Lentil seed aquaporins form a hetero-oligomer which is phosphorylated by a Mg^{2+}-dependent and Ca^{2+}-regulated kinase

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In plants, aquaporins regulate the water flow through membranes during growth, development and stress responses. We have isolated two isoforms of the aquaporin family from the protein-storage vacuoles of lentil (*Lens culinaris* Med.) seeds. Chemical cross-linking experiments showed that both isoforms belong to the same oligomer in the membrane and are phosphorylated by a membrane-bound protein kinase. We assigned the kinase activity to a 52 kDa protein that is magnesium-dependent and calcium-regulated.

Key words: calcium-dependent protein kinase, cross-linking, protein body, membrane, water.

INTRODUCTION

Aquaporins belong to the major integral protein (MIP) superfamily of transmembrane channel proteins [1]. Aquaporin-1 (AQP-1) from red blood cells and renal proximal tubules has been characterized extensively, and structural studies have led to a common structural model for all members of the aquaporin family: aquaporins exhibit at least six closely associated α-helices spanning the lipid membrane [2–4]. The loops located between helices 2 and 3 and between helices 5 and 6 each include a highly conserved Asn-Pro-Ala motif that could be essential for the protein's activity. It is likely that this motif lines the water-permeation path throughout the protein [5–7].

Plant aquaporins have been identified in both plasmalemma and tonoplast [8]. In plants, aquaporins are thought to regulate the water flow through membranes during growth, development and stress responses [7]. Actually, the pattern of gene expression is highly regulated by developmental or physiological programmes [8]. For instance, specific isoforms are expressed in seeds [9], in elongating zones of roots and shoots [10] and in vascular tissues of shoots and roots [11].

During seed maturation, prior to desiccation, storage proteins synthesized in the endoplasmic reticulum are sequestered in small spherical organelles (protein-storage vacuoles or PSVs) of parenchyma cells of both developing cotyledons and endosperm [12]. These proteins provide the nitrogen required for the seedling growth during the early stage of germination, as the seed rehydrates. Exchanges of water and solutes across the tonoplast of PSVs are required in order to make these nitrogen reserves available for the cellular metabolism [13]. It has been shown that PSV membranes of *Phaseolus* seeds contained one MIP named α-TIP (tonoplast intrinsic protein) [14–16]. α-TIP is a member of the aquaporin protein family [15]. Early studies on native tonoplasts have shown that α-TIP is phosphorylated by an unidentified calcium-dependent membrane-bound protein kinase [17]. Heterologous expression of a site-specific mutant of α-TIP in *Xenopus* oocytes has shown that phosphorylation is required to activate water transport [18].

Two closely related TIP cDNAs have been cloned from bean [9] and *Arabidopsis* seeds [10]. They were called α- and β-TIP respectively, suggesting that at least two TIPs are expressed in plant seeds. However, these two TIPs have not been purified so far. Moreover, β-TIP has not yet been identified in a tissue. Therefore, it is not known if these two homologous proteins are expressed simultaneously and targeted to the same organelles.

In this work, we isolated two TIP isoforms from the PSV membrane of *Lens culinaris* seeds. Chemical cross-linking experiments revealed that lentil TIP isoforms exist as oligomers in the membrane. We identified a 52 kDa magnesium-dependent protein kinase capable of phosphorylating each isoform.

EXPERIMENTAL

Purification of the PSV membranes from lentil seeds

Membranes of PSVs from lentil (*L. culinaris* Med.) seeds that had imbibed for 18 h in water were prepared as described by Mäder and Chrispeels [15], except that the final membrane pellet was resuspended in distilled water at a protein concentration of 5 mg/ml and stored at −70 °C. Protein concentration was determined using the BCA kit (Pierce).

TIP enrichment of PSV membranes

PSV membranes were incubated at 4 °C for 1 h in the presence of deoxycholate (DOC) at a detergent/protein ratio of 2 (w/w) and then centrifuged for 1 h at 150000 ***g*** (SW-60 rotor, Beckman L7 ultracentrifuge). This step removed most membrane proteins except TIPs. The pellet was resuspended in 25 mM Tris, pH 8.5, with 150 mM NaCl and stored at −70 °C.

Computation of free ion concentration

Concentrations of free metal ions were calculated with the Solcon software developed by D. C. S. White (University of York, York, U.K.) and Dr Y. E. Goldman (University of

Abbreviations used: MIP, major integral protein; PSV, protein-storage vacuole; TIP, tonoplast intrinsic protein; AQP-1, aquaporin-1; DOC, deoxycholate; DTSP, dithiobis-succinimidylpropionate; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; SUV, small unilamellar vesicle; W-7, *N*-(6-aminohexyl)-5-chloropentene-1-sulphonamide hydrochloride; CDPK, calcium-dependent protein kinase.

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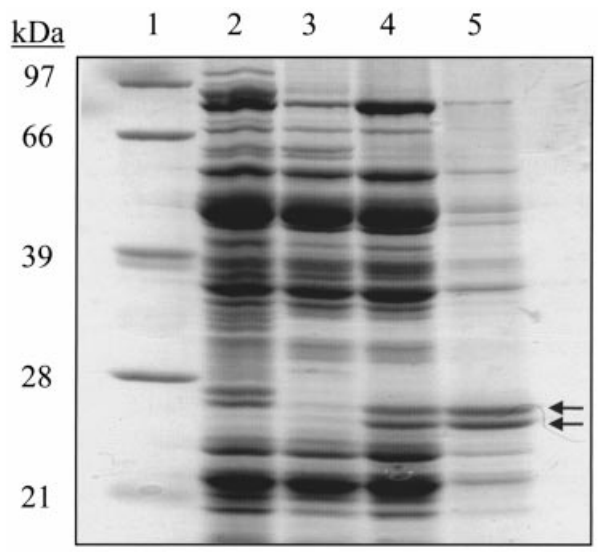

Figure 1 Analysis by SDS/PAGE of polypeptides associated with PSVs and PSV membrane of *L. culinaris*

Lanes 1–5 (in that order): molecular-mass markers, total seed fraction, supernatant after osmotic disruption of the PSVs, PSV membrane and PSV membrane after DOC treatment.

