Tonoplast-Bound Protein Kinase Phosphorylates Tonoplast Intrinsic Protein

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ABSTRACT

Tonoplast intrinsic protein (TIP) is a member of a family of putative membrane channels found in bacteria, animals, and plants. Plants have seed-specific, vegetative/reproductive organ-specific, and water-stress-induced forms of TIP. Here, we report that the seed-specific TIP is a phosphoprotein whose phosphorylation can be monitored in vivo by allowing bean cotyledons to take up [*32P]orthophosphate and in vitro by incubating purified tonoplasts with γ-labeled [*32P]ATP. Characterization of the in vitro phosphorylation of TIP indicates that a membrane-bound protein kinase phosphorylates TIP in a Ca2+-dependent manner. The capacity of the isolated tonoplast membranes to phosphorylate TIP declined markedly during seed germination, and this decline occurred well before the development-mediated decrease in TIP occurs. Phosphoamino acid analysis of purified, radiolabeled TIP showed that serine is the major, if not only, phosphorylated residue, and cyanobrome cleavage yielded a single radioactive peptide peak on a reverse-phase high-performance liquid chromatogram. Estimation of the molecular mass of the cyanobrome phosphopeptide by laser desorption mass spectroscopy led to its identification as the hydrophilic N-terminal domain of TIP. The putative phosphate-accepting serine residue occurs in a consensus phosphorylation site for serine/threonine protein kinases.

The plant vacuole is a multifunctional cellular compartment that acts as a reservoir for various ions and metabolites, contributes to the maintenance of cellular pH and osmotic homeostasis, and, with its complement of acid hydrolases, serves as the lytic compartment of the plant cell (for a review, see ref. 1). In the storage tissues of seeds, vacuoles take on the specialized role of sequestering storage proteins, which are digested in situ during early seedling development. All of these functions depend on the capacity of the vacuolar membrane, or tonoplast, to regulate the passage of materials between the vacuolar and cytosolic compartments.

Transport systems for the movement of small metabolites and ions across the tonoplast have been well characterized by physiological experiments, but relatively little is known about the molecular mechanisms of transport. As part of our goal toward understanding vacuolar transport mechanisms, we have recently characterized an abundant, seed-specific, integral membrane protein called TIP, which is highly conserved among seed plants (15). The amino acid sequence deduced from a bean seed cDNA indicates that TIP is a 27-kD protein with six membrane-spanning domains (16). Other TIP clones have since been isolated from an Arabidopsis genomic library, indicating the existence of a small multigene family (14). The seed-specific form of TIP present in both bean and Arabidopsis is now referred to as αTIP.

As to the general function of αTIP, we can for the moment only draw inference from the fact that αTIP shows significant homology to other proteins in the so-called MIP family of membrane proteins (14, 27). The MIP of mammalian lens fiber junctional membranes, the best characterized member of this family, has been shown to exhibit channel activity in artificial lipid bilayers (3). The glyceral facilitator (GlpF) of Escherichia coli has been implicated in the rapid equilibration of small molecules across the bacterial inner membrane (11). Also, there is recent evidence to suggest that NOD26, the first plant member of the MIP family to be identified, may function as a dicarboxylate transporter in the symbiosome membrane of soybean root nodules (26). There is less information regarding the function of other homologs, including the bib gene product in Drosophila (30), a root-specific gene in tobacco (38), a water-stress-induced transcript in pea shoots (7), and a vegetative tissue-specific tonoplast protein (γTIP) in Arabidopsis (14).

The most common mechanism for regulating the activity of proteins in cells is phosphorylation/dephosphorylation. Many ion channels in animal cells are known to be modulated by phosphorylation (18, 34), and membrane channels in plant cells may be regulated in a similar manner. For example, the plasma membrane H+-ATPase has been shown to be phosphorylated by a CDPK (33). The role of phosphorylation of this proton ATPase is unknown, but phosphorylation of the yeast plasma membrane H+-ATPase has been reported to stimulate its activity (20).

