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Expression and distribution of a vacuolar aquaporin in young and mature leaf tissues of *Brassica napus* in relation to water fluxes

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Abstract. Recently, it has been shown that water fluxes across biological membranes occur not only through the lipid bilayer but also through specialized water-conducting proteins, the so called aquaporins. In the present study, we investigated in young and mature leaves of *Brassica napus* L. the expression and localization of a vacuolar aquaporin homologous to radish γ-tonoplast intrinsic protein/vacuolar-membrane integral protein of 23 kDa (TIP/VM 23). In-situ hybridization showed that these tonoplast aquaporins are highly expressed not only in developing but also in mature leaves, which export photosynthates. No substantial differences could be observed between different tissues of young and mature leaves. However, independent of the developmental stage, an immunohistochemical approach revealed that the vacuolar membrane of bundle-sheath cells contained more protein cross-reacting with antibodies raised against radish γ-TIP/VM 23 than the mesophyll cells. The lowest labeling was detected in phloem cells. We compared these results with the distribution of plasma-membrane aquaporins cross-reacting with antibodies detecting a domain conserved among members of the plasma-membrane intrinsic protein 1 (PIP1) subfamily. We observed the same picture as for the vacuolar aquaporins. Furthermore, a high density of gold particles labeling proteins of the PIP1 group could be observed in plasmalemmasomes of the vascular parenchyma. Our results indicate that γ-TIP/VM 23 and PIP1 homologous proteins show a similar expression pattern. Based on these results it is tempting to speculate that bundle-sheath cells play an important role in facilitating water fluxes between the apoplastic and symplastic compartments in close proximity to the vascular tissue.

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Abbreviations: PIP = plasma membrane intrinsic protein; TIP = tonoplast intrinsic protein; VM23 = Vacuolar-membrane integral protein of 23 kDa

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Introduction

Water fluxes in plant leaves are complex. During leaf development, water is imported not only by the xylem but also by the phloem (Schmalstig and Geiger 1985). Part of this water is used for plant growth and taken up by vacuoles, which account for the rapid expansion of young leaf cells. Mature leaves play a central role in ascent of the vascular sap and phloem sap translocation. During daytime, as water evaporates and withdraws into the interstices between mesophyll cells, the water surface develops acute menisci and the hydrostatic pressure becomes more and more negative. Thus, the motive force for vascular sap ascent from the root system to the leaves is generated at the air-water interfaces within leaf parenchyma according to the cohesion theory (Dixon 1914; Milburn 1996). However, when transpiration is low, as it is at night, many herbaceous plants exhibit root pressure and droplets of water may be forced out of the leaves (guttation process).

A part of the water derived from the xylem enters the symplastic compartment due to the osmotic gradient across the plasma membrane. This water is required for metabolism and for phloem transport. The respective roles of water fluxes have been visualized by nuclear magnetic resonance microimaging in castor bean seedlings (Köckenberger et al. 1997). It has been shown that from a vascular sap flux of 38 $\mu L\ h^{-1}$, 5 $\mu L\ h^{-1}$ is used for growth of the shoot of the seedling, 16 $\mu L\ h^{-1}$ is evaporated and 17 $\mu L\ h^{-1}$ is recycled through the phloem. The water transport pathways to the companion cell and sieve tube have not been particularly well studied. However, it is likely that this water transport is species-specific, as already demonstrated for the loading of photosynthetic

products. Symplastic loading of sugars has been shown to occur in a considerable number of plants (Van Bel 1987, 1993; Gamalei 1991). For such plants it is tempting to suggest that water flow also occurs through the symplastic pathway. However, in many annual herbs, mainly in temperate regions, loading of solutes occurs by the apoplastic pathway (Delrot 1987; Bourquin et al. 1990; Gamalei 1991).

Recently, it has been shown that water fluxes across biological membranes occur not only through the lipid bilayer but also through specialized water-conducting proteins, the aquaporins (Maurel 1997). Aquaporins are members of a large gene family and different forms have been identified in vacuolar and plasma membranes (Maurel et al. 1993; Daniels et al. 1994; Kammerloher et al. 1994; Dozolme et al. 1995; Kaldenhoff et al. 1995; Maurel 1997; Schäffner 1998). Different observations indicate that differential expression of aquaporins regulates water fluxes in plants. Yamamoto et al. (1991) showed that TobRB7, a putative plasma-membrane water channel of tobacco, is highly expressed in the meristem and the immature central cylinder of roots. The tonoplast aquaporin γ-tonoplast intrinsic protein (γ-TIP; Ludevid et al. 1992) and AthH2/AtPIP1b (Kaldenhoff et al. 1995), a plasma-membrane aquaporin, are both expressed in young, developing tissue. Most recently, it has been shown that *ZmTIP1* (Barrieu et al. 1998) is expressed in root epidermis, endodermis and conducting tissues. Using a quantitative electron-microscopic approach, Fleurat-Lessard et al. (1997) showed that γ-TIPs homologues are present at much higher amounts in functional than in non-functional pulvini motor cells of *Mimosa*. Interestingly, a higher expression of the vacuolar ATPase was also observed, indicating that increased solute fluxes in the motor cells of *Mimosa* require an enhanced vacuolar energization. This observation suggests that plants can regulate the water permeability by modulating the amount of aquaporins in a membrane. However, for bean α-TIP (but not *Arabidopsis* γ-TIP), which is associated with storage vacuoles, it has additionally been shown that phosphorylation is also implicated in regulation of water fluxes (Maurel et al. 1995).