Pennsylvania, Philadelphia, PA, U.S.A.). The following dissociation constants (pK_m values) were used: $[H^+][Mes]/[H.Mes]$, 6.1; $[Ca^{2+}][Mes]/[Ca.Mes]$, 0.7; $[Mg^{2+}][Mes]/[Mg.Mes]$, 0.8; $[H^+][EGTA]/[H.EGTA]$, 9.47; $[H^+]^2[EGTA]/[H_2.EGTA]$, 8.85; $[H^+]^3[EGTA]/[H_3.EGTA]$, 2.66; $[H^+]^4[EGTA]/[H_4.EGTA]$, 2; $[K^+][EGTA]/[K.EGTA]$, 0.96; $[Na^+][EGTA]/[Na.EGTA]$, 1.66; $[Mg^{2+}][EGTA]/[Mg.EGTA]$, 5.209; $[Mg^{2+}][H^+][EGTA]/[Mg.H.EGTA]$, 12.921; $[Ca^{2+}][EGTA]/[Ca.EGTA]$, 9.47; $[Ca^{2+}][H^+][EGTA]/[Ca.H.EGTA]$, 9.47; $[H^+][ATP]/[H.ATP]$, 6.536; $[H^+]^2[ATP]/[H_2.ATP]$, 4.076; $[K^+][ATP]/[K.ATP]$, 0.903; $[Na^+][ATP]/[Na.ATP]$, 1.2; $[Mg^{2+}][ATP]/[Mg.ATP]$, 4.516; $[Mg^{2+}][H][ATP]/[Mg.H.ATP]$, 8.715; $[Ca^{2+}][ATP]/[Ca.ATP]$, 3.994; and $[Ca^{2+}][H][ATP]/[Ca.H.ATP]$, 8.731.

Phosphorylation assay *in vitro*

Unless otherwise stated, the standard reaction mixture contained: 50 mM Mes (final pH 6.5), 5 mM dithiothreitol, 36 μM ATP (including 10 μCi of [γ-^{32}P]ATP), 0.2 mM EGTA, 10 mM $MgCl_2$, $CaCl_2$ to yield 5 μM free Ca^{2+} and 50 μg of PSV-membrane proteins in a total volume of 40 μl. The reaction was started by addition of ATP, and the sample was mixed and incubated for 30 min at 30 °C. The reaction was stopped by addition of 10 μl of 4-fold-concentrated SDS sample buffer (60 mM Tris, pH 6.8, 2% SDS, 7.5% glycerol, 5% β-mercaptoethanol and 0.06% Bromophenol Blue). In experiments carried out at different pHs, Mes was replaced by Hepes (pH 7 and 7.5), Tricine (pH 8 and 8.5), BTP {1,3-bis[Tris(hydroxymethyl)-methylamino]propane; pH 9 and 9.5} or Caps [3-(cyclohexylamino)propane-1-sulphonic acid; pH 10].

SDS/PAGE, autoradiography and radioactivity quantification

Protein separation was carried out on 10.5% polyacrylamide gels according to the method of Laemmli [19] with a constant current of 22 mA at room temperature. Polypeptides were further stained with Coomassie Brilliant Blue [20]. After staining, the gel was dried and sealed between two plastic sheets. Autoradiography was carried out by exposing the sealed gel to Kodak X-OMAT AR film at −70 °C for 16 h. To quantify the radioactivity associated with each protein, the corresponding bands were cut out of the gel and incubated in 5 ml of scintillation liquid (Ecolite, ICN) for 1 h. Radiolabelling intensity was measured using a liquid-scintillation counter (Rackbeta, LKB).

In-gel protein kinase assay

The protein kinase activity in the gel was detected using the protocol of Kameshita and Fujisawa [21] with a few modifications: the pH of the 10% running gel was decreased to 7.0 and the SDS concentration was reduced by 30%. Either 0.8 mg/ml calf histone H1 or 1 mg/ml PSV membrane incubated at pH 12 to remove any residual phosphatase activity was added to the gel solution before polymerization. After protein kinase reactivation, the gel was incubated for 2 h in 10 ml of the reaction buffer: 50 mM Mes (pH 6.2), 25 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM EGTA, 10 μM ATP (containing 4 μCi of [γ-^{32}P]ATP) and 5 μM Ca^{2+}. The reaction was stopped by extensive washing in a solution of 5% (w/v) trichloroacetic acid and 1% (w/v) $Na_4P_2O_7$, 10 H_2O to remove the unreacted ATP. The gel was autoradiographed using Kodak X-OMAT AR film at room temperature for 24 h.

Western blotting and microsequencing

Proteins were transferred from a 12% polyacrylamide gel to a PVDF membrane by electroblotting in semi-dry conditions (Trans-Blot SD, Bio-Rad) at 22 V for 1 h. The membrane was then stained with Coomassie Brilliant Blue and dried. The protein bands on the PVDF membrane were excised, and the protein N-terminal amino acid sequences were determined at the pmol level by automated Edman degradation using a Beckman LF3400D protein-peptide microsequencer equipped with an on-line model 126 Gold system microgradient HPLC and a model 168 diode-array detector (Beckman Instruments, Palto Alto, CA, U.S.A.). All sequencing reagents were from Beckman. For internal sequencing, samples were cleaved *in situ* with CNBr and peptides were purified by capillary reversed-phase HPLC [22].