Among members of the MIP family, both MIP (17) and NOD26 (37) are known to be phosphorylated. Recently, Ouyang et al. (26) reported a correlation between the in vitro

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2 Abbreviations: TIP, tonoplast intrinsic protein; CDPK, calcium-dependent protein kinase; CNBr, cyanobrome; MIP, major intrinsic protein; PVDF, polyvinylidene difluoride; NOD26, nodulin 26.
phosphorylation of NOD26 and the rate of malate uptake into isolated symbiosomes. If TIP follows this pattern, then the design of experiments intended to examine the rate of TIP’s transport function should take into account the possible requirement of phosphorylation for activity and/or specificity of transport. Toward that goal, we now present evidence that αTIP is phosphorylated both in vivo and in vitro, and phosphorylation is mediated by a tonoplast-bound CDPK. This protein kinase phosphorylates a single serine residue near the N terminus of TIP.

MATERIALS AND METHODS

Plant Material and Chemicals

Plants of Phaseolus vulgaris L. cv Greensleeves were grown in a greenhouse. Carrier-free $^{32}$Pi (10 mCi/mL) and $[\gamma-^{32}P]ATP$ (3000 Ci/mmol) were purchased from Amersham; Protein A Sepharose CL-4B was supplied by Pharmacia; ultrapure grade Triton X-100 and acrylamide were purchased from BRL; ultrapure SDS was obtained from International Biotechnologies, Inc. Unless otherwise stated, all other organic chemicals were obtained from Sigma Chemical Co.; solvents and inorganic chemicals were supplied by Fisher Scientific.

Isolation of Tonoplasts from Bean Cotyledons

Membranes of protein storage vacuoles (protein bodies) from bean seeds that had imbibed for 18 h were prepared as described by Mader and Chrispeels (24) with the following modifications. After the final water wash of the enriched tonoplast pellet, the membranes were resuspended in 10 mM Tris (pH 7.5), 12% (w/v) sucrose, 1 mM EDTA, 1 mM DTT, and 0.01% (v/v) Triton X-100 using a Teflon pestle tissue homogenizer. The suspended tonoplasts were pelleted by centrifugation for 60 min at 95,000 g and washed once by resuspension in the same buffer minus the EDTA and Triton X-100, followed by recentrifugation. The final membrane pellet was resuspended in the wash buffer, aliquoted to Eppendorf tubes, and stored at −70°C until use.

Preparation of Microsomes

Because protein bodies in developing seeds cannot be isolated by the procedure described above, microsomal membranes were used in the developmental experiment (Fig. 7). Ten cotyledons from the midmaturation stage (18 DAP) or mature bean seeds imbibed for 1, 2, or 3 d were ground in a prechilled mortar with 15 mL of 100 mM Tris (pH 7.8), 12% sucrose, 1 mM EDTA, and 1 mM DTT (homogenization buffer). The homogenate was squeezed through cheese cloth and centrifuged at 200g for 5 min to remove cell walls and debris. The supernatant was then centrifuged at 12,500g for 10 min to remove mitochondria, and microsomes were pelleted by centrifugation at 150,000g for 60 min. The microsomes were resuspended in the homogenization buffer minus EDTA, aliquoted to Eppendorf tubes, and frozen at −70°C until use.

Preparation of Vacuoles from Tobacco Leaves

Vacuoles from normal and αTIP-transfected tobacco leaves were isolated on a Ficoll/betaine step gradient from mechanically lysed mesophyll protoplasts, as described by Höfte et al. (14). The transgenic tobacco expresses high levels of bean αTIP, which has been localized to the tonoplast of the mesophyll cells (13).

In Vivo Phosphorylation Measurement

Each of four cotyledons from developing bean seeds (18 DAP) was placed abaxial surface down onto 10 μL (100 μCi) of $^{32}$Pi on Parafilm for 2 h at room temperature. After the 2-h labeling period (pulse), the cotyledons were rinsed twice with 50 μL of 100 mM NaHPO$_4$ and two of them were incubated another 2 h on 50 μL of 100 mM NaHPO$_4$ (chase). A 2-mm-thick slice from the abaxial side of each cotyledon was then taken, and the pulsed and chased slices were ground separately in a 2-mL Teflon tissue homogenizer with 1 mL of homogenization buffer. The homogenates were transferred to Eppendorf tubes and spun at 10,000 rpm for 10 min in a microcentrifuge. The supernatants were transferred to Oak Ridge tubes and spun for 2 h at 95,000g in a 50 Ti rotor to pellet the microsomes. The microsomal pellets were rinsed briefly and then resuspended in 400 μL of homogenization buffer. Equal aliquots (30 μL) of the resuspended membrane samples were either extracted with 90% acetone to precipitate total protein or immunoprecipitated with TIP antiserum (see below) for analysis by SDS-PAGE autoradiography. Of the original 30 μL of resuspended membranes that was precipitated with acetone, 6 μL was loaded on the gel, whereas the entire immunoprecipitated sample was loaded.

