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Cloning and characterization of a cytosolic isoform of triosephosphate isomerase developmentally regulated in potato leaves

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Abstract

A full-length cDNA encoding a triosephosphate isomerase (TPI) was cloned from the wild potato *Solanum chacoense*. Sequence analysis showed that the cDNA has high levels of homology to sequences coding for cytoplasmic TPIs (cTPIs) and lower homology to plastidic TPI (pTPI) sequences. A specific polyclonal antiserum was generated against recombinant (6 × His) epitope-tagged cTPI expressed in *Escherichia coli*. In *Solanum tuberosum* this immune-serum recognized a single TPI polypeptide present in crude leaf extracts while an antigenic signal was not evident in purified chloroplasts. Immunoblot analysis demonstrated that cTPI was distributed in all vegetative and reproductive tissues. TPI activity was followed over a 3 months time course encompassing the whole development of potato plants. Activity expressed on a fresh weight basis was significantly higher in expanding leaves compared to mature and senescing leaves during the course of the experiment. Consistent with this, there was a gradient of TPI activity and cTPI protein along the shoot axis with the highest levels found in the youngest tissues. Analysis of TPI isoforms profiles by anion exchange chromatography demonstrated that (i) photosynthetic and non-photosynthetic tissues express 2 TPI isoforms and (ii) cTPI always represents the bulk of extractable TPI activity. Of all tissues surveyed, expanding leaves had the highest cTPI to pTPI ratio. The results are discussed in relation to the hypothesis that leaf cTPI plays a role in growing tissues, where glycolysis and respiration fulfill a key function in production of energy and C skeletons for biosynthetic purposes.

Keywords: Triosephosphate isomerase; Potato; Glycolysis; Carbohydrate metabolism; Plant development; Triose phosphate

1. Introduction

Triosephosphate isomerase (TPI, EC 5.3.1.1) catalyzes the interconversion of the glycolytic intermediates dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP). TPI activity is ubiquitous in prokaryotes and eukaryotes. TPI is generally considered as an extremely efficient enzyme, and is even referred to as a perfect catalyst [1]. In vascular plants, only a small number of studies have been conducted on TPI expression. Two nuclear-encoded TPIs have been described [2–4]. The product of the first gene corresponds to the plastidic isoform, pTPI, a Calvin cycle enzyme. pTPI has been purified from several sources including leaves of lettuce [5] and spinach [4]. It is not known if the gene encoding pTPI is also expressed at high levels in non-photosynthetic tissues. However, TPI activity is found in non-green plastids [6]. The second gene encodes the cytosol-localized isoform, cTPI. This isoform is found in photosynthetic and non-photosynthetic tissues. It is not clear however, if cTPI gene expression is higher in roots [2] or in leaves [3]. cTPI has been purified from leaves of spinach and lettuce [4,5] and from pea seeds [7]. The gene encoding cTPI is expressed in roots [2,3,8], stem and corollas [8]. In *Petunia hybrida* corollas, the steady-state level of cTPI

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mRNA varies during the development of the tissue and is induced by GA_3 [8].

There is an abundance of literature on the role, structure and regulation of animal TPIs. Indeed, a rare but severe form of haemolytic anaemia caused by mutations inducing TPI deficiency in humans has prompted extensive research on animal TPIs [9–12]. This situation is in sharp contrast to the current research on plant TPIs. Despite the fact that TPI catalyzes a very important step in carbohydrate metabolism, information on the biochemical or physiological regulation of the cytosolic or chloroplastic isoforms is still scarce. Recently, a genetic analysis of the maize *opaque-2* mutant has revealed that the high levels of free amino acids phenotype associated with this mutation could be linked to a cTPI locus [13]. Hence, cTPI activity could play a role in the balance of metabolic fluxes in plant primary metabolism. Plant TPIs are homodimers composed of subunits of approximately 27 kDa, similarly to animal TPIs [5]. TPIs have a higher affinity towards GAP than DHAP [7,14] and display a narrow substrate specificity since DHAP and GAP are the only known substrates of this enzyme [15]. Available data on plant TPI kinetic properties indicate that the pea seed enzyme may be inhibited by physiological concentrations of phospho*enol*pyruvate [7]. A recent report suggests that cTPI activity is also regulated by glutathionylation in suspension cell cultures of *Arabidopsis thaliana* [16].