Chemical cross-linking

TIP-enriched membranes were diluted to a final concentration of 0.5 mg/ml with 50 mM Hepes, pH 8.5, 100 mM KCl and 20 mM EDTA in a total volume of 300 μl. Some glutaraldehyde stock solution [2.5% (v/v), 15 μl] was added to the mixture and the reaction was allowed to proceed at room temperature for time intervals ranging from 1 s to 16 h. The reaction was stopped by addition of 4-fold-concentrated SDS sample buffer and freezing in liquid nitrogen. A similar protocol was used with two other cross-linkers: DTSP (dithiobis-succinimidylpropionate) solubilized in DMSO and DFDNB (1,5-difluoro-2,4-dinitrobenzene) solubilized in methanol.

Cleavage of DTSP-treated proteins

Cross-linked proteins were resolved by SDS/PAGE (12% gel) in the absence of β-mercaptoethanol in the sample buffer. Proteins were revealed by reverse staining [23], and bands corresponding to monomers, dimers, trimers and tetramers were cut out of the gel. Gel slides were then incubated at room temperature for 1 h in sample buffer containing β-mercaptoethanol. Gel slides were

then deposited in the wells of an SDS/12% polyacrylamide gel and proteins bands were revealed by silver staining.

Chemical cross-linking on small unilamellar vesicles (SUVs) containing PSV-membrane proteins

Azolectin (150 mg; Sigma) dissolved in 3 ml of chloroform was dried as a thin film under N_2. The lipid film was then vacuum-dried overnight to remove residual traces of solvent. SUVs were formed at room temperature by dispersion of the thin film in 3 ml of buffer (50 mM Hepes, pH 8.5, 100 mM KCl and 20 mM EDTA) by sonication. Proteoliposomes were formed after rapid mixing of the SUV sample with the TIP-enriched membranes by two cycles of freeze–thawing. Volumes of the SUV and protein samples were adjusted to increase the lipid/protein ratio by 10- and 100-fold. Cross-linking was carried out with glutaraldehyde (see above for details) and proteins were resolved by SDS/PAGE.

RESULTS

Identification of two TIP isoforms in lentil PSV membrane

Most of the polypeptides contained in *Lens* seeds were storage proteins of PSVs (Figure 1, lanes 2 and 3). Although a large fraction of soluble protein was associated with the membrane, two proteins (arrows) were present in the membrane fraction only (Figure 1, lane 4). The membrane fraction obtained after solubilization of the peripheral proteins with DOC at a 2:1 (w/w) detergent/protein ratio (Figure 1, lane 5) was significantly enriched in the two proteins. They accounted for 55% of the membrane proteins and for about 5% of the total PSV-membrane proteins (before the DOC treatment), as estimated by scanner densitometry of SDS/polyacrylamide gels stained with Coomassie Brilliant Blue. The N-terminal sequence of the 25 kDa protein (PRRAYGFGRADEATH) shared 87% identity with β-TIP of *Arabidopsis thaliana* and 60% identity with α-TIP of *Phaseolus vulgaris* [16]. The N-terminal end of the 26 kDa polypeptide was blocked, and an internal sequence (ATVA-EFASTAIFLFAGEGS) was determined after trypsinolysis. It shared significant amino acid identity with both *Arabidopsis* β-TIP (73%) and *Phaseolus* α-TIP (78%). Bands of 25 and 26 kDa were blotted on to a PVDF membrane and cleaved with CNBr. Comparison of the sequences of five internal peptides revealed a high sequence similarity between the two proteins (Figure 2, boxes III–VII). Such strong similarity has already been described in the case of *Phaseolus* α- and β-TIP cDNAs, which displayed 75% sequence identity [16].