In this study we were interested to analyze the expression and distribution of a tonoplast aquaporin homologous to radish γ-TIP/VM 23, in young and mature leaves of *Brassica napus*, a plant with an apoplastic loading system. The data were compared with the distribution of a homologous *Arabidopsis thaliana* plasma membrane intrinsic protein 1a (PIP1a) aquaporin in mature leaves.

Materials and methods

Plant material

Brassica napus L. cv. Samourai (Station de Génétique et d'Amélioration des Plantes, INRA, Rennes, France) plants were grown in climate-controlled chambers at 22 ± 1 °C and 65 ± 5% relative humidity with a 16-h photoperiod. For our studies we used leaf number 2 of 4-week-old plants (mature leaf, 7 cm length) and leaf number 2 (young leaf, 0.5 cm length) from 2-week-old plants. Leaves were collected 2 h after the beginning of the photoperiod.

In-situ RNA hybridization

Sense and antisense *VM 23* RNA probes were synthesized by in vitro transcription using digoxigenin-UTP and, respectively, T7 and T3 RNA polymerases, according to the manufacturer's instructions (Boehringer Mannheim). The RNA probes were submitted to partial alkaline hydrolysis for 22 min at 60 °C. In-situ hybridizations were performed essentially according to the method of Fleming et al. (1992). Briefly, leaves (young and mature) of *Brassica napus* L. were fixed in 4% formaldehyde (v/v), 0.25% (v/v) glutaraldehyde in phosphate buffer (pH 7.2). After dehydration in ethanol, the probes were transferred to Histoclear II (Prolabo, Fontenay-sous-Bois, France) and embedded in Paraplast Plus (Labonord, Blonay, Switzerland). Sections (7 μm) were cut and attached to poly-L-lysine-coated slides. The slides were subjected to a prehybridization and treatment with proteinase K, and washed with acetic anhydride. Hybridizations were conducted overnight at 50 °C. The slides were washed twice for 30 min with 0.2 × saline sodium citrate buffer (SSC; 1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 55 °C, twice with NTE buffer (2.5 mM NaCl; 50 mM Tris, pH 8.0; 5 mM EDTA) at 37 °C, once for 30 min in a 20 μg mL^{-1} solution of RNase A in NTE buffer at 37 °C, and finally washed for 60 min at 55 °C in 0.2 × SSC and for 5 min at room temperature in phosphate-buffered saline (PBS) buffer. For immunolocalization of hybridized transcripts, the slides were incubated at room temperature for 40 min, first in 1% blocking reagent (Blocking Reagent; Boehringer) in buffer 1 (0.1 M Tris, 0.15 M NaCl, pH 7.5) and subsequently in buffer 2 (1% BSA, 0.3% Triton X-100 in buffer 1). Subsequently, the slides were incubated for 90 min at room temperature with anti-digoxigenin–alkaline phosphatase conjugate at a 1:1250 dilution in buffer 2. The unbound conjugate was washed four times for 15 min with buffer 2 and 10 min with buffer 3 (0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). For color development, the slides were immersed in 175 μg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 350 μg mL^{-1} nitroblue tetrazolium chloride in buffer 3 for 48 h at room temperature. After development the slides were washed in water, mounted in 50% glycergel (Dako, Carpinteria, Calif., USA) and covered with a coverslip.

Chemical fixation

For immunolocalization, young and mature leaf tissues were cut into 1- or 2-mm-thick pieces and fixed for 25–45 min in a mixture of 1.5% (w/v) paraformaldehyde, 0.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2). Abundant washing (at least six baths during 1 h) in the same buffer was followed by a postfixation of 4 min in 1% (v/v) OsO_4, rapid dehydration in an ethanol series, and overnight embedding in London White Resin (TAAB Laboratories, J Delville St. Germain en Laye, France). Polymerization was allowed to occur in gelatin capsules at 55 °C for 24 h. Semi-thin sections were used to select veins. Thin sections, carefully spread with toluene vapor, were collected on parlodion-coated gold grids.

Antibodies

Polyclonal antibodies against the purified VM 23 of radish were raised in rabbits (Maeshima 1992). This antibody does not recognize α-TIPs and δ-TIPs. A recombinant peptide comprising the first 42 amino acids of *Arabidopsis* AtPIP1a was expressed as a fusion to glutathione-*S*-transferase GST-AtPIP1a-N in *Escherichia*

coli and used to immunize chickens. Specific antibodies were affinity-purified using the same PIP1a-antigen expressed as a fusion protein to maltose-binding protein.

