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Rapid Communication

PIP1 Aquaporins Are Concentrated in Plasmalemmasomes of *Arabidopsis thaliana* Mesophyll[1]

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The PIP1 subfamily of water channel proteins (aquaporins) constitute about 1% of the plasma membrane (PM) proteins from *Arabidopsis thaliana* leaves. Immunogold electron microscopy has confirmed their localization at the PM of mesophyll cells. Very high labeling density at PM invaginations known as plasmalemmasomes was observed. Therefore, we suggest that these subcellular structures are involved in water transport between the apoplast and the vacuole.

Until recently, biophysical investigations into water movement in plants gave no indication of a specific or even a protein-mediated mechanism for water transport other than simple diffusion in response to osmotic gradients (see Steudle and Henzler, 1995, for review). Thus, the discovery of a class of intrinsic membrane proteins that facilitate the passive exchange of water across membranes has evoked great interest among plant physiologists.

The first protein of this type, functionally characterized by heterologous expression in *Xenopus laevis* oocytes, was the erythrocyte protein CHIP28, subsequently named aquaporin1 (Preston et al., 1992). In the meantime, four additional mammalian aquaporins have been identified, one of which is also capable of transporting small solutes such as glycerol and urea in addition to water (see Knepper, 1994, for review; Raina et al., 1995).

The first aquaporin in plants to be identified was γ-TIP of *Arabidopsis thaliana* (Maurel et al., 1993). Subsequently, TIP homologs named PIPs were found to be present in the PM of *A. thaliana* (Daniels et al., 1994; Kammerloher et al., 1994). On the basis of N- and C-terminal sequences, two subfamilies of PM aquaporins in *A. thaliana*, designated PIP1 and PIP2, can be distinguished. At least five PIP1 and six PIP2 genes are expressed in *A. thaliana* (Weig and Chrispeels, 1995). Homologous aquaporins from tobacco (Oppermann et al., 1994) and the common ice plant (Yamada et al., 1995) have been functionally characterized. Thus, aquaporins are a feature common to the membranes of both plant and animal cells.

Aquaporins in the PM of mammalian cells are clearly the cause of the high water permeabilities of erythrocytes and renal epithelia, but they are now known to play important roles in numerous secretory processes, in osmoperception, and in highly active, near-isoosmotic transport situations as well (Knepper, 1994; Raina et al., 1995). By contrast, the physiological role of aquaporins in plants is less well understood. The preferential expression of γ-TIP and one member of PIP1 (Ludevid et al., 1992; Kaldenhoff et al., 1995) in elongating rather than in meristematic cells, however, does suggest that they may be important for cell enlargement by promoting water uptake. In this report, we show that aquaporins in *A. thaliana* mesophyll are found to be concentrated in specific invaginating domains of the PM called plasmalemmasomes. By protruding deep into the vacuole, these structures may allow for a rapid exchange of water with the apoplast.

MATERIALS AND METHODS

Materials

Arabidopsis thaliana ecotype Landsberg *erecta* were grown in soil. Leaves from rosette-stage plants were harvested and fixed for immunocytochemistry or used for PM preparations. Secondary anti-chicken IgY antibodies were obtained from B. Kaspers (Tierärztliche Institut, Universität München, Germany; monoclonal, peroxidase-coupled), from Promega (alkaline phosphatase-coupled), or from Dianova (Hamburg, Germany; 12-nm gold-coupled rabbit anti-chicken IgY [IgG, IgH, IgL]). All other reagents were of the highest purity grade available and were from Sigma, Merck (Darmstadt, Germany), or Boehringer Mannheim.

Purification of PM

PM from *A. thaliana* leaves was prepared by aqueous two-phase partitioning as previously described by Kammerloher et al. (1994). Protein concentrations were determined according to Markwell et al. (1978) using BSA as a standard.

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Abbreviations: GST, glutathione-*S*-transferase; PIP, plasma membrane intrinsic protein; PM, plasma membrane; TIP, tonoplast intrinsic protein.

Recombinant Expression of a PIP1-Specific Epitope

An epitope specific for the hydrophilic N terminus of the *A. thaliana* PIP1 subfamily was expressed as a fusion protein to GST, GST-PIP1a/N42, using pGEX-3X as vector (Pharmacia) (Kammerloher et al., 1994). After lysing isopropyl-thio-galactoside-induced bacteria in the presence of 1% Triton X-100 by sonication, the recombinant protein was purified by affinity chromatography according to Pharmacia. The protein concentration was determined as above.