In Vitro Phosphorylation Assay

Unless otherwise stated, the in vitro phosphorylation reaction mixture contained 50 mM Mes (pH 6.5), 2 mM MgCl$_2$, 5 mM DTT, 10 μM ATP (including 10 μCi of $[\gamma-^{32}P]ATP$), 1 mM NaF, 0.2 mM EGTA, 0.24 mM CaCl$_2$ (to yield 50 μM free Ca$^{2+}$), and 30 to 60 μg of tonoplast (or microsomal) protein in a total volume of 40 μL. The reaction was started with the addition of membranes and allowed to proceed for 30 min at 30°C with gentle orbital shaking. For analysis of total radio-labeled polypeptides, the reaction was stopped by the addition of 40 μL of denaturing buffer (60 mM Tris [pH 6.8], 4% SDS, 10% glycerol, 100 mM DTT, and 0.04% bromphenol blue). For samples that were to be immunoprecipitated with TIP antiserum, the reaction was stopped by the addition of 200 μL of Tris-buffered saline (20 mM Tris [pH 7.5], 500 mM NaCl) containing 1% (w/v) SDS in preparation for immunoprecipitation.

For assays utilizing whole vacuoles, the reaction mixtures contained, in addition to the chemicals listed above, 0.6 mM betaine, 5 mM Hepes, 4.3 mM EGTA, 75 μg/mL of BSA, 5 μg/mL of leupeptin, 3 μg/mL of pepstatin, 100 μg/mL of PMSF, CaCl$_2$ to yield the indicated free Ca$^{2+}$ concentrations, and 25,000 vacuoles in a total volume of 100 μL. The reaction was allowed to proceed for 15 min at 22°C. Immunoprecipitation of TIP was performed on a 50-μL sample of the reaction mixture.
Protein Determination, Immunoprecipitation, SDS-PAGE, and Autoradiography

Quantitative measurement of protein concentration was carried out as previously described (15). TIP was immunoprecipitated with TIP antiserum according to the procedure of Faye and Chrispeels (5). For gel electrophoresis, protein samples were dissolved in denaturing buffer and incubated for 5 min at 50°C. Protein separation was carried out on 15% acrylamide gels according to the method of Laemmli and Favre (22). The gels were air dried between sheets of BioGelWrap (BioDesign, New York), and autoradiography was carried out by exposing the dried gel to Kodak X-Omat film (Eastman Kodak) at room temperature.

Phosphoamino Acid Analysis

Radiolabeled TIP was immunoprecipitated from an in vitro phosphorylation reaction mixture containing tonoplasts. The immunoprecipitate was resolved by SDS-PAGE and transferred to a PVDF membrane (Immobilon, Millipore Corp.) according to the method of Matsudaira (25), and the labeled TIP band was detected by autoradiography. The labeled band was excised, rinsed with water, dried, and then incubated in 200 μL of 6 N HCl for 1 h at 110°C in a capped microcentrifuge tube. The tube was then centrifuged, and the supernatant was transferred to a new tube and lyophilized. The dry residue was dissolved in 10 μL of pH 1.9 electrophoresis buffer (88% formic acid: glacial acetic acid: water, 2.5:7.8:89.7, v/v) containing 1 μg each of phosphoserine, phosphothreonine, and phosphotyrosine, centrifuged 10 min in a microcentrifuge to remove any undissolved material, and then applied to a cellulose thin-layer plate. The mixture was separated in two dimensions by electrophoresis, followed by chromatography according to the method of Boyle et al. (2). The authentic standards were detected by spraying the dried plate with 0.25% ninhydrin in acetone, and radioactivity was detected by autoradiography.