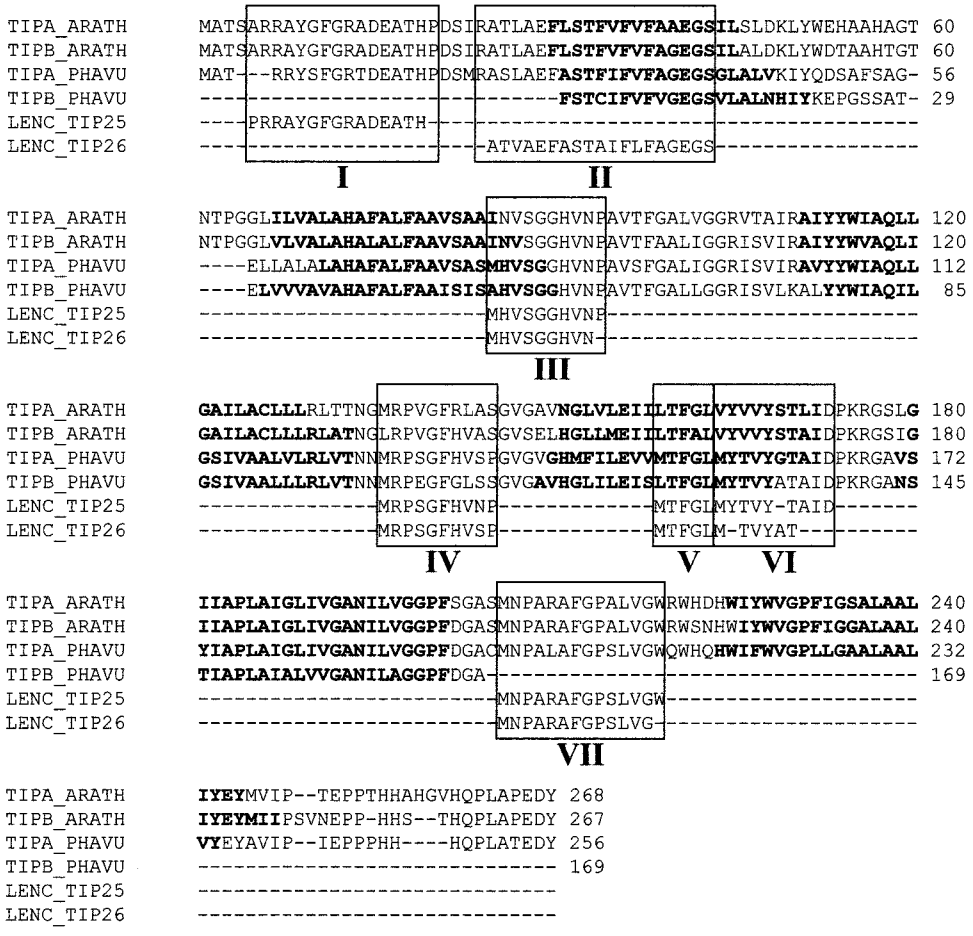

Figure 2 Sequence comparison of TIPs from *Arabidopsis*, *Phaseolus* and *Lens*

Sequence alignment and comparison (boxed residues) between sequences from *L. culinaris* TIPs (LENC-TIP25 and LENC-TIP26), from *A. thaliana* (TIPA-ARATH and TIPB-ARATH) and *P. vulgaris* (TIPA-PHAVU and TIPB-PHAVU) cDNA. Residues in box I correspond to the N-terminal sequence of the 25 kDa TIP, residues in box II to an internal sequence obtained after trypsinolysis of the 26 kDa TIP, and residues in boxes III–VII to peptides obtained after cleavage of each of the *Lens* TIPs with CNBr. Dashes indicate gaps introduced to maximize sequence identity and bold residues represent predicted transmembrane regions.

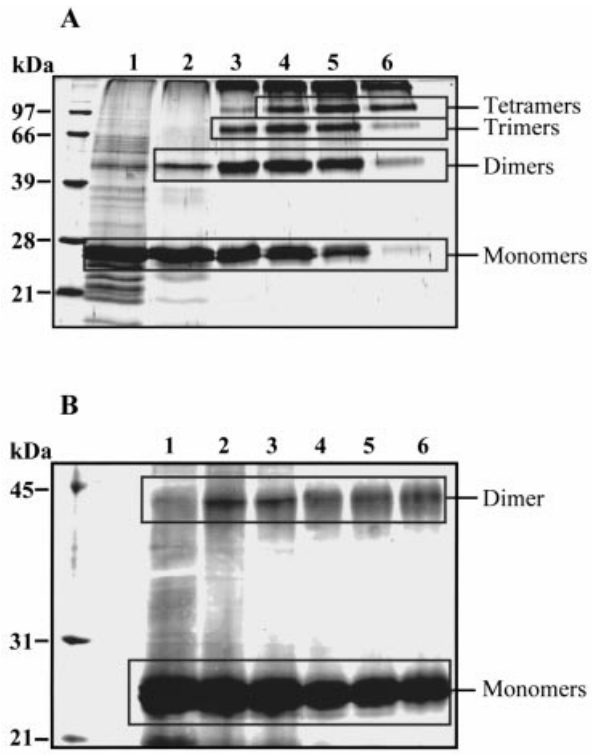

Figure 3 SDS/PAGE analysis of the DOC-treated PSV membranes after cross-linking with glutaraldehyde

TIP proteins were cross-linked in the presence of either 0.125 (**A**) or 0.03% (v/v; **B**) glutaraldehyde. (**A**) Lane 1, DOC-treated PSV membrane before cross-linking ; lanes 2–6, cross-linking of TIP proteins after incubation with glutaraldehyde for 10 s, 10 min, 1 h, 3 h and 16 h respectively. (**B**) Lane 1, DOC-treated PSV membrane before addition of glutaraldehyde; lanes 2–6, incubation of protein sample with glutaraldehyde for 10 s, 30 s, 1 min, 10 min and 30 min respectively.

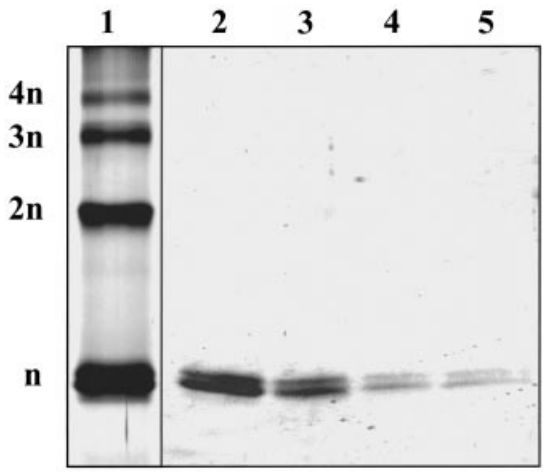

Figure 5 Composition of the multimeric aggregates

DOC-treated PSV membranes were cross-linked with DTSP in the absence of β-mercaptoethanol and analysed by SDS/PAGE. Dimers (2n), trimers (3n) and tetramers (4n) were cut out of the gel and incubated for 1 h in the presence of β-mercaptoethanol. Each gel slide was loaded on an SDS/12% polyacrylamide gel. Lane 1, oligomers formed after cross-linking with DTSP. Lanes 2–5, monomers released from samples of n (lane 2), 2n (lane 3), 3n (lane 4) and 4n (lane 5) after cleavage of the cross-linking agent.

Chemical cross-linking

The recent cloning, sequencing and functional expression of *Phaseolus* α-TIP show that this protein is very similar to AQP-1, which is now reasonably well characterized [24]. AQP-1 is known to form tetramers in the membrane, even though the monomer can function as an aquaporin [25]. Cross-linking experiments were performed in order to show that close physical association occurs between different TIP molecules within the PSV membranes. DTSP, DFDNB and glutaraldehyde were used as cross-linkers. TIP-enriched membranes were incubated in 0.125% (v/v) glutaraldehyde for different periods of time. SDS/PAGE revealed that as incubation time increased, both 25 and 26 kDa proteins disappeared with a concomitant production of higher-molecular-mass bands (Figure 3A). The presence of a dimer is observed after only 10 s of incubation. Oligomers larger than tetramers did not migrate on an SDS/12% polyacrylamide gel. To increase the gel resolution in the region of the dimer, proteins

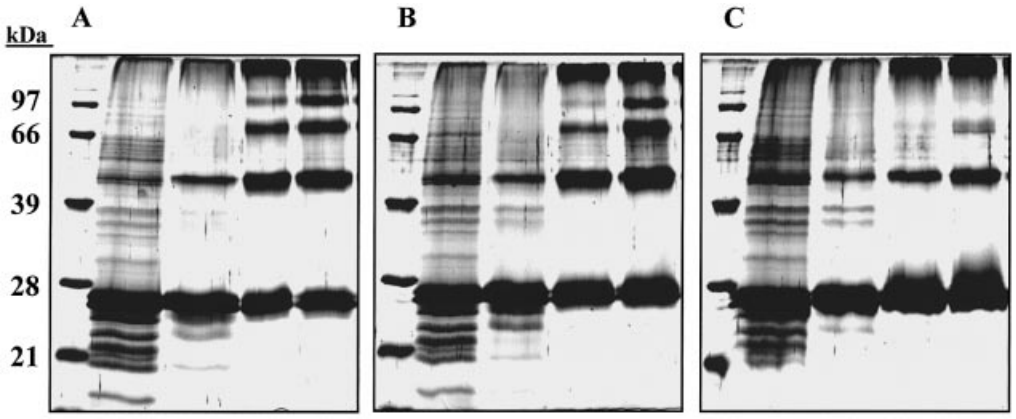

Figure 4 Time course of cross-linking of TIP proteins inserted into SUVs

PSV membranes treated with DOC were fused with SUVs. Proteoliposomes were incubated with 0.125% (v/v) glutaraldehyde for increasing times and proteins were resolved on SDS/PAGE. (**A**) Control experiment performed on the DOC-treated membrane without added lipid. Proteins and lipid amounts were adjusted to increase the lipid/protein ratio by 10- (**B**) or 100-fold (**C**). For each panel, reading left to right, the first lane represents molecular-mass markers; the next lanes represent incubation of protein samples with glutaraldehyde for 10 s, 10 min, 1 h and 3 h respectively.

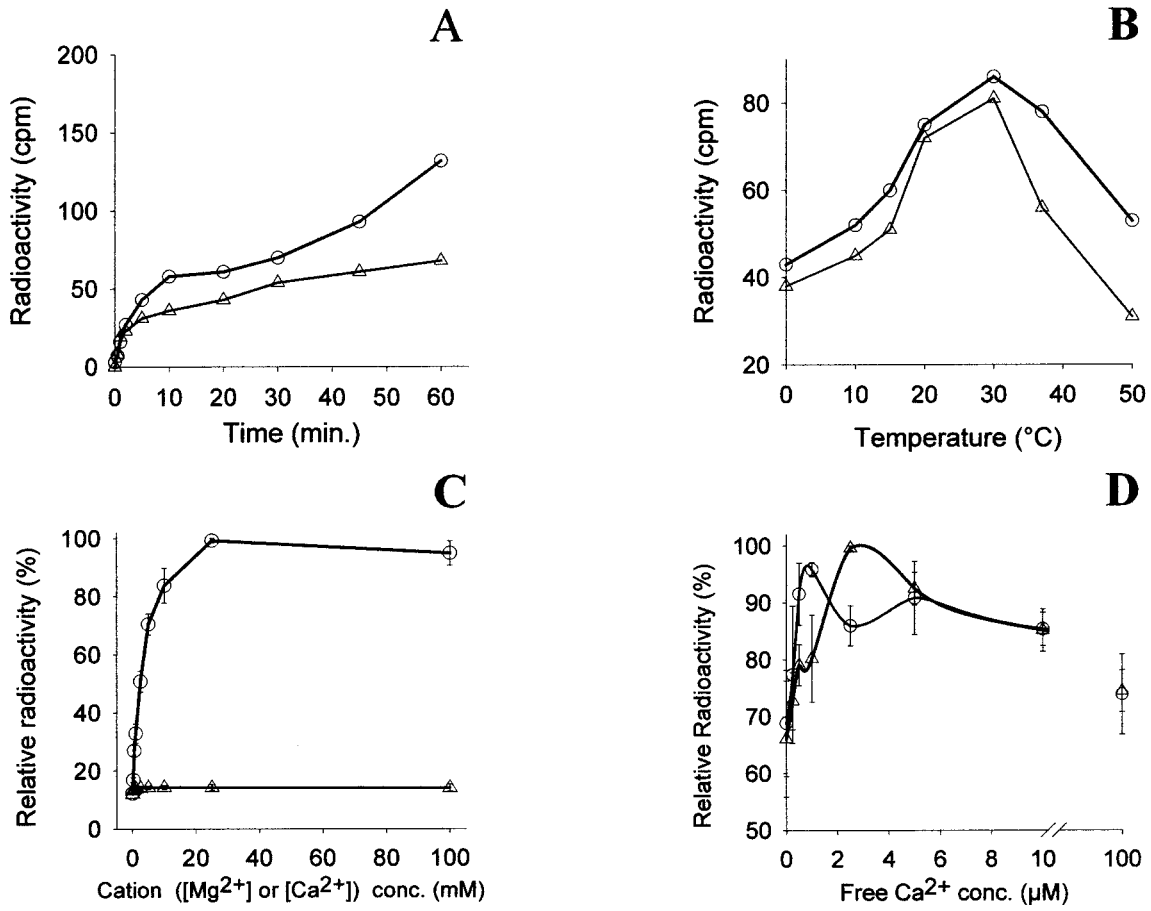

Figure 6 Phosphorylation properties of lentil seed TIPs by PSV-membrane kinase

(**A**) Time course of γ-^{32}P incorporation into TIPs. PSV membranes were incubated in the presence of 10 μM [γ-^{32}P]ATP, and proteins were further resolved by SDS/PAGE. Experiments were performed in the standard reaction mixture. (**B**) Temperature-dependence of the kinase activity. PSV membranes were incubated at various temperatures for 30 min in the presence of radioactive ATP. Proteins were then resolved by SDS/PAGE. Experiments were performed in the standard reaction mixture. (**C**) Effect of bivalent cations on the kinase activity. PSV membranes were incubated at 30 °C for 30 min, pH 6.5, in the standard reaction mixture containing either Mg^{2+} (○) or Ca^{2+} (△). (**D**) Ca^{2+} stimulation of the kinase activity in the presence of a constant Mg^{2+} concentration. PSV membranes were incubated in the standard phosphorylation mixture. The Ca^{2+} concentration was adjusted to the indicated values and the Mg^{2+} concentration was constant (2 mM). For all panels, radioactivity was determined by Cerenkov counting on excised gel slides. For (**A**), (**B**) and (**D**), ○ represents the radioactivity associated with the 25 kDa TIP and △ indicates that associated with the 26 kDa TIP.

were allowed to migrate in an SDS/9 % polyacrylamide gel and glutaraldehyde concentration was reduced to 0.03 % (v/v). As shown in Figure 3(B), only a single band of about 44 kDa was observed. This result indicates that two homodimers do not form after cross-linking. Similar patterns were obtained with DTSP and DFDNB (results not shown).

Molecular diffusion is known to favour the formation of oligomers [26]. This phenomenon would be especially relevant at high membrane-protein density. To minimize an aspecific intermolecular coupling, membrane proteins were diluted in their lipidic environment. With this aim, liposomes were fused with the TIP-enriched membranes in order to increase the lipid/protein ratio. Increasing this ratio delayed the formation of trimers and tetramers but not that of dimers (Figure 4), illustrating the specificity of monomer–monomer interaction.

Oligomers can be formed from either 25 or 26 kDa proteins (homo-oligomers) or from both 25 and 26 kDa proteins (hetero-oligomers). DTSP was used to distinguish homo-oligomers from hetero-oligomers. DTSP consists of two molecules of thiopropionic acid (*N*-succinimidyl ester) linked by a covalent disulphide bond. In the presence of a reducing agent (in this case β-mercaptoethanol) DTSP is cleaved into two thiopropionic acid molecules. In the absence of β-mercaptoethanol, DTSP induced the formation of dimers, trimers and tetramers (Figure 5) similar to those observed with glutaraldehyde (Figures 3A and 3B). To separate the monomers forming each oligomer, each band observed in Figure 5 (lane 1) was cut out of the gel and incubated for 1 h in the presence of β-mercaptoethanol. Each gel slide was loaded on to an SDS/12 % polyacrylamide gel. As shown in Figure 5 (lanes 2–4), each oligomer was made of both 25 and 26 kDa proteins.

Phosphorylation of TIP

For some plant and animal MIP homologues, it has been shown that the phosphorylation/dephosphorylation of serine residues regulates the protein activity [18,27]. More precisely, α-TIP of *Phaseolus* seeds is regulated by a membrane-bound protein kinase [17]. This prompted us to determine whether or not both TIPs of *Lens* were phosphorylatable and to investigate the regulation of the phosphorylation process. *In vitro* phosphorylation experiments were performed with the membrane fraction