Protein Separation, Immunoblot, and Quantification

Proteins were separated on 12% SDS polyacrylamide gels. Denaturation was performed for 20 min at 56°C with 4% (w/v) SDS and 100 mM DTT. Polypeptides were electrophoretically transferred to nitrocellulose and bound antibodies were detected with alkaline phosphatase-coupled secondary antibodies. Peroxidase-coupled antibodies were used for chemiluminescence detection (ECL, Amersham). Signals were densitometrically quantified using an Eagle Eye camera system (Stratagene) and the WinCam 2.2 (Cybertech, Berlin, Germany) program. A Triton X-114 separation of leaf PM proteins was performed as described by Kammerloher et al. (1994).

Immunoelectron Microscopy

Leaf segments were prefixed with 0.1% (w/v) paraformaldehyde, 0.05% (v/v) glutaraldehyde in 50 mM potassium phosphate buffer, pH 7.0, at 4°C for 4 h, and then postfixed in phosphate-buffered 0.01% (w/v) OsO_4 at room temperature for 2 h. The segments were washed and immersed in aqueous 0.1% (w/v) uranyl acetate for 1 h at room temperature. These fixation conditions were determined on the basis of a dot-blot screening procedure for assessing antigen stability (Riederer, 1989). Dehydration, embedding, and on-section immunogold staining were carried out by standard procedures (Hoh et al., 1995). Affinity-purified PIP1 antibodies (chicken IgG, 6 ng mL^{-1}) were presented to sections at a dilution of 1:1 to 1:5, and secondary colloidal gold-coupled rabbit anti-chicken IgY antibodies were presented at a dilution of 1:20. Sections were poststained with uranyl acetate and lead citrate before they were observed with a Philips (Eindhoven, The Netherlands) CM 10 electron microscope at 80 kV.

RESULTS AND DISCUSSION

Affinity-purified antibodies against PIP1 aquaporins (Kammerloher et al., 1994) specifically recognized proteins at 26 kD in the PM of *A. thaliana* mesophyll (Fig. 1b). To estimate the relative abundance of these aquaporins in the PM, known amounts of a recombinant PIP1-specific epitope, expressed as a fusion protein to GST, were electrophoresed along with leaf PM proteins and subsequently blotted for immunodetection (Fig. 1b). Densitometric evaluation of the blot indicated that PIP1 represents 1.4% of total leaf PM proteins. This high abundance of PIP1 aquaporins was confirmed in independent experiments using a chemiluminescence detection system as well. Similarly

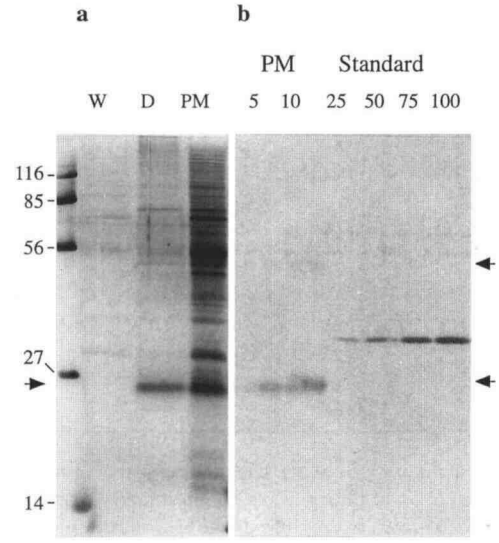

Figure 1. Abundance of PIP1 in leaf PM from *A. thaliana*. a, Silver staining of leaf PM. Whole-leaf PM (4 μg, PM) and aqueous (W) versus detergent phases (D) of Triton X-114-partitioned leaf PM are shown. b, Immunoblot with affinity-purified PIP1 antibodies. On the same gel as in a, 5- and 10-μg leaf PM proteins (PM, 5 and 10) were electrophoresed along with 25, 50, 75, and 100 ng of GST-PIP1/N42 (Standards 25, 50, 75, 100) for comparison. Although the molecular mass of the latter protein (31 kD) was very similar to that of PIP1 (30 kD, indicated by lower arrows), the intrinsic membrane protein electrophoresed with a higher mobility. The faint band at about 50 kD in the PM lane (right upper arrow) is a residual dimeric form of PIP1 (Kammerloher et al., 1994). Molecular masses of markers are indicated on the left (kD).

high values have been recorded for aquaporin1 in total renal cortex membranes and are presumed to be the reason for the high water permeability of these cells (Nielsen et al., 1993b). In accordance with the high proportion of PIP1 aquaporins, a strong silver-staining band was observed at the expected position in an SDS polyacrylamide gel of leaf PM proteins, with its integral membrane character being supported by partitioning into a Triton X-114 detergent phase (Fig. 1a).