Phosphopeptide Analysis

32P-labeled TIP was prepared, isolated, and transferred to a PVDF membrane as described above for phosphoamino acid analysis. Membrane strips (0.2 × 12 cm) containing the phosphorylated TIP were then subjected to CNBr cleavage according to the method of Yuen et al. (39). Briefly, the dried strips were cut into 2-× 4-mm strips and placed into 1.5-mL Eppendorf tubes. The strips in each tube were incubated in 200 μL of 70% formic acid containing 1.5 mg of CNBr for 24 h in the dark. More than 70% of the bound radioactivity was solubilized by this cleavage procedure. The digests were then diluted 10-fold with water, pooled, dried under vacuum, and then redissolved in 5 μL of water. The molecular weight of the phosphopeptide was determined on a matrix-assisted laser desorption mass spectrometer (12) operated by a technical representative from Finnigan MAT, Ltd.

RESULTS AND DISCUSSION

In Vivo and in Vitro Phosphorylation of TIP

Close inspection of the deduced amino acid sequence of a bean cDNA for αTIP revealed several potential phosphorylation sites. To determine whether TIP is phosphorylated in vivo, developing bean cotyledons were incubated with 32Pi. After a 2-h labeling period (pulse), half the cotyledons were harvested; the other half were incubated for an additional 2 h in unlabeled Pi (chase) before harvesting. Microsomal membranes were isolated and washed once by ultracentrifugation, and the membrane proteins were dissolved in denaturing buffer and separated by SDS-PAGE, followed by autoradiography. Many high mol wt microsomal polypeptides became phosphorylated during the pulse (Fig. 1, lane 1), but no labeled band corresponding to the migration position of TIP could be discerned. The labeling of TIP became evident, however, after TIP was enriched by immunoprecipitation (Fig. 1, lane 2). About half of the radioactivity apparent in TIP after the 2-h pulse period was lost during the 2-h chase (Fig. 1, lane 4), indicating that in vivo TIP dephosphorylation occurs as well.

Plant cells contain both soluble and membrane-bound

![Figure 1. SDS-PAGE autoradiogram of in vivo phosphorylated TIP. The abaxial surfaces of four developing cotyledons (20 DAF) were exposed to 32Pi for 2 h (PULSE), and then two of the cotyledons were chased with unlabeled Pi for an additional 2 h (CHASE). Postmitochondrial microsomes were prepared from each set of labeled cotyledons, rinsed, and resuspended. Polypeptides were either acetone precipitated to obtain total polypeptides (lanes 1 and 3) or immunoprecipitated with TIP antiserum (lanes 2 and 4) before separation by SDS-PAGE and autoradiography.](https://plantphysiol.org)
protein kinases (29). To determine whether the TIP-containing tonoplasts have a protein kinase capable of phosphorylating TIP, we incubated the tonoplast-enriched membranes from bean cotyledons in an in vitro phosphorylation reaction mixture containing [γ-32P]ATP and then fractionated the polypeptides by SDS-PAGE. The autoradiogram of this gel (Fig. 2) shows about 10 to 15 labeled polypeptides, including a major radioactive band with an apparent molecular mass of 25 kD (Fig. 2, lane 1). This major band comigrates with TIP, as indicated by the position of TIP immunoprecipitated from the same reaction mixture (Fig. 2, lane 2).

**Effect of Ca²⁺ Concentration and pH on TIP Phosphorylation**

Because the activities of many plant protein kinases are Ca²⁺ dependent (28), we wished to determine whether in vitro TIP phosphorylation was similarly dependent on the presence of Ca²⁺. Tonoplast-enriched membranes were incubated in the presence of EGTA and CaCl₂ mixtures that yielded known concentrations of free Ca²⁺ (4). The reaction mixtures were immunoprecipitated and total radioactivity in each immunoprecipitate was determined by Cherenkov counting before the samples were analyzed by SDS-PAGE autoradiography. The results are shown in Figure 3. In reaction mixtures containing 0.1 μM or less free Ca²⁺, we could not detect labeled TIP. Phosphorylation activity increased at higher Ca²⁺ concentrations up to 10 μM, plateaued between 10 and 200 μM, and then decreased at higher free Ca²⁺ concentrations. Thus, the half-maximal rate of TIP phosphorylation occurs between 1 and 10 μM Ca²⁺, which is similar to the Ca²⁺ dosage dependence of other CDPKs in plants (9, 31) and Paramecium (8). The addition of spinach calmodulin (1 μM final concentration) had no effect on TIP phosphorylation (data not shown). Although it is possible that our tonoplast preparations contained an endogenous level of calmodulin sufficient to activate the CDPK-catalyzed phosphorylation of TIP, there is mounting evidence for the existence of calmodulin-independent CDPKs in plants (9, 31). Calmodulin independence could be attributable to the presence of a calmodulin-like domain in the kinase itself, as has been shown recently for the CDPK of soybean cells (10). Although the soybean CDPK is a soluble enzyme, its cloning revealed the existence of multiple molecular forms. Thus, it is quite possible that the calmodulin-independent CDPK gene family may include membrane-associated forms, such as the plasma membrane-associated CDPK in oat roots (32).

The effect of pH on in vitro TIP phosphorylation (at 50 μM free Ca²⁺) is shown in Figure 4. There is a rather sharp optimum at pH 6.5 with a shoulder at pH 7.5. If it is assumed that protein storage vacuoles maintain an acidic internal pH (pH 5–6) like other plant cell vacuoles (1), the active site of the TIP-phosphorylating, tonoplast-bound protein kinase is probably oriented toward the cytoplasm. It is interesting that the phosphorylation of the plasma membrane proton ATPase in oat roots also occurs optimally at slightly acidic pH values (33).

**TIP Is Phosphorylated in Vitro by a Membrane-Bound Protein Kinase**

When we examined the amino acid sequence of TIP, we could not find any domain homologous to the highly conserved sequences (e.g. ATP-binding and catalytic domains) of known protein kinases. Thus, it seems unlikely that TIP is a protein kinase that undergoes autophosphorylation. To test the notion that TIP serves as a substrate for a distinct, membrane-bound protein kinase, we extracted the tonoplast-enriched membranes with various agents known to differentially solubilize peripheral and integral membrane proteins.

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**Figure 2.** SDS-PAGE autoradiogram of in vitro phosphorylated tonoplast polypeptides. Protein body membranes were allowed to phosphorylate in vitro. The labeled polypeptides were then separated by SDS-PAGE before (lane 1) or after (lane 2) immunoprecipitation using TIP antiserum, followed by autoradiography. Mₙ markers are shown on the left.

**Figure 3.** Effect of Ca²⁺ concentration on in vitro phosphorylation of TIP. Reaction mixtures containing various Ca²⁺ concentrations were immunoprecipitated with TIP antiserum. Radioactivity in each immunoprecipitate was determined by Cherenkov counting, and the values are shown at the top of the autoradiogram. (Each value was adjusted by subtracting 1060 cpm, the value determined for the minus-Ca²⁺ sample shown in lane 1.) Equal aliquots of the samples were then run on SDS-PAGE and autoradiographed. Beginning with lane 1, the free Ca²⁺ concentrations in μM were: <0.01, 0.1, 1.0, 10, 50, 200, 1000, and 3000, respectively. Mₙ markers are indicated on the right.
Figure 4. Effect of pH on in vitro phosphorylation of TIP. Reaction mixtures at variable pH (50 mM Mes-NaOH for pH 5.5–6.5; 50 mM Tris-HCl for pH 7.0–8.5) were immunoprecipitated, counted (see graph), and then subjected to SDS-PAGE to obtain the autoradiogram.

We then washed the membranes free of the extraction agent and assayed the washed membranes for both TIP abundance and endogenous phosphorylation activity. Some of the results are shown in Figure 5. Extraction with 10 mM NaOH (Fig. 5B, lane OH−) or 0.1% Triton X-100 (data not shown) had no effect on either TIP abundance or phosphorylation. After extraction of the tonoplasts with 0.5% Triton X-100 or octylglucoside, however, endogenous TIP phosphorylation was reduced dramatically (Fig. 5B, lanes Tx and OG). This was not due to the loss of TIP from the membranes, because its level in each membrane pellet remained unaltered by these treatments (Fig. 5A). These data are consistent with the conclusion that extraction of the membranes with 0.5% Triton X-100 or octylglucoside removes a significant amount of the protein kinase that is responsible for TIP phosphorylation. Solubilization of a plasma membrane-bound CDPK from oat roots capable of phosphorylating the H+-ATPase was achieved using 0.9% Triton X-100 (32).