Immunogold staining

The immunogold reaction was performed at 20 °C on thin sections collected on gold grids. Solutions were filtered (Millipore MFVCWP, 0.1-mm pores) or centrifuged (500*g*). The sections were hydrated in deionized water and the next steps were performed in the dark. Sections were etched using 0.56 M $NaIO_4$ and 0.1 N HCl, and subsequently placed for 15 min on PBS, 0.1% (v/v) Triton X-100, 0.2% (v/v) glycine, at pH 7.2. After washing in the PBS-Triton medium, non-specific sites were saturated for 45 min with goat serum in PBS, 0.2% (v/v) Triton X-100, 0.2% (v/v) Tween, 0.1% (w/v) BSA. The slides were incubated overnight with the antibodies against the water-channel proteins VM 23 and PIP1a diluted 1:50 and 1:5, respectively. After washing in PBS, the sections were placed for 40 min on Tris-buffered saline (TBS), pH 8.2, 0.2% (v/v) Tween, 0.2% (v/v) Triton X-100, 1% (w/v) BSA, and goat serum. Secondary antibodies diluted 1:40 (goat anti-rabbit IgG and rabbit anti-chicken IgY) labeled with 15-nm and 18-nm gold particles, respectively, were applied for 2 h. The samples were then washed in TBS and deionized water, and were subsequently treated for contrast in a saturated uranyl acetate solution (8 min) and in lead citrate (3 min).

Two types of control were done: (i) omission of the first antibodies (VM 23 and PIP1a), (ii) saturation of the antibodies with the corresponding purified antigens (VM 23 and GST-AtPIP1a-N).

Observations were made under 80 kV with a 100C Jeol microscope (Tokyo, Japan). Samples for quantification included tissues from five independent chemical fixations for each organ (young and mature leaves). At least eight immunoreactions were performed from five mature leaves and five young leaves.

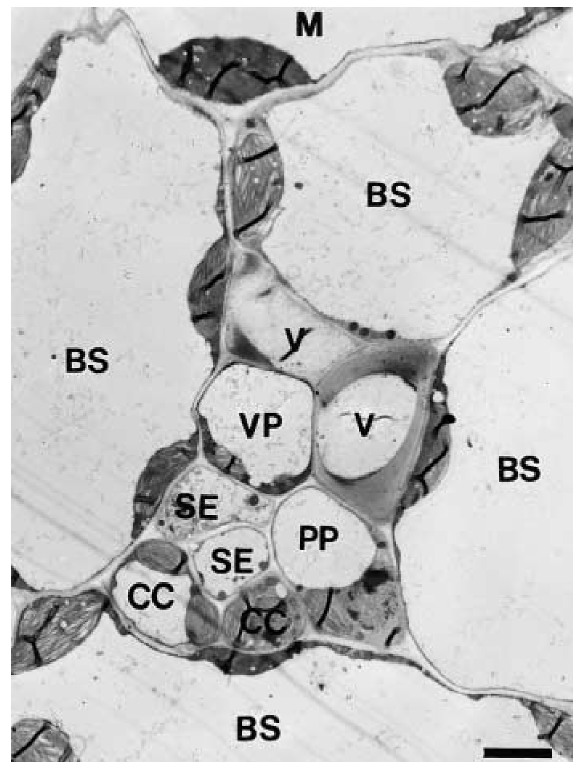

Fig. 1. Structure of a *Brassica napus* vein from a mature leaf. *BS*, bundle sheath; *CC*, companion cell; *M*, mesophyll; *PP*, phloem parenchyma; *SE*, sieve element; *V*, vessel; *VP*, vascular parenchyma. Bar = 4 μm

Results

Structure of the investigated tissues

The spatial relationships between the various cell types of a *Brassica napus* minor vein (Fig. 1) show that these cells are compactly arranged and, due to the absence of intercellular spaces, evaporation is strongly reduced, in contrast to the mesophyll. They are surrounded by the bundle-sheath cells, which contain very large vacuoles. Similar to all the other plants described so far (Van Bel and Gamalei 1991) plasmodesmata linking phloem parenchyma and bundle-sheath cells can be observed in *B. napus*. In the young leaves studied (data not shown), phloem and xylem cells of minor veins were not differentiated, whereas, the bundle-sheath cells were clearly distinguishable.

In-situ hybridization

In order to verify whether a *VM 23* cDNA clone of radish can recognize the corresponding γ-TIP(s) of *B. napus*, we performed a northern blot analysis with leaf number 2 of a 4-week-old plant (data not shown). Indeed, a single band with the correct molecular mass was evident. This result indicates that *γ-TIP/VM 23* homologues exist in *Brassica*. At least one γ-TIP member has been shown to be expressed during germination (Gao et al. 1999). To localize in more detail

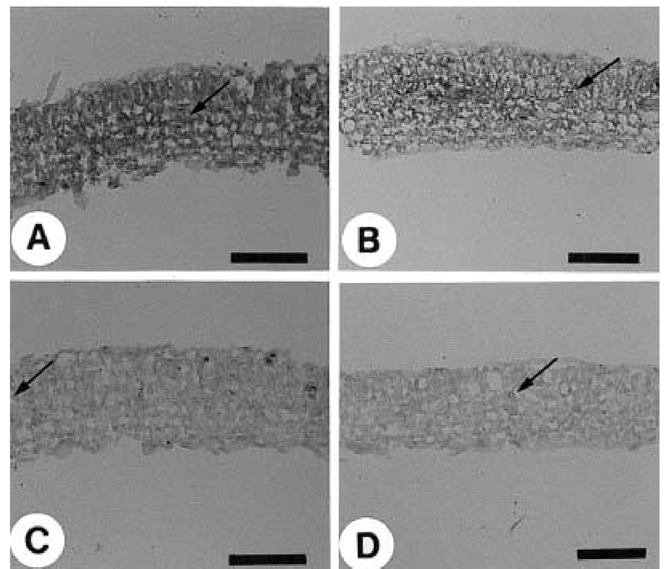

Fig. 2A–D. Localization of γ-TIP VM 23 mRNA by in-situ hybridization. **A,B** Transverse sections of young (**A**) and mature (**B**) leaves hybridized with antisense RNA. The purple color indicates the presence of mRNA corresponding to γ-TIP. **C,D** Controls, showing transverse sections of young (**C**) and mature (**D**) leaves hybridized with sense RNA. Veins are indicated by *black arrowheads*. Bar = 100 μm