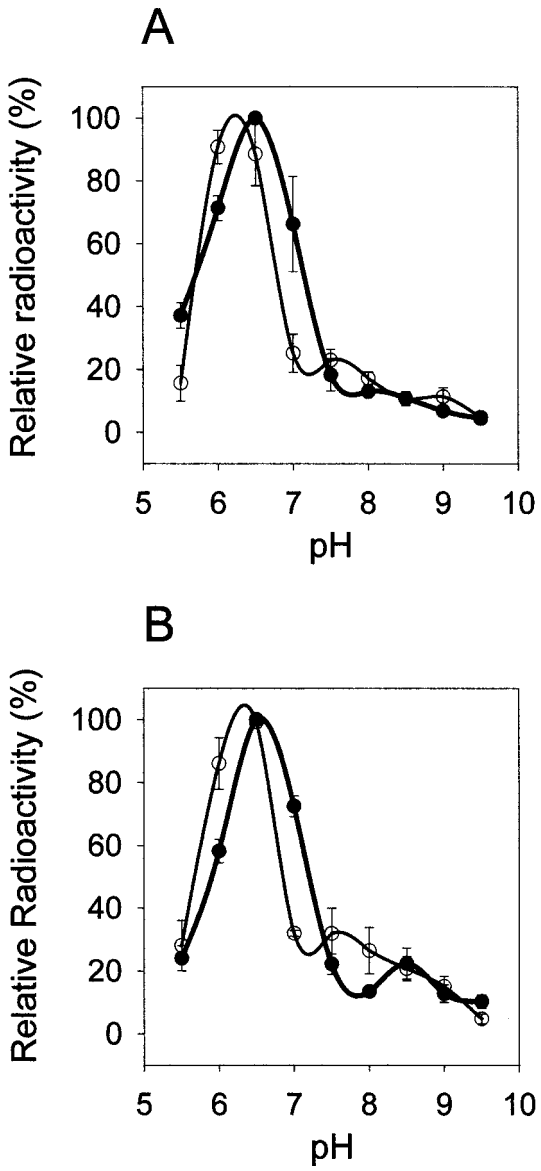

Figure 7 Effect of pH on the kinase activity

PSV membranes were incubated for 30 min at 30 °C in the appropriate buffer (see the Experimental section) in the presence of either 2 (●) or 10 mM Mg^{2+} (○). After SDS/PAGE, the gel was stained with Coomassie Brilliant Blue R-250, bands were excised and ^{32}P was determined by Cerenkov counting. Radioactivity associated with the 25 (**A**) and 26 kDa (**B**) bands was determined by Cerenkov counting on excised SDS/PAGE gel slides.

not treated with DOC. After incubation in the presence of [γ-^{32}P]ATP, membrane proteins were separated on a 10.5% polyacrylamide gel and the bands corresponding to the 25 and 26 kDa TIPs were cut out of the gel to quantify the radioactivity.

As shown in Figure 6(A), the time course of ^{32}P incorporation reached a plateau after 10 min. The radioactivity associated with the 25 kDa band was always higher than that of the 26 kDa band (Figures 6A and 6B). For both proteins, the incorporation of ^{32}P was maximal at 30 °C and at pH 6.5 (Figures 6B and 7). The pH-dependence was slightly affected by the $MgCl_2$ concentration. Increasing the $MgCl_2$ concentration from 2 to 10 mM shifted the pH response curve to a lower pH by about 0.5 pH unit (Figure 7).

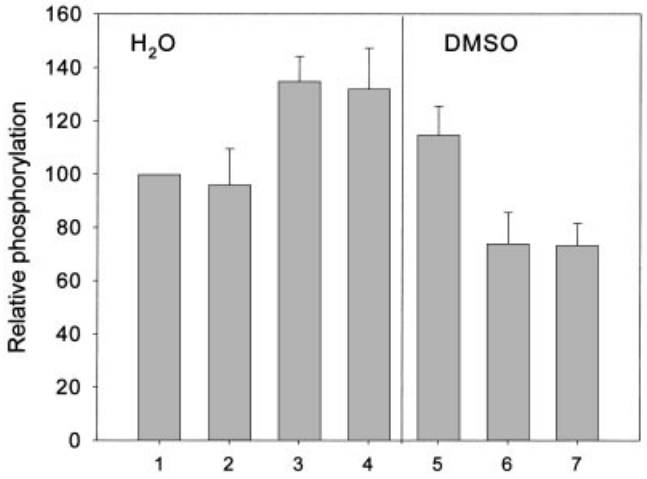

Figure 8 Effect of kinase stimulators and inhibitors on the kinase activity

PSV membranes were incubated in the standard reaction mixture and 2 μl of the following compounds was added: 1, water (control with no stimulators/inhibitors added); 2, 100 μM phorbol ester; 3, 1 mM Mn^{2+}; 4, 10 mM Mn^{2+}; 5, 5% DMSO; 6, 50 μM W-7 [*N*-(6-aminohexyl)-5-chloropentene-1-sulphonamide hydrochloride]; 7, 100 μM W-7. Results presented in bars 2–4 refer to compounds that are water-soluble and therefore must be compared with the control (bar 1). Bars 6 and 7 show results obtained with DMSO-soluble compounds and must be compared with bar 5 (DMSO alone).

In the absence of calcium, ^{32}P incorporation into the 25 kDa TIP is magnesium-dependent (Figure 6C). Saturation of the phosphorylation reaction occurred at 10 mM Mg^{2+}, whereas 1 mM $MgCl_2$ yielded half saturation. However, in the absence of magnesium, calcium did not affect ^{32}P incorporation. This lack of effect also suggests that $MgCl_2$ stimulation of the phosphorylation reaction is not attributable to chloride. In our experiments, free Mg-ATP concentration is nearly constant over the range of Mg^{2+} concentrations used. Thus an excess of free Mg^{2+} is essential for kinase activation.

At a constant magnesium concentration, the kinase activity is stimulated by micromolar amounts of free calcium (Figure 6D). Maximal stimulation of ^{32}P incorporation was achieved at 1 and 2.5 μM free calcium with the 25 and 26 kDa TIP, respectively (Figure 6D). Kinase activity was further characterized by addition of kinase effectors and inhibitors (Figure 8). Concentrations of 1 or 10 mM Mn^{2+} resulted in a 30% increase in kinase activity (Figure 8, bars 3 and 4). Phorbol ester did not affect the activity (Figure 8, bar 2). Addition of W-7 [*N*-(6-aminohexyl)-5-chloropentene-1-sulphonamide hydrochloride], a calmodulin antagonist also known as a calcium-dependent protein kinase (CDPK) inhibitor [28,29], to the reaction mixture resulted in a 30% loss in kinase activity (Figure 8, bars 6 and 7). W-7 was solubilized in DMSO, which itself induces an enhancement of the kinase activity (Figure 8, bar 5).