Recent phosphorylation studies of tonoplast fractions from monocot roots have cast doubt on the existence of protein kinases intrinsic to the vacuolar membrane (6, 21). Because αTIP is found exclusively in the protein storage vacuolar membranes of seeds (15), and washing these membranes with 10 mM NaOH or 0.1% Triton X-100 fails to diminish TIP phosphorylation in vitro, we conclude that seed tonoplasts contain an integral protein kinase.

As part of an ongoing study to examine the function of TIP in the tonoplast, we inserted the full-length bean cDNA for αTIP into tobacco and showed that this protein is highly expressed and correctly targeted to the tonoplasts in the transgenic mesophyll cells (13). When leaves of control and transgenic plants were fed 32Pi via their petioles, we could detect immunoprecipitable, 32P-labeled TIP in the total leaf extract of transgenic plants only (data not shown). Moreover, purified vacuoles isolated from αTIP-expressing tobacco mesophyll protoplasts phosphorylate TIP in vitro in a Ca2+-

Figure 5. Effect of membrane extraction agents on TIP abundance and phosphorylation. Equal aliquots of protein body membranes were suspended in either Tris/sucrose buffer (C), 10 mM NaOH/sucrose (OH−), or Tris/sucrose buffer containing 0.5% Triton X-100 (Tx) or 0.5% octylglucoside (OG). The membranes were spun down, washed once, and then resuspended in Tris/sucrose buffer. A, Immunoblot using TIP antiserum; B, SDS-PAGE autoradiogram of immunoprecipitated TIP.

Figure 6. Phosphorylation of TIP in transgenic tobacco vacuoles. Vacuoles were prepared from untransfected (lane 1) and αTIP-transfected (lanes 2–4) tobacco mesophyll protoplasts and assayed for phosphorylation in vitro in the presence of 90 μM (lanes 1 and 2), 10 μM (lane 3), or 0.1 μM (lane 4) free Ca2+ (based on added CaCl2 only). The reaction mixtures were immunoprecipitated with TIP antiserum, and the vacuolar polypeptides were separated by SDS-PAGE and visualized by autoradiography.
prepared from cotyledons of developing (18 DAF) and germinating bean seeds. Figure 7 shows that on a per-mg-of-protein basis, TIP phosphorylation was highest in microsomes derived from the developing seeds (Fig. 7, lane M), and this activity declined steadily during imbibition by dry seeds for 1, 2, and 3 d (Fig. 7, lanes 1, 2, and 3).

The simplest explanation of these results is that the in vivo phosphorylation status of TIP changes during development. If TIP is progressively phosphorylated in vivo during seed germination, there would be correspondingly less unphosphorylated TIP available for incorporation of $^{32}$P in vitro. Thus, the decline in in vitro TIP phosphorylation during seed germination could reflect an enhanced phosphorylation of TIP in vivo during the same period. A similar argument has been used to explain differences in the effects of blue light on the phosphorylation of a 120-kD plasma membrane protein, a putative intermediate in the signal transduction chain of phototropism in pea stem segments (35). Relative to untreated controls, blue light irradiation of stem segments caused a reduction in the ability of the 120-kD protein to become phosphorylated in vitro in microsomes prepared from this tissue. On the other hand, when microsomes prepared from untreated pea stems were irradiated in phosphorylation buffer, a large enhancement in phosphorylation of this protein occurred. Thus, in this system as in ours, in vivo utilization of phosphorylation sites could lead to an apparent

dependent manner (Fig. 6). When calculating the free Ca$^{2+}$ concentration in the vacuole phosphorylation reaction mixtures, we ignored any Ca$^{2+}$ contribution from the added vacuoles. With this caveat in mind, 90 $\mu$M Ca$^{2+}$ (Fig. 6, lane 2) is supraoptimal and 0.1 $\mu$M Ca$^{2+}$ (Fig. 6, lane 4) is suboptimal for TIP phosphorylation. However, mesophyll vacuoles may store significant levels of Ca$^{2+}$, which could leak across the tonoplast or become released by inadvertent vacuole rupture during the phosphorylation assay. (The latter cannot be a major source of extravascular Ca$^{2+}$ because hemacytometer counts of the vacuolar reaction mixtures at the end of the phosphorylation incubation period indicate that loss of intact vacuoles never exceeds 5%). Nevertheless, our calculations represent minimum estimates of the actual free Ca$^{2+}$ concentrations. The point we wish to make here is that the tobacco mesophyll tonoplast also contains a Ca$^{2+}$-stimulated protein kinase capable of phosphorylating TIP, which reaffirms our conclusion that plant tonoplasts contain protein kinase activity.