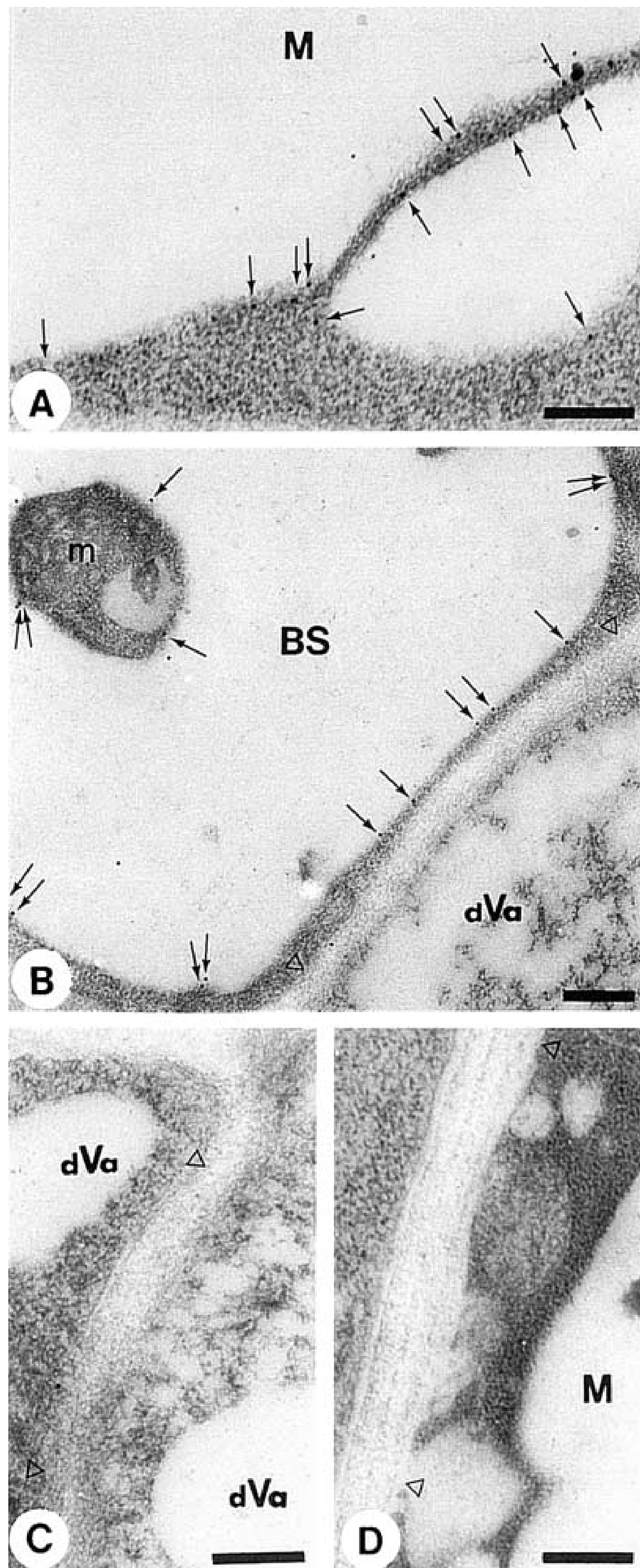

Fig. 3A–D. Distribution of the tonoplast aquaporin in young leaves of *Brassica napus*. **A,B** The immunolabeling for tonoplast aquaporins (*black arrowheads*) is high in the vacuolar membrane of mesophyll (**A**) and bundle-sheath (**B**) cells. **C** The immunolabeling is very low in differentiating vascular cells. **D** Nonspecific labeling using the antibody saturated by the purified VM 23 protein. *BS*, bundle sheath; *dVa*, differentiating vascular cell; *M*, mesophyll; *m*, mitochondrion; Δ, plasma membrane. Bar = 0.5 μm

the expression of this (these) aquaporin(s), we performed in-situ hybridization using digoxigenin-labeled probes.

To determine whether expression in mature leaf sections might be different from that in sections of young leaves, we compared the expression pattern of γ-TIPs in leaf number 2 of 4-week-old plants and leaf number 2 of 2-week-old plants.

In young leaves, thin sections hybridized with the sense probe (control) showed a very low staining, which was due to unspecific labeling (Fig. 2C). In contrast, hybridization of the *VM 23* antisense probe to the same sections (Fig. 2A) resulted in a high intensity of staining in all parts of the leaf. No difference in staining could be observed in epidermal, mesophyll and vein cells, suggesting that expression is similar in the different cell types.