We undertook the present study to better understand the function and regulation of TPI activity and enzyme level in plants. As part of this effort, we cloned a cTPI cDNA, raised a specific polyclonal anti-cTPI immune-serum and investigated the expression pattern of this enzyme in potato. We show here that cTPI is expressed in vegetative and reproductive tissues. cTPI activity and protein levels appear to be regulated during the development of potato leaves, with highest levels of expression in expanding leaves. These results suggest that cTPI may be important in the glycolytic pathway for the supply of C to respiratory and biosynthetic pathways during active growth.

2. Materials and methods

2.1. Chemicals, biological materials and plant culture conditions

All buffers, chemicals and reagents were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Nepean, ON, Canada), except when otherwise mentioned. Percoll was from Amersham Biosciences (Baie d'Urfé, QC, Canada) and Fractogel EMD DEAE-650(S) was from VWR (Mississauga, ON, Canada). Hen anti-PsbA serum was from Agrisera (Vännäs, Sweden). Seed potatoes (cv Russet Burbank) were obtained from Propur Inc. (Saint-Ambroise, QC, Canada). Potatoes were planted in potting mixture (Pro-Mix-BX, Tourbières Premier, Rivière-du-Loup, QC, Canada): loam: perlite (2:2:1, v/v/v) in a growth chamber (12 h day, 22 °C, 200–300 μmol PAR quanta m^2 s^{-1} and 12 h night, 22 °C). 20-20-20 (NPK) fertilizer was applied weekly (200 ppm). In experiments carried out on leaves during potato plant development, leaves were numbered from 1 to 12 in the order they appeared. Potato cell cultures were maintained as described previously [17] and harvested by centrifugation after 11 days of culture. Potato root tips (1 cm long) were harvested on 1–2-week-old plants. All plant samples were collected, weighed, frozen in liquid nitrogen and stored at −80 °C until used.

2.2. cDNA cloning and sequencing

A search for a full-length cTPI was made in a *S. chacoense* EST collection generated from weakly expressed mRNAs in pistil tissues [18]. To generate the EST collection, cDNA libraries were made from 5 μg of poly(A) + mRNA isolated from compatibly-pollinated pistils 48 h post-pollination in the ZAP express pBK vector following the manufacturer's instructions (Stratagene, LaJolla, CA). In order to enrich for weakly expressed mRNAs in pistil tissues, a negative selection screen was performed using either labeled first strand cDNAs made from leaf mRNA or from flower tissues. Colonies representing weakly expressed cDNAs in pistil tissues were selected and sequenced from the 5′ end. A search of the resulting ESTs led to the identification of two identical cDNAs encoding for cTPI. No pTPI sequence was found in the collection. One of the cTPI cDNAs was completely sequenced on both strands using the Big Dye Terminator 2.0 sequencing kit (Perkin-Elmer, Montréal, QC, Canada) and an ABI 310 automatic sequencer.

2.3. Generation of a construct for expression of recombinant cTPI in bacteria and recombinant protein purification

We took advantage of the fact that the untranslated 5′ sequence of the TPI cDNA contained a *Bgl*II restriction site. pBKCMV carrying the cTPI cDNA was digested with *Bgl*II and *Xho*I and the resulting restriction fragment carrying the cDNA was cloned into *Bam*HI/*Xho*I digested pProEx HTb (Invitrogen Canada Inc., Burlington, ON, Canada). The ligated plasmid was used to transform competent *E. coli* cells (DH5α strain). Restriction digestions were used to confirm the presence and correct orientation of the insert. In addition, induced cells carrying the construct for the recombinant protein had elevated TPI activity (>100-fold) compared to induced control cells carrying the empty plasmid. The resulting expression plasmid carried the entire coding sequence of *S. chacoense* cTPI in frame with a 44 amino acids extension at the N-terminus. This extension was encoded partly by the sequence of the (6 × His) tag on the expression vector and partly by the sequence of the untranslated 5′ end of the cDNA. The deduced amino acid