**Capacity to Phosphorylate TIP Declines during Seedling Growth**

Immunoblots of total seed protein from bean cotyledons of developing and germinating seeds have shown that the level of aTIP remains relatively constant from 18 DAF through the first 3 d of seed germination and then declines gradually to low levels during the next 4 d of seedling development (15). To determine whether the capacity to phosphorylate TIP changes during development, we measured TIP phosphorylation in vitro in microsomal fractions

**Figure 7.** Effect of developmental stage on in vitro TIP phosphorylation. Postmitochondrial microsomes were prepared from bean cotyledons at the midmaturaion seed stage (lane M; 18 DAF) and after imbibition by dry seeds for 24 h (lane 1), 48 h (lane 2), and 72 h (lane 3). Protein (40 $\mu$g) from each microsomal preparation was used in in vitro phosphorylation reaction mixtures, which was immunoprecipitated with TIP antiserum and analyzed by SDS-PAGE autoradiography.

**Figure 8.** Identification of phosphoserine in in vitro labeled TIP. An acid hydrolysate of gel-purified $^{32}$P-TIP was separated by two-dimensional cellulose thin-layer electrophoresis/chromatography and then visualized by autoradiography. The plus sign (+) marks the origin. pSer, pThr, and pTyr indicate migration positions for the corresponding phosphorylated amino acid standards detected with ninhydrin.
The two peak CNBr-cleaved peptides of αTIP were eluted from a PVDF membrane during CNBr treatment. The eluted peptides were then dissolved in acetonitrile containing 0.1% TFA with a flow rate of 1 mL/min. Fractions from the elution were collected, and radioactivity was determined by Cherenkov counting.

An alternate explanation for the results shown in Figure 7 is that the activity of the enzyme is limiting the phosphorylation of TIP and that germination is accompanied by a rapid decline in the level of activity of the tonoplast-bound protein kinase.

**TIP Is Phosphorylated on a Serine Residue**

To ascertain the nature of the phosphorylated residue(s) in TIP, tonoplast-enriched membranes were labeled with [32P]ATP and TIP was immunoprecipitated, purified by SDS-PAGE, and transferred to a PVDF membrane. The radioactive TIP band was detected by autoradiography, excised, and subjected to acid hydrolysis in 6 N HCl. Analysis of the hydrolysate by two-dimensional, thin-layer electrophoresis/chromatography, as shown in Figure 8, indicates the presence of labeled phosphoserine. Radioactive phosphothreonine and phosphotyrosine were undetectable.

**TIP Phosphorylation Site Is Located in the N-Terminal Domain**

There are 16 serine residues in αTIP. According to the topological model of TIP, however, only four of these serines occur in domains that would be accessible to Ca2+-regulated phosphorylation on the cytosolic side of the tonoplast (16). Two of them are located in the N-terminal domain, and the other two appear in the cytoplasmic loop linking the second and third transmembrane domains. To determine which serine residue(s) becomes phosphorylated in vitro, we took advantage of the seven internal methionine residues in αTIP to generate peptides by CNBr cleavage. 32P-labeled TIP was isolated from tonoplast membranes by immunoprecipitation,

**Figure 9.** HPLC chromatogram of CNBr-cleaved peptides of αTIP. Peptides released from a PVDF membrane during CNBr treatment were concentrated to dryness by vacuum centrifugation, dissolved in 0.3 mL of 0.1% TFA, loaded onto a Vydac C18 column, and eluted with a linear gradient of 0 to 50% acetonitrile in 0.1% TFA with a flow rate of 1 mL/min. One-milliliter fractions were collected, and radioactivity was determined by Cherenkov counting.