A transverse section of mature tissues hybridized with the sense probe (control) is shown in (Fig. 2D). Hybridization of the *VM 23* antisense probe to a transverse section (Fig. 2B) showed the same intensity of staining as in the young leaves. Mesophyll and vein cells seemed to have the same expression.

Immunogold labeling of γ-TIPs and comparison with localization of PIP1a aquaporins

In-situ hybridizations showed that *VM 23* homologues are highly expressed in young and mature leaves. Therefore, the corresponding gene product seems to exhibit an important function. However, the sensitivity of the in-situ technique does not allow detection of minor changes in expression between the different cells. Moreover, mRNA abundance does not always correlate with the protein content, due to post-transcriptional regulation and different turnover rates. To obtain more insight into the protein content at the cellular level, we performed immunolocalization studies to compare the distribution of the VM 23 aquaporins in different cell types of a leaf (Fig. 1).

To verify the cross-reactivity of antibodies, we performed a Western blot analysis. Immunoblots (data not shown) of microsomes and purified plasmalemma from *B. napus* leaves showed that polypeptides of 24 kDa and 27 kDa (and the corresponding dimer) are specifically recognized by antibodies directed against the radish tonoplast γ-TIP/VM 23 and antibodies directed

Table 1. Determination of the distribution of γ-TIP sites in differentiating young leaves of *Brassica napus* using immunogold labeling. The number of gold particles is given per 10 μm of membrane (mean ± SE). Five independent fixations where made per leaf and 23–90 measurements were performed, each one for a length of 10 μm. The control consisted of VM 23 antibodies saturated with the purified protein

Treatment	Gold particles in		
	Mesophyll	Bundle sheath	Differentiating vascular cells
VM 23 antibodies	9.80 ± 0.14	13.2 ± 0.25	2.22 ± 0.11
Control	0.08 ± 0.03	0.10 ± 0.06	0.00 ± 0.00

against a PIP1-specific epitope derived from *Arabidopsis* AtPIP1a, respectively.

In the case of young leaves, we studied the distribution of aquaporins in minor veins of leaves where xylem and phloem cells were not yet differentiated. Quantification of gold particles revealed that their density differed according to the cell type (Fig. 3). This density was slightly higher in bundle-sheath cells (13.2 particles per 10 μm; Table 1) than in mesophyll cells (9.8 particles per 10 μm). Differentiating vein cells showed a low number of gold particles (2.2 particles per 10 μm). Treatment of the sections with VM 23 antibodies previously incubated in the presence of a 10-fold excess of purified protein, resulted in a very low, nonspecific labeling, indicating that the reaction observed in the presence of VM 23 antibodies alone can be attributed to a specific interac-