sequence of the construct for the resulting recombinant protein was 298 amino acids in length and encoded a 32,422.86 Da product. The transformed *E. coli* clone carrying pProEx HTb with the construct encoding recombinant cTPI was grown in Luria-Bertani broth medium (1 L total volume) at 37 °C to an A_{600} of 0.5. Isopropyl ß-D-thiogalactoside was then added to the culture at a final concentration of 0.6 mM. Bacteria were grown as described above until the culture reached an A_{600} of 0.9–1. Cells were harvested by centrifugation (10 min, 5000 × *g*) and the pellets frozen at −80 °C until used. For the purification of recombinant cTPI, the steps were performed at room temperature in the presence of urea. Cell pellets were thawed in 10 mL of extraction buffer containing 100 mM NaH_2PO_4, 10 mM Tris–Cl, 1 mM PMSF, 6 M urea and adjusted to pH 8 with NaOH. Cells were disrupted using a French press (18,000 psi) and the extract centrifuged for 15 min at 10,000 × *g*. The supernatant was loaded on a 1.2 mL column of Ni-NTA (Invitrogen Canada Inc., Burlington, ON, Canada) equilibrated with extraction buffer. The column was washed with 10 mL of extraction buffer followed by 10 mL of extraction buffer adjusted to pH 6.3. The bound proteins were eluted from the column with 4 mL of extraction buffer adjusted to pH 5.9 with HCl and 5 mL of extraction buffer adjusted to pH 4.5 with HCl. During elution, the column eluate was collected in 1 mL fractions. Aliquots of the fractions were analyzed by SDS/PAGE as described below and staining of the gel with Coomassie blue. Judging from the expected subunit M_r of recombinant cTPI, the protein eluted mainly during the step at pH 4.5. This was confirmed when aliquots of the fractions were renatured and assayed for TPI activity. Dialysis was done against a buffer containing 50 mM Tris–Cl, pH 8.0, 1 mM EDTA, 1 mM DTT. TPI activity was measured as described below. The peak fractions (3 mL total) were pooled and used to prepare the anti-cTPI immune-serum.

2.4. Production of an anti-cTPI immune-serum

Affinity-purified (6 × His)-epitope-tagged cTPI was subjected to preparative SDS/PAGE. After electrophoresis, polypeptides were stained with Coomassie blue for 5 min followed by 15 min destaining. The band with a M_r corresponding to the recombinant cTPI was excised. The protein was then electroeluted from the gel using a Bio-Rad electroeluter according to the directions of the manufacturer. The electroeluted protein solution (approximately 250 μg protein in 1 mL) was stored at −20 °C until used for antiserum production. Antibodies were raised using a 2 kg New-Zealand rabbit. After collection of the preimmune-serum, recombinant TPI (100 μg, emulsified in complete Freund's adjuvant) was injected subcutaneously into the back of the rabbit. Booster injections were performed at days 14, 21 and 35 with 50 μg recombinant TPI freshly emulsified in incomplete Freund's adjuvant. Fifteen days after the final injection, blood was collected by cardiac puncture. The serum was collected after centrifugation at 1500 × *g*, frozen in aliquots in liquid N_2 and kept at −80 °C until used.

2.5. Preparation of glyceraldehyde-3-phosphate

DL-Glyceraldehyde-3-phosphate used as substrate for TPI was routinely prepared by hydrolysing of the diethylacetal form of the compound in H_2O. The monobarium salt of the diethylacetal (100 mg) was thoroughly mixed with 750 mg DOWEX-50H$^+$ (Bio-Rad Laboratories, Mississauga, ON, Canada) in a 30 mL Corex tube and in a final volume of 6 mL. The tube was placed in boiling water for 3 min with intermittent shaking, and then quickly transferred to an ice bath for 5 min followed by centrifugation for 5 min at 12,000 × *g*. The supernatant containing the substrate was collected. The resin was washed by two cycles of resuspension in 2 mL H_2O followed by centrifugation. These washes were pooled with the first supernatant to give the substrate stock. The yield (usually ≥95%) was estimated by determination of the concentration of the GAP solution in an enzyme assay with rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.59).

