td>
<td>PCR on genomic DNA</td>
</tr>
<tr>
<td>GeneTIP−</td>
<td>5′-ATGCCGCAGCGGATGAATA-3′</td>
<td>PCR on genomic DNA</td>
</tr>
<tr>
<td>PoPIP1:1a+</td>
<td>as GenePIP+</td>
<td>Generic PoPIP1:1 riboprobe</td>
</tr>
<tr>
<td>PoPIP1:1a−</td>
<td>5′-CAGCAGTCAGTAAGACAGAGC-3′</td>
<td>Generic PoPIP1:1 riboprobe</td>
</tr>
<tr>
<td>PoPIP1:1b+</td>
<td>5′-TATGTTGTTGTGCTTTGAATCA-3′</td>
<td>Specific PoPIP1 riboprobe</td>
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<tr>
<td>PoPIP1:1b−</td>
<td>as GenePIP−</td>
<td>Specific PoPIP1 riboprobe</td>
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<tr>
<td>PoTIP1:1a+</td>
<td>5′-GGGCCATGGCTCTCTGGGTTTT-3′</td>
<td>Specific PoTIP1 riboprobe</td>
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<tr>
<td>TIPX−</td>
<td>5′-ATTTAGGATCTTCTCAGCAATTCAACA-3′</td>
<td>PCR on cDNA</td>
</tr>
</tbody>
</table>

+: sense primers; −: antisense primers.

**Isolation of DNA and RNA**

45% salinity, or with distilled water up to 35% and 30% salinity. Salinity was checked every day using a refractometer.

DNA was purified from leaves according to the Cetyltrimethylammonium bromide method devised by Doyle and Doyle (1989) modified by Giordani et al. (2000). For further purification, solid CsCl and ethidium bromide were added to the nucleic acid solution up to final concentrations of about 0.8 mg ml⁻¹ (refraction index 1.3890–1.3895) and 200 µg ml⁻¹, respectively. The solution was centrifuged at 44,000 rpm in a Beckman L5-65 ultracentrifuge for 30 h at 15°C using the 65 Ti rotor, and the DNA band, visualized under long-wave UV illumination, was collected. Ethidium bromide was then removed by gentle inversion of the solution with n-butanol and dialysed against water at 4°C for 3 h. Finally, DNA was ethanol precipitated and resuspended in the appropriate buffer.

Total RNA extraction was performed according to the protocol described in Giordani et al. (2000). Contaminant DNA was removed from the samples by digestion with RQ1 DNase (Promega). RNA was then purified by phenol/chloroform extraction, ethanol precipitated and solubilized in diethylpyrocarbonate-treated water. RNA integrity was assayed on agarose gels, before Northern blotting. Analyses were performed on three leaf portions: a basal meristematic region, undifferentiated, light-green; an intermediate region, with differentiating cells, green; and a subapical region, with differentiated cells, green. The leaf tip was not used because of the presence of epiphytes.

**Isolation of sequences encoding putative aquaporins**

DNA sequences homologous to aquaporin-coding genes were isolated by RACE (Frohman et al. 1988) on *P. oceanica* total RNA, using the 3′-RACE System and 5′-RACE System kits (Invitrogen) according to the manufacturer’s instructions. Degenerated primers were designed on conserved transmembrane domain 2–NPA box (forward primers) and transmembrane domain 6 (reverse primers) of aquaporin-encoding genes from monocotyledons (*Oryza sativa*, *Zea mays*, Allium cepa). These primers and others used in PCR experiments are reported in Table 1. For 3′-RACE, first-strand cDNA was synthesized using total RNA from control plants as a template. RNA (2.5 µg) was heated for 10 min at 70°C and retrotranscribed in a 25 µl volume reaction using 500 µM each deoxynucleotide triphosphate (dNTP), 500 nM AP-adapter primer, 1× PCR buffer, 10 mM DTT, 200 U SuperScript II reverse transcriptase, 2.5 mM MgCl₂, Incubation time was 50 min at 42°C. After retrotranscription, RNase Mix was added to the mixture for 20 min at 37°C. Retrotranscribed cDNA was used for PCR amplification: PCR was performed using 2 µl retrotranscribed cDNA, 1.5 mM MgCl₂, 200 µM of each dNTP, 200 nM PIP− or TIP− primer in conjunction with 200 nM AP-adapter primer, 1× PCR buffer, 10 mM DTT, 200 U SuperScript II reverse transcriptase, 2.5 mM MgCl₂, Incubation time was 50 min at 42°C.