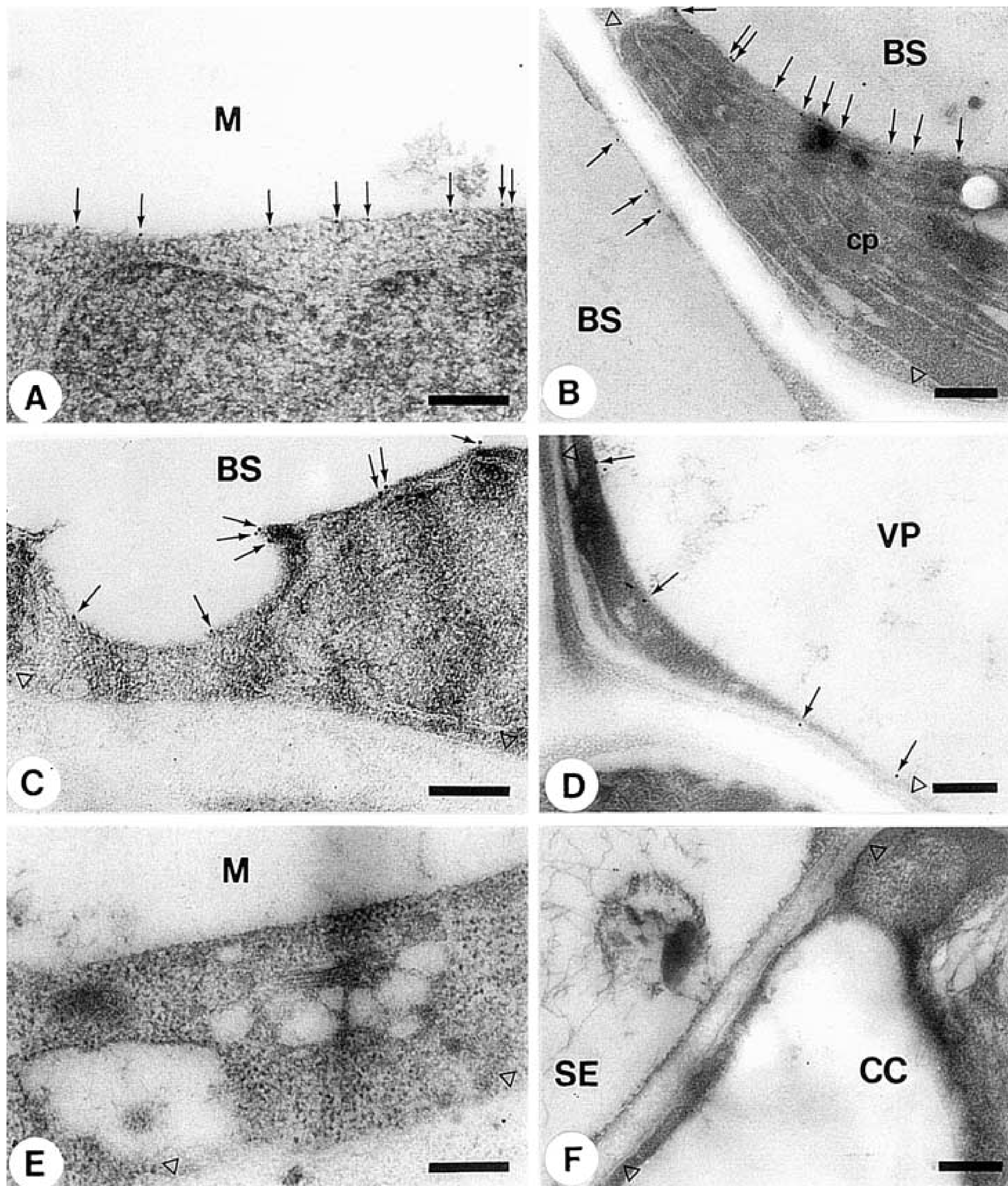

Fig. 4A–F. Distribution of the tonoplast aquaporin in mature leaves of *Brassica napus*. **A–C** The immunolabeling for tonoplast (*black arrowheads*) aquaporin is high in mesophyll (**A**) and bundle-sheath (**B**,**C**) cells. **D**,**E** A low labeling can be observed in vascular parenchyma cells (**D**) and very low labeling in phloem cells (**F**). **E** Nonspecific labeling using the antibody saturated by the purified VM 23 protein. *BS*, bundle sheath; *CC*, companion cell; *cp*, chloroplast; *M*, mesophyll; *SE*, sieve element; *VP*, vascular parenchyma; Δ, plasma membrane. Bar = 0.5 μm

Table 2. Determination of the distribution of γ-TIP sites in mature leaves of *Brassica napus* using immunogold labeling. The number of gold particles is given per 10 μm of membrane (mean ± SE). Five independent fixations where made per leaf and 23–110 measurements were performed, each one for a length of 10 μm. The control consisted of VM 23 antibodies saturated with the purified protein

Treatment	Gold particles in				
	Mesophyll	Bundle sheath	Vascular parenchyma	Phloem parenchyma	Companion cells
VM 23 antibodies	7.64 ± 0.44	14.81 ± 0.35	3.84 ± 0.31	1.10 ± 0.22	1.20 ± 0.22
Control	0.10 ± 0.02	0.05 ± 0.04	0.09 ± 0.01	0.00 ± 0.00	0.00 ± 0.00

Table 3. Determination of the distribution of PIP1 sites in mature leaves of *Brassica napus* using immunogold labeling. The number of gold particles was given per 10 μm of membrane (mean ± SE). Five independent fixations where made per leaf and 23–110 measurements were performed, each one for a length of 10 μm. The control consisted of an antibodies PIP1 saturated with the purified protein

Treatment	Gold particles in					
	Mesophyll	Bundle sheath	Vascular parenchyma	Phloem parenchyma	Companion cells	Sieve tube
PIP1 antibodies	3.36 ± 0.44	4.16 ± 0.35	3.24 ± 0.44	1.20 ± 0.18	1.20 ± 0.18	0.88 ± 0.04
Control	0.09 ± 0.04	0.10 ± 0.05	0.09 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

tion of VM 23 antibodies with the corresponding protein in vacuolar membranes (Fig. 3D; Table 1).

In the case of mature leaves (Fig. 4), the density of sites in bundle sheath cells was 2-fold higher than in mesophyll cells (Table 2), 4-fold higher than in the vascular parenchyma cells and 14-fold higher than in phloem cells (phloem parenchyma and companion cells). The distribution of sites corresponding to γ-TIP aquaporin in the different cell types as well as their amount were therefore similar in young and mature leaves, a result which corresponds to our in-situ hybridization analysis.

In order to compare our data on γ-TIP-like aquaporin distribution with one class of plasma-membrane aquaporins, we quantified members of the PIP1 subfamily in mature leaves of *B. napus* (Table 3). A similar relative distribution of gold particles could be detected for PIP1- and γ-TIP aquaporins (Fig. 5; Table 3). Sections treated with PIP1a antibody exhibited a higher density of gold particles in the bundle sheath, the mesophyll and the vascular parenchyma cells. However, labeling was more uniform (Fig. 5; Table 3). In vascular parenchyma cells, a high labeling of plasmalemmasomes (Fig. 5D) could be observed. A similar observation was reported for *Arabidopsis* mesophyll by Robinson et al. (1996). Phloem cells showed a low density of labeling, particularly in the sieve tubes (Fig. 5E).