Postembedding immunogold labeling confirmed the presence of PIP1 aquaporins at the PM of *A. thaliana* mesophyll (Fig. 2). Labeling was restricted to the PM with virtually no background in other compartments of the cell (Fig. 2a). The density of labeling was quite low, except at convoluted invaginations of the PM, where high concentrations of gold particles were observed (Fig. 2b). These structures have been described on numerous occasions in higher plant cells and have been termed paramural bodies and, more frequently, plasmalemmasomes (e.g. Marchant and Robards, 1968; Nishigawa and Mori, 1977; Willie and Lucas, 1984). Plasmalemmasomes represent one of two possibilities for locally increasing the surface area of the PM. The other is a specialized form of evaginations known as plasmatubules (Harris et al., 1982). Since tubular profiles are occasionally seen in sectioned plasmalemmasomes (e.g. Willie and Lucas, 1984), plasma tubules and plasmalemmasomes are not necessarily mutually exclusive structures and could simply reflect the turgor status of

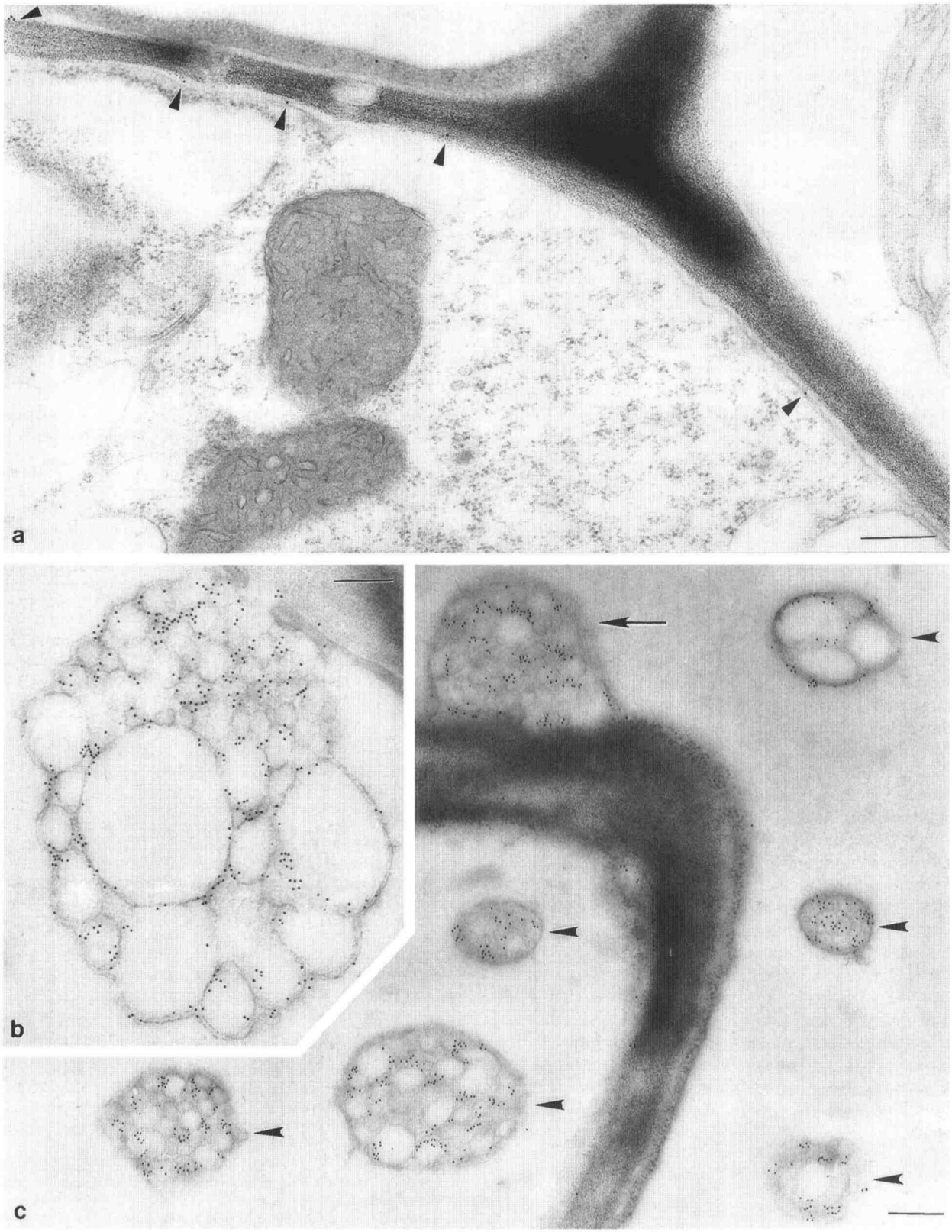

Figure 2. PIP1 immunocytochemistry. a, Specific but relatively low labeling density (indicated by arrowheads) of the PM in *A. thaliana* mesophyll. ×49,000. b, Highly convoluted plasmalemmasome showing intense gold labeling of vesicle membrane. The entire structure protrudes inwardly into the vacuole lumen, bordered by the tonoplast. ×45,000. c, Tangential section showing high and specific labeling of a plasmalemmasome (arrow) and intravacuolar multivesicular bodies (arrowheads). ×33,000. Bars = 0.25 μm.

the cell in question. These structures have been discussed as artifacts of chemical fixation, but their preservation after cryofixation (Chaffey and Harris, 1985) and the specific dense labeling in immunocytochemical studies provide evidence of their genuine nature (Herman and Lamb, 1992; this study).

At those areas of Arabidopsis mesophyll cells where the cytoplasm is only a relatively thin layer, the plasmalemmasome protrudes some distance into the vacuole (Fig. 2b). As a result, PM and tonoplast come into close contact with one another, thus allowing for an accelerated exchange of water from the vacuole to the apoplast and vice versa. When such deeply protruding plasmalemmasomes are sectioned in a plane parallel to the PM, profiles that have been described as "intravacuolar multivesicular bodies" (Herman and Lamb, 1992) are obtained. These, too, are highly labeled with PIP1 antibodies (Fig. 2c). However, in the absence of serial sectioning data we cannot refute the claim of Herman and Lamb (1992) that such profiles represent internalized plasmalemmasomes that are subsequently sequestered and degraded within the vacuole.

Although plasmalemmasomes have been discussed as transitory structures (Harris and Chaffey, 1985), we know little about their formation and fate beyond what was revealed by Herman and Lamb (1992). In this respect it is intriguing to note that aquaporins have been detected at high densities in multivesicular bodies in renal-collecting duct cells (Nielsen et al., 1993a). During vasopressin-regulated changes in water permeability, aquaporins apparently shuttle back and forth from these structures to the PM (Sabolic et al., 1995).

Plasmalemmasomes are often seen in situations where high transport fluxes have been presumed, e.g. at the host-parasite interface in the dwarf mistletoe (Coetzee and Fineran, 1987), in the scutellar epithelia of developing barley embryos (Harris et al., 1982), and in transfer cells of young pea leaves (Harris and Chaffey, 1985, and refs. therein). Analogous structures termed charasomes, which are apparently involved in chloride transport, have also been recorded at the PM in the giant algae *Chara corallina* (Lucas et al., 1986). The high density of PIP1 aquaporins in plasmalemmasomes, as shown here, provides the first molecular clue to the role of these structures in plant transport processes. Clustering of aquaporins may provide the means for achieving a rapid osmotic balance, and therefore, turgor maintenance in mesophyll cells. It is interesting that Höfte et al. (1991) found that a presumptive tonoplastic aquaporin (*Phaseolus* α-TIP) is located in multivesicular bodies in transgenic tobacco that express *Phaseolus* α-TIP. Neither the mechanism responsible for sequestering PIP1 at plasmalemmasomes is known, nor is the benefit of a high local aquaporin concentration instead of a generally increased expression at the plasma membrane apparent.

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