Identification of the protein kinase

The results of Johnson and Chrispeels [17] and those reported here indicate that the protein kinase that phosphorylates α-TIP is membrane-bound. This kinase has not yet been identified. In order to determine the apparent molecular mass of the kinase, we used an in-gel assay [21,30]. Calf histone H1 or denaturated PSV-membrane proteins were used as substrates for the kinase and mixed with the acrylamide solution of an SDS/12% polyacrylamide gel containing 30% less SDS (see the Experimental

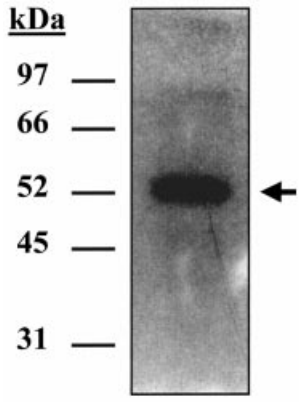

Figure 9 In-gel detection of lentil PSV-membrane kinase

Aliquots of total PSV membrane were run on electrophoresis gels containing 0.8 mg/ml calf histone H1. Protein kinase activity was detected by autoradiography of gel incubated in the reaction buffer containing [γ-^{32}P]ATP and then washed extensively in a buffer containing sodium pyrophosphate (see the Experimental section for details).

section). Membrane proteins were then separated in the gel by electrophoresis. The gel was soaked in a solution containing [γ-^{32}P]ATP. In the presence of [γ-^{32}P]ATP, kinase induced the phosphorylation of calf histone H1 (and of PSV-membrane proteins; results not shown), which permitted its localization at 52 kDa by autoradiography (Figure 9). In a control experiment, it was confirmed that the lower SDS concentration did not affect the migration of proteins in the gel during electrophoresis.

DISCUSSION

In beans, the screening of an expression library from mid-mature cotyledons has led to the identification of two distinct TIP cDNA clones [16]. Similar results have been obtained with *Arabidopsis* seeds [10]. In these studies, the gene coding for α-TIP has been fully sequenced while only a partial sequence for a second gene (coding for β-TIP) has been published. This suggests that all plants do potentially contain two seed-specific forms of TIP. However, until now, all attempts to isolate both proteins from seeds have been fruitless. We show here that two proteins highly homologous to α- and β-TIP are present in the membrane of PSVs in lentil. Lentil seed TIP isoforms represent 5% of PSV-membrane-associated proteins, as determined by scanner densitometry. The two TIPs identified in *Lens* are highly homologous (Figure 2) but distinct. First, their apparent molecular masses are significantly different. Second, the 26 kDa TIP N-terminal sequence was not accessible to Edman degradation, and this was not the case for the 25 kDa TIP. Third, after trypsinolysis, peptides IV and VI of each TIP are not fully identical. The occurrence of two distinct TIP proteins in seeds is consistent with the identification of two cDNAs coding for TIP in plant seeds [10,16].

Consistent with other reports on AQP-1 [31,32], cross-linking experiments showed that TIPs from lentil seeds form oligomers. As for AQP-1, the most probable oligomeric assembly of plant TIPs in PSV membrane is a tetramer [33]. The physiological significance of the occurrence of a hetero-oligomer in PSV membrane remains unclear. The functional expression of mRNA encoding α-TIP from *P. vulgaris* in *Xenopus* oocytes indicates that at least one of the isoforms is able to form a water channel [18]. This does not preclude the possibility that the oligomeric structure is required for the transport function. Co-injection of mRNA of each seed TIP isoform in oocytes would allow us to investigate the role of the heterodimer in water transport.

In the kidney collecting duct, two aquaporin isoforms (AQP-2 and -3) have been co-localized. Their physiological roles are different. AQP-2 is mainly involved in water transport whereas AQP-3 is involved in the flow of both solutes and water [34]. Only two amino acid substitutions allow the transformation of an aquaporin into a solute channel [35]. Therefore, even though the two isoforms identified in *Lens* are highly homologous, we cannot exclude the possibility that they have separate, specific functions. Of course, such a hypothesis remains to be tested.

α-TIP of *Phaseolus* is a phosphoprotein [17], and its phosphorylation has been shown to increase the water-transport activity [18]. We investigated the *in vitro* phosphorylation properties of two TIPs from lentil seeds. Our data provide strong evidence that a protein kinase is associated with the PSV membrane. Its activity is strongly increased between 15 and 40 °C, which corresponds to the range of temperatures required for seed germination in this species. In *Phaseolus*, endogenous phosphorylation of α-TIP is activated by calcium and it has been suggested therefore that this kinase activity could be associated with a protein belonging to the CDPK family [17]. Our results are consistent with this hypothesis: (i) the kinase is activated by calcium, (ii) it requires Mg^{2+}, (iii) it has an apparent molecular mass between 50 and 60 kDa and (4) it is inhibited by calmodulin antagonists.

We provide strong evidence that the 52 kDa protein kinase activity is Mg^{2+}-regulated in the absence of Ca^{2+}. This was not reported for the membrane-bound kinase of *Phaseolus* seeds [17]. Lentil PSV-membrane kinase requires Mg^{2+} at a concentration greater than that needed to form a stable Mg-ATP complex, indicating the presence of a Mg^{2+}-binding site. At magnesium concentrations higher than 10 mM, soluble CDPKs are inhibited [36,37]. This is not observed with the membrane-bound CDPK. To the best of our knowledge this effect of Mg^{2+} has not been investigated previously for the membrane-bound CDPK. Under physiological conditions (micromolar amounts of Ca^{2+} and millimolar amounts of Mg^{2+}) the kinase will work at its maximum level of activity (Figure 6D).

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