Discussion

Although lipid bilayers exhibit a rather high basal water permeability, biological membranes have been shown to harbor specialized water-conducting proteins or aquaporins. Such aquaporins can be localized in the plasma (PIPs) and vacuolar (TIPs) membranes. In both cases, multigene families can be subdivided into subclasses by distinct amino acid signature motifs, α-TIP, γ-TIP, and δ-TIP in the tonoplast and PIP1 and PIP2 in the plasmalemma (Maurel 1997; Schäffner 1998). The expression of some aquaporins is organ- and tissue-specific whereas others are induced by environmental conditions (Ludevid et al. 1992; Daniels et al. 1994; Maurel 1997; Sarda et al. 1997; Weig et al. 1997; Barrieu et al. 1998). In the case of TIPs it has been shown that γ-TIPs are associated with the lytic vacuole, α-TIPs with the storage vacuoles and δ-TIPs localized on different types of vacuole (Paris et al. 1996; Jauh et al. 1998). In adult cells, the vacuole occupies more than 80% of the cell volume, constituting a large water reserve. A high water permeability of the tonoplast may help to facilitate water fluxes. Nevertheless, whole-cell water resistance is dependent on the permeability of the plasmalemma, which may be enhanced or regulated by PIPs. Only a few publications, so far, have reported on the cellular localization of PIPs; Kaldenhoff et al. (1995) demonstrated a ubiquitous expression of AtH2/AtPIP1b, mostly in differentiating cells.

It has been shown that fast-growing tissues exhibit a higher level of aquaporins than slow-growing tissues (Ludevid et al. 1992). Specialized cell types exhibiting slow movements like stomata (Kaldenhoff et al. 1995; Sarda et al. 1997) or rapid movements like motor cells of pulvini (Fleurat-Lessard et al. 1997) exhibit a high expression of PIPs, δ-TIP or γ-TIP. Electron-microscopical techniques in combination with immunolocalization and quantification of the gold particles permit comparison of the abundance of recognized membrane proteins in different tissues. Based on our in-situ hybridization analysis, no consistent difference in the expression of γ-TIP homologues could be observed in the different cell types or between leaves of different ages. This result is surprising since Ludevid et al. (1992) showed a higher expression in young leaves of *Arabidopsis* for an individual γ-TIP by promoter reporter analysis. However, the highest expression signals reported by these

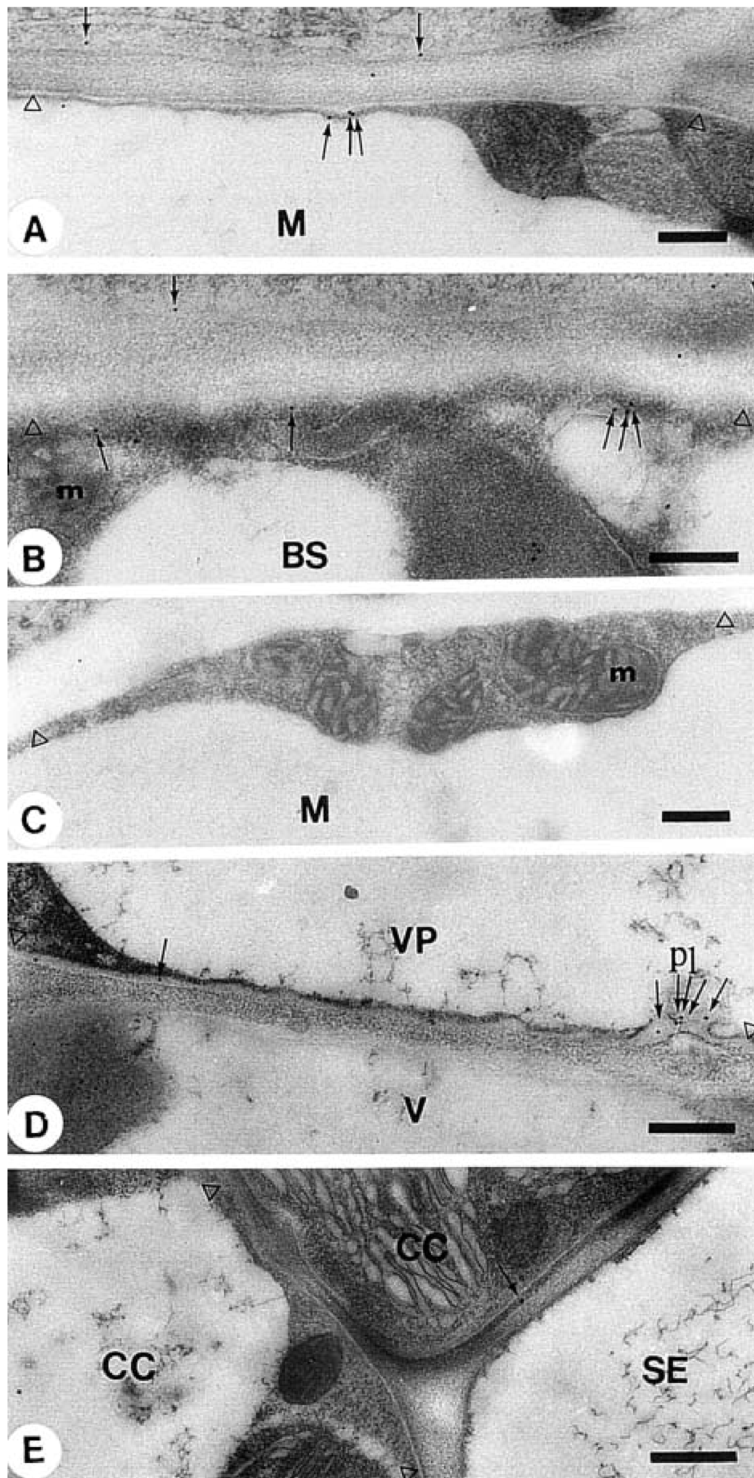

Fig. 5A–E. Distribution of the plasma-membrane aquaporin PIP1 in mature leaves of *Brassica napus*. **A,B** The immunolabeling (*black arrowheads*) is high in mesophyll (**A**) and bundle-sheath (**B**) cells. **C** Nonspecific labeling using the antibody saturated by the purified PIP1 protein. **D** High labeling is present in a plasmalemmasome of the vascular parenchyma.
E There is a very low labeling in phloem cells. *BS*, bundle sheath; *CC*, companion cell; *M*, mesophyll; *m*, mitochondrion; *Pl*, plasmalemmasome; *SE*, sieve element; *VP*, vascular parenchyma; *V*, vessel; Δ, plasma membrane. Bar = 0.5 μm

authors were in hypocotyls and the elongation zone of roots, not in young leaves. Since in our experiments similar results were obtained by northern blot analysis, in-situ hybridizations and immunocytochemistry, we conclude that in *Brassica* the abundance of γ-TIP homologues in young and adult leaves is similar. However, it should be mentioned that a similar picture for in-situ hybridization and immunolocalization may not necessarily be expected for genes and their products, depending on the specificities of either the hybridization probe or the antibodies. Thus, a slightly different picture than for *Brassica* was observed in *Arabidopsis* where apparently a higher density of gold particles could be observed in young leaves (data not shown). Using in-situ hybridization, Barrieu et al. (1998) showed that in *Zea mays* the highest expression of *ZmTIP1*, a maize γ-TIP, was mainly found in rootlets. This aquaporin is mainly expressed in the rhizodermis, endodermis and pericycle. At the electron-microscope level, differences in γ-TIP homologue contents were also detectable in leaves of

B. napus. Bundle-sheath cells contained the highest density of γ-TIP sites while phloem parenchyma cells almost lacked aquaporins cross-reacting with the VM 23 antibody. A similar pattern of distribution could be observed for PIP1a sites in the plasma membrane although the ubiquitous expression was more uniform than γ-TIP homologue distribution (Tables 2, 3). Whether the lower amount of gold particles detected for PIP1 compared with γ-TIP homologues indicates a lower abundance of the plasma-membrane aquaporin can not be deduced from our results since the cross-reactivity of the two antibodies may differ. Our observations indicate that the tissue exhibiting the highest content of γ-TIP and PIP1a sites in leaves (bundle-sheath) corresponds to that in the roots (endodermis/pericycle). It is interesting that a similar distribution has also been reported for soybean nodules (Serraj et al. 1998).

Based on these results it is tempting to speculate that water flux is facilitated mainly at the level of the bundle-sheath and not directly at the phloem cells. The strategic position and the large size of these cells suggest that they may play a role as a water reserve in mature leaves. The fact that the ratio between γ-TIP homologue sites observed in bundle-sheath and mesophyll cells increases when the volume of the bundle-sheath cells gets larger during leaf expansion, may be further support this hypothesis. However, in our study we have investigated only one subclass each of tonoplast and plasma-membrane aquaporin and it cannot be excluded that other aquaporins exhibit a different expression pattern and, thus, play potentially different roles. Different types of vacuole have been identified and it was shown that γ and δ-TIPs may reside on the same vacuole (Jauh et al. 1999). On the other hand, we could not detect α-TIPs in *Brassica* leaves (data not shown), indicating that this aquaporin does not play a role in water fluxes in leaf cells. The aquaporins PIP1 and PIP2, each subfamily encoded by several members e.g. in *Arabidopsis* (Weig et al. 1997), may control whole-cell water flux at the plasma membrane. The antibodies used in this study detected PIP1 subfamily members of *B. napus* as in other species, like e.g. *Lotus japonicus* (Henzler et al. 1999) since they had been raised against a recombinant peptide representing the conserved PIP1 N-terminal amino acid sequence. Members of the PIP2 subclass will not be detected by these antibodies, and further studies with other aquaporins have to be performed in order to be able to establish a complete picture of the role of aquaporins in leaf tissues. To establish a model which takes into account the different factors, the distribution of plasmodesmata should be included. In *Brassica* leaves, many plasmodesmata are present between the phloem parenchyma and the bundle-sheath cells (data not shown), indicating that water fluxes between these cells are facilitated by both symplastic connections and aquaporins. Finally, plasmalemmasomes containing a high density of PIP1a homologues could be detected in *Brassica*. While Robinson et al. (1996) found these structures in the mesophyll, we observed them in the vascular parenchyma. It is well known that in renal tubules, the aquaporin density in the plasma membrane can be modulated by fusion and retraction (Nielsen et al. 1993; Ward et al. 1999). The observed putative aquaporins in plasmalemmasomes indicate that such a mechanism could also occur in plants. However, presently we are unable to speculate what conditions are required for such a fast regulation to take place.

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