2.6. Enzyme extraction from plant tissues, TPI activity and protein assays

All steps were carried out at 4 °C. All plant tissues except cell cultures were ground with a pestle and mortar using a ratio of 2:1 (mL extraction buffer/g fresh weight) in a buffer containing 50 mM Tris–Cl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol, 5 mM DTT and 5% (w/v) insoluble PVPP. Potato cell cultures were homogenized in the above buffer using a polytron as described, previously [17]. The homogenate was centrifuged for 15 min at 12,000 × *g*. The resulting supernatant was used for TPI activity measurements and protein assays after being diluted five-fold in a buffer containing 50 mM Tris–Cl, pH 8.0, 1 mM EDTA, 10% (v/v) glycerol and 1 mM DTT. In cases where proteins were being analyzed by immunoblot, an aliquot of the extract was frozen immediately after extraction and heat-denatured in SDS sample buffer and stored frozen until used. TPI activity recovery from fresh extracts was checked in preliminary trials. First, commercial rabbit muscle TPI (Sigma Chemical Co., St. Louis, MO) was added to various plant extracts. In all cases, recoveries were >95%. Fresh and frozen tissues as well as desalted and non-desalted extracts were also compared for TPI activity recovery. Identical specific activities were obtained in all cases. The enzyme was therefore routinely assayed in non-desalted extracts obtained from frozen tissues. Under these conditions, TPI activity in the extract stored on ice was stable for more than 4 h without any loss of activity. TPI was assayed in the GAP to DHAP direction in a coupled enzyme assay performed with ∝-glycerophosphate dehydrogenase (∝-GPDH, EC 11.1.8)

according to [7] with the following modifications (standard assay). Reactions were carried out in 96-well microplates in a final volume of 200 μL. The assay mixture was optimized for the potato enzyme and contained 0.1 M Tris–Cl pH 7.5, 1 mM GAP, 0.5 mM EDTA, 2.5 U/mL ∝-GPDH and 0.2 mM NADH. NADH disappearance over time was followed at 340 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA). Blank reaction rates were also recorded in assay mixtures lacking GAP and were subtracted from rates obtained in the presence of GAP. The reaction rates were linear with time and proportional to the amount of protein added to the assay. One unit (U) of enzyme activity corresponds to the disappearance of 1 μmol GAP per minute. pH profile of TPI isoforms was done using a mixture of three buffers (25 mM acetic acid, 25 mM MES, 50 mM Tris) adjusted with 1N NaOH or 1N HCl to reach the desired values [19]. Data for K_m determinations were processed using a nonlinear regression analysis software (SigmaPlot 8.0, SPSS Inc., Chicago, Il).

2.7. Chloroplast isolation from potato leaves

All procedures were carried out at 4 °C. The isolation method [20] was optimized for the recovery of pure and intact potato leaf chloroplasts. Mature leaves were de-starched by leaving the plants in the dark for 48 h prior to extraction. Ten g of de-veined leaves were sliced in pieces of approximately 2 mm^2 and homogenized in 40 mL of buffer containing 50 mM Hepes-KOH, pH 7.6, 540 mM sorbitol, 5 mM DTT, 2 mM EDTA, 1 mM $MgCl_2$, and 1 mg/mL defatted BSA using a Polytron homogenizer (Brinkmann Instruments, Mississauga, ON, Canada) equipped with at PTA 20S probe (5 s, medium speed). The homogenate was then filtered through two layers of cheesecloth and two layers of Miracloth to yield the crude extract. Four mL of the crude extract were layered on the top of a tube containing two 5 mL layers of Percoll prepared in the above homogenization buffer (25%, w/v at the top and 35%, w/v at the bottom). After centrifugation for 15 min at 2510 × *g* in a swing-out rotor, the chloroplast band was removed from the 25 to 35% interface and used for further studies. Organelle purity was evaluated by assaying phospho*enol*-pyruvate carboxylase (EC 4.1.1.31) as cytosolic marker [21] and NADP-dependent glyceraldehyde dehydrogenase (NADP-GAPDH, EC 1.2.1.13) as a chloroplastic marker [22]. No PEPC activity was detected in Percoll-purified chloroplasts. Chloroplast intactness was assessed using NADP-GAPDH before and after breaking the chloroplasts in homogenization medium without sorbitol. Chloroplast intactness was between 70 and 75%. For native PAGE analysis of the sub-cellular distribution of TPI isoforms, aliquots of crude extract and Percoll-purified chloroplasts were diluted three-fold in homogenization medium without sorbitol and loaded on native gels, which were either stained for TPI activity or subjected to immunoblot analysis as described below.