**Figure 10.** Predicted CNBr cleavage pattern for αTIP. The numbers on the left refer to the number of residues from the N terminus of the first amino acid in each peptide. Underlined sequences are predicted transmembrane domains. Mol wts take into account that each C-terminal methionine residue has been converted to homoserine lactone.

**Figure 11.** Laser desorption mass analysis of the phosphorylated TIP peptide. The two peak radioactive HPLC fractions (41–42; see Fig. 9) were dried by vacuum centrifugation and then redissolved in a small amount of water for mass spectrometry.

![Image](https://plantphysiol.org/download/1793.png)
followed by preparative SDS-PAGE and transfer to a PVDF membrane. The membrane band corresponding to TIP was excised and treated with CNBr, which caused the release of about 70% of the total radioactivity into soluble form. This material was then applied to a reverse-phase HPLC column, and all the recoverable radioactivity appeared in a single peak, which coeluted with a major peptide peak (Fig. 9). This radioactive peak accounted for 78% of the original radioactivity applied to the column, and there were no minor peaks detected. Thus, a single CNBr peptide contains the major, if not only, phosphorylation site(s).

Figure 10 shows the CNBr fragmentation pattern predicted for αTIP. Peptide A corresponds to the N-terminal domain and peptide C contains the first cytoplasmic loop. Because both of these peptides contain a serine residue flanked by nearby arginines (Ser^7 and Ser^90), a feature common to several consensus phosphorylation sites used by known serine/threonine kinases (19), these two serines became the leading candidates for the phosphate acceptor. After several unsuccessful attempts to microsequence the radioactive, HPLC-purified peptide, we turned to matrix-assisted laser desorption mass spectrometry, which provides a very accurate molecular mass estimation for peptides (12). Figure 11 shows that the phosphorylated peptide has an estimated molecular mass of 2223 m/z. The predicted mass of peptide A containing a single phosphate group is 2225 m/z. There are no other predicted CNBr peptides having similar masses (see Fig. 10), leading us to conclude that the phosphorylated CNBr peptide is peptide A with a single phosphate group. According to the A214 profile in Figure 9, the radioactive peak corresponds to the most abundant peptide. Of all the predicted CNBr peptides, peptide A is overall the most hydrophilic and, thus, would be expected to be the most easily solubilized from the hydrophobic PVDF membrane.

Of the two serine residues in peptide A, Ser^7 is the more likely candidate for the phosphate acceptor. It lies in a motif (RRYS) that matches the consensus phosphorylation sites known for certain serine/threonine protein kinases in animal tissues, including the multifunctional calmodulin-dependent protein kinase II (RXXS) and cAMP-dependent protein kinase (RXXS) (19). It also matches the consensus motif of R/KXXS/T described for several protein and synthetic peptides that are phosphorylated by CDPKs in plants (28) and algae (31). It is interesting that the vegetative tissue-specific γTIP from Arabidopsis has a phosphorylation substrate motif similar to that of αTIP at the same N-terminal location, but the sequence of key amino acids is reversed: SARR (14). We have not yet tested whether this isoform of TIP is phosphorylated.

The phosphorylation sites for NOD26 by a CDPK (37) and MIP by cAMP-dependent protein kinase (23) both occur in the cytoplasmically oriented, C-terminal domains of these TIP homologs, whereas αTIP phosphorylation occurs in the N-terminal domain. Adding a highly charged phosphate group to the terminal domains of each of these intrinsic membrane proteins could alter its conformation and activity or, possibly, its interaction with some yet unknown cytoplasmic factor. The specific functions of these proteins have yet to be conclusively demonstrated, and until a definite activity can be measured, the role of phosphorylation in each case will remain unknown.

**SUMMARY**

The isoforms of TIP that occur in the tonoplasts of seeds (αTIP) and nonseed tissues (γTIP) are members of a superfamily (the MIP family) of putative membrane channels. The exact nature of the substances that are transported by each of these proteins remains to be determined. In this paper we show that αTIP is phosphorylated by a CDPK intrinsic to the tonoplast. There is a single phosphorylation site that is located in the N-terminal domain of TIP. We are now in a position to screen potential transport metabolites of TIP expressed in transgenic vacuoles and to assess the effect, if any, of phosphorylation on this function.

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**LITERATURE CITED**