For 5′-RACE, first-strand cDNA was synthesized as follows: 2.5 µg RNA was heated for 10 min at 70°C and retrotranscribed in a 25 µl volume reaction using 400 µM each dNTP, 100 nM PIP− or TIP− primer, 1× PCR buffer, 10 mM DTT, 200 U SuperScript II reverse transcriptase, 2.5 mM MgCl₂, Incubation time was 50 min at 42°C.
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After retrotranscription, RNase Mix was added to the mixture for 30 min at 37°C. cDNA was then purified and a poly(C) tail was added to denatured cDNA 3'-ends according to manufacturer’s instructions. This cDNA was used for PCR amplification: PCR was performed using 5 μM purified cDNA, 1.5 mM MgCl₂, 200 μM each dNTP, 400 nM AAP-ubiquitously-anchor primer in conjunction with 400 nM PIP- or TIP- primer, 1× thermostable buffer, 2.5 U Tag DNA polymerase. The reaction mixture (50 μl) was heated to 94°C for 2 min, then 35 cycles, each consisting of 30 s at 94°C, 30 s at 55°C, 45 s at 72°C, were used. The final step at 72°C was extended for 7 min.

3'- and 5'-RACE fragments were cloned into a pGEM-T Easy plasmid vector (Promega). The cloned fragments were sequenced by the dideoxy chain termination method, and overlapped to obtain the complete cDNA sequences.

Primers were then designed on the 5'- and 3'-ends of the complete cDNA sequences and used on genomic DNA. PCR amplification was performed using 100 ng of genomic DNA, 200 μM each dNTP, 1.25 U Pfu DNA polymerase in 1× buffer, 1 μM GenePIP+ or GeneTIP+ primer in conjunction with 1 μM GenePIP– or GeneTIP– primer, respectively. The reaction mixture (50 μl) was heated to 94°C for 4 min, then 30 cycles, each consisting of 30 s at 94°C, 30 s at 62°C, 120 s at 72°C, were used. The final step at 72°C was extended for 7 min.

After an A-tailing procedure performed using Taq DNA polymerase, the amplified fragments were cloned into a pGEM-T Easy plasmid vector. The cloned fragments were sequenced by the dideoxy chain termination method. Experiments of amplification of genomic DNA, cloning and sequencing of the genes were repeated three times. Three clones were sequenced per experiment: no sequence variation was observed.

Alignment of PoPIP1;1 and PoTIP1;1 to corresponding genes of other species are reported in the Supplementary Material.

Southern blot analysis

Ten micrograms of genomic DNA was digested with the restriction endonucleases BamHI, EcoRI and KpnI in 5-fold excess according to the supplier’s instructions (Roche). Southern blotting of digested DNAs was performed according to standard protocols (Sambrook et al. 1989) and hybridizations were made using various labelled riboprobes, PoPIP1;1a and PoPIP1;1b, or PoTIP1;1a and PoTIP1;1b. PoPIP1;1a and PoTIP1;1a correspond to conserved protein domains, while PoPIP1;1b and PoTIP1;1b correspond to 3′-UTR regions. These sequences were obtained by PCR using primers reported in Table 1 in the same experimental conditions as described above. Amplified fragments were cloned in pGEM-T Easy, sequenced as above and used to obtain labelled riboprobes.

All probes were digoxigenin-labelled using the DIG RNA labelling kit SP6/T7 (Roche). After hybridization in 50% deionized formamide, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), 2% blocking reagent (Roche), 0.02% SDS, 0.1% SLS, filters were washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature, once in 1× SSC, 0.1% SDS for 30 min at 68°C and, in some experiments, once in 0.3× SSC, 0.1% SDS for 30 min at 68°C. Detection of hybridization signals was performed using the DIG-Nucleic Acid Detection Kit (Roche) according to the manufacturer’s instructions.

Expression analysis in Xenopus oocytes

To obtain the entire sequences of aquaporin genes, a PCR amplification was performed using 2 μl retrotranscribed cDNA from 3′-RACE as template, 200 μM each dNTP, 1.25 U Pfu DNA polymerase in 1× Buffer (Promega), 1 μM PIPX+ or TIPX+ primer in conjunction with 1 μM PIPX– or TIPX– primer, respectively. The reaction mixture (50 μl) was heated to 94°C for 4 min, then 30 cycles, each consisting of 30 s at 94°C, 30 s at 65°C, 100 s at 72°C, were used. The final step at 72°C was extended for 7 min. After an A-tailing procedure performed using Taq DNA polymerase, the amplified fragments were cloned into a pGEM-T Easy plasmid vector.

Primers PIPX+, PIPX–, TIPX+ and TIPX– contained BstII sites at their 5′-ends for insertion in expression vector. After checking the sequence of the cloned fragments, plasmids containing the entire PoPIP1;1 and PoTIP1;1 were digested with BstII and the inserts were cloned into the pXGev2 expression vector carrying 5′ and 3′-untranslated sequences of a β-globin gene from X. laevis.

After linearization of the vectors with BamHII, capped complementary RNAs (cRNAs) were synthesized in vitro using T3 RNA polymerase (Roche) following the manufacturer’s instructions, yielding PoPIP1;1 and PoTIP1;1 protein-encoding cRNAs.

Fully grown oocytes (stage V and VI) were isolated from X. laevis, defolliculated with 1 mg ml−1 collagenase type I (Sigma) for 2 h in Barth’s solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄· 10 mM HEPES–NaOH, pH 7.4). The defolliculated oocytes were maintained for 12 h in Barth’s solution supplemented with 50 μM 1× gentamicin sulfate at 18°C before injection. In vitro transcripts of PoPIP1;1, PoTIP1;1 and ZnPi2P2.5 (2 μg μl⁻¹) or nuclelease-free water (each treatment 28 nl) were microinjected into five oocytes per treatment. Microinjected oocytes were kept at 18°C. Three days after injection, the oocytes were transferred from Barth’s solution (200 mosmol) to the same solution diluted to 40 mosmol.

Changes in cell volume were surveyed under a microscope by taking photographs at 20 s intervals. The oocyte diameters were measured four times along two sets of perpendicular axes. The volume was estimated as the mean of two spherical volumes and they were used for the calculation of oocyte relative volume.

Northern blot analysis

Total RNAs (20 μg) were separated by 1% formaldehyde–agarose gel electrophoresis and blotted onto positively charged nylon membranes (Roche). The integrity and the equal amount of RNA loading were confirmed by ethidium bromide staining and subsequent densitometric image analysis and by re-hybridization with a Phaseolus coccineus rDNA probe. The ribosomal probe was an EcoRI–SacII 25S rDNA fragment of clone pPH1, subcloned in pBluescript II SK+ and labelled as the other probes.

Hybridizations were performed using PoPIP1;1b or PoTIP1;1b labelled RNA probes as above. Filters were washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature, once in 1× SSC, 0.1% SDS for 30 min at 68°C and once in 0.3× SSC, 0.1% SDS for 30 min at 68°C. Detection of hybridization signals was performed as above. Northern blots were repeated three times per experiment. When hybridization signals seemed over saturation, experiments were repeated loading reduced quantities of RNA.

Sequence analysis

Intron delimitation within genomic sequences was made by comparing with cDNA sequences. Database searches were conducted using the program BLAST (National Center for Biotechnology Information, U.S.A.). Alignment of deduced amino acid sequences of PoPIP1;1 and PoTIP1;1 with other aquaporin-encoding sequences was obtained using the program CLUSTALW (GenomeNet WWW Server, Japan). Some adjustments were made by eye.

Hydropathicity profiles of the deduced proteins were constructed by the program Protscal, at the Expasy server (Switzerland), according to amino acid scale values by Kyte and Doolittle (1982).
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Using deduced protein sequences, aligned as above, a phylogenetic tree was constructed by the neighbour-joining method (distance algorithm after Kimura 1980), using the PHYLIP program package version 3.572 (Felsenstein 1989); using the SEQBOOT program, 1,000 versions of the original alignment were generated; then, trees were generated using PROTDIST and NEIGHBOR programs. A strict consensus tree was obtained from the available trees using the CONSENSE program.

Supplementary material
Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

Acknowledgments
We thank Prof. François Chaumont (Université Catholique de Louvain, Belgium) for his generous gift of plasmids pXBGenv2 and pxZmPIP2-5 and his useful suggestions, and Prof. Luciana Dente (Università di Pisa, Italy) for her help during expression studies in oocytes and critical reading of the manuscript. This research work was supported by CNR, Italy, Target Project on Biotechnology.

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(Received July 1, 2004; Accepted October 11, 2004)