2.8. SDS/PAGE, LDS/PAGE, native PAGE, isozyme staining and immunoblot analysis

SDS/PAGE analysis was performed according to Laemmli [23] on 12% acrylamide gels. Subunit M_r were estimated using the following protein standards (Bio-Rad, Mississauga, ON, Canada): egg white lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase b (97.4 kDa). Native PAGE was performed on 10% acrylamide gels according to Laemmli [23] except that SDS was omitted from gels and electrode buffers. A technique for TPI activity staining was developed based on a recently described method [24]. Briefly, after native electrophoresis, gels were incubated for 20 min in equilibration buffer (0.1 M Tris–Cl pH 7.5, 0.5 mM EDTA and 0.2 mM NADH) with gentle agitation. The gel was then transferred to the developing buffer containing 0.1 M Tris–Cl pH 7.5, 1 mM GAP, 0.5 mM EDTA, 0.2 mM NADH and 3 U/mL ∝-GPDH. When viewed over a UV transilluminator, TPI isozymes appeared within 2–5 min as dark bands over a fluorescent background. Control gels were incubated without GAP and did not show any activity band even when incubated for up to 60 min. SDS and native gels were electroblotted onto PVDF or nitrocellulose membranes for 60 min at 100 V constant voltage. Transfer efficiency was followed by staining the membrane with Ponceau S [25]. Blots were incubated with anti-cTPI polyclonal immune-serum (1/500 dilution) or affinity-purified anti-cytosolic castor oil seed endosperm aldolase IgG [26] (1/100 dilution) for 1 h at room temperature. Polypeptides were detected using an alkaline phosphatase-tagged secondary antibody (1/10,000 dilution) (Promega, Nepean, ON, Canada). The phosphatase reaction was visualized using 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium and was routinely allowed to develop for 5–10 min at room temperature. Immunoblots incubated with the preimmune-serum gave negative results. For PAGE and immunodetection analysis of the photosystem II protein PsbA (chloroplast marker), a different procedure was used due to the high hydrophobicity of the protein. Extracts were denatured in a buffer containing 100 mM Tris–Cl, pH 7.8, 2% (w/v) LDS and 10% (v/v) glycerol. Denaturation was achieved by three rounds of freezing/thawing followed by 20 s sonication. DTT was then added to the sample at a final concentration of 50 mM. The sample was incubated 30 min at 25 °C then centrifuged for 5 min at 12,000 × *g* prior to PAGE. The supernatant was used for PAGE analysis. PsbA protein was resolved on 10% acrylamide gel in which SDS was replaced by LDS. After transfer onto nitrocellulose, membranes were incubated with hen anti-PsbA serum (1/7000 dilution) overnight at 4 °C. PsbA antigen–antibody complexes were visualized using a rabbit anti-chicken IgY (H + L) immune-serum conjugated to horseradish peroxidase (1/8,000 dilution) followed by reaction with the Lumi-light ECL kit from Pierce (Rockland, IL).

2.9. Analytical anion exchange chromatography

TPI activity was analyzed by Fractogel EMD DEAE-650 (S) ion-exchange chromatography. TPI was extracted and clarified by centrifugation as described above for leaf tissue. The clarified extract was desalted in buffer A (Tris–Cl 20 mM pH 8, EDTA 1 mM, $MgCl_2$ 1 mM, glycerol 10% (v/v), DTT 1 mM) using PD-10 columns (Pharmacia). This step was found useful to avoid clogging of the column. Desalted extract (approximately 2 mg protein) was loaded at 0.5 mL/min onto a column (1 cm × 8 cm) of Fractogel EMD DEAE-650 (S) pre-equilibrated in buffer A. The column was connected to a Pharmacia FPLC system and washed with 18 mL buffer A. The flow through did not contain any TPI activity. TPI was eluted with a 72 mL linear gradient of (0–500) mM KCl in buffer A followed by a step gradient to 1 M KCl in buffer A. Fractions of 1 mL were collected and assayed. Activity recovery from the column was high (>85%). TPI activity was resolved in two discrete peaks eluting between 100 and 200 mM KCl. Both peaks typically eluted over one column volume. These results were reproduced three times with different plants, and similar results were obtained. Representative elution profiles of leaves from one plant are shown. An aliquot of fractions containing TPI activity was further analyzed by SDS/PAGE and immunoblot using the anti-cTPI immune-serum. Another aliquot was analyzed by native PAGE followed by TPI activity staining. Representative blots and gels are shown.

3. Results

3.1. Cloning and sequence analysis of a full-length cDNA encoding for cTPI

We searched the *S. chacoense* EST collection to recover TPI cDNAs. Two clones encoding for cTPI were found. Sequencing of the cDNAs followed by a BLAST database search revealed that they encoded the same full-length sequence (accession number AY438596). Conceptual translation and analysis of the cDNA sequence led to a 254-amino acid ORF encoding a 27,039.9 kDa polypeptide with an isoelectric point of 5.73. Sequence analysis of *S. chacoense* cTPI using BLASTP (Fig. 1) revealed a high degree of homology (80–91%) with other plant cTPIs and 59–60% homology with pTPIs. Further analysis showed that amino acid residues previously identified as part of the protozoan parasite *Leishmania mexicana* TPI active site [15] are conserved in *S. chacoense* and other plant cTPIs and pTPIs. In *S. chacoense*, these residues correspond to Asn10, Lys12, His96 and Glu166. In addition, 2 Cys residues at positions 13 and 127 are conserved in all plant TPIs whereas Cys67 is conserved only in cTPIs. Analysis of the phylogenetic tree constructed with plant TPI sequences (Fig. 2) showed that the *S. chacoense* sequence was grouped with other dicotyledon cTPI sequences. Amino acid sequences follow known evolutionary relationships with cTPIs forming two groups (mono- and dicotyledons) and pTPIs forming a third, distinct group.

3.2. Subcellular compartmentation of TPI isoforms, expression of cTPI in various potato tissues and during the light/dark period

TPI activity was extracted from mature potato leaves and analyzed by native gel (Fig. 3A). In absence of GAP in the development buffer, no TPI activity band could be detected (lane 1). Two TPI activity bands were detected in the presence of substrate (lane 2). These two bands possibly correspond to cTPI and pTPI isoforms. The cDNA encoding for *S. chacoense* cTPI was used to generate a recombinant protein using the bacterial expression vector pProEx HTb. The 32,423 kDa recombinant protein was affinity-purified on a Ni-NTA column and used to generate an anti-cTPI polyclonal immune-serum in rabbits. This serum was used for immunoblot analysis of the two TPI isoforms separated by native gel electrophoresis (Fig. 3A, lane 3). Only the slower moving (cathodal) band was recognized by the anti-cTPI immune-serum. These results indicate that the serum is specific to one isoform, probably cTPI. In order to further demonstrate the specificity of the serum towards cTPI, we investigated the sub-cellular localization of the two TPI isoforms detected by native PAGE. Potato chloroplasts were purified from mature leaves using a Percoll gradient. Crude and chloroplast extracts were compared for their contents in TPI isoforms, cytosolic aldolase (cytosolic marker) and PsbA (chloroplastic marker) (Fig. 3B). Native PAGE analysis followed by TPI activity stain and immunoblot analysis of the extracts demonstrated that only the anodal TPI activity band was present in chloroplasts and that this band was not recognized by the anti-cTPI immune-serum. Sub-cellular distribution of the TPI isoform recognized by the anti-cTPI antibody coincided with that of the cytosolic aldolase (Fig. 3B), suggesting a cytosolic localization. The other TPI isoform probably corresponds to pTPI since it was found in the chloroplast fraction together with the PsbA protein (Fig. 3B). Taken together, these results strongly support the view that the immune-serum generated against recombinant cTPI is isoform-specific and recognizes cTPI. Immunoblot experiments were performed to investigate the expression of cTPI in various potato tissues (Fig. 3C). A single band with a M_r of 27 kDa was detected in all tissues tested. This corresponds to the expected M_r of the polypeptide encoding cTPI. The cTPI signal was easily detectable in all vegetative tissues. The highest levels of cTPI protein were observed in cold-stored tuber tissues and in expanding leaves. Interestingly, in the three types of leaves surveyed in this study, the levels of cTPI protein decreased with tissue age (see below). In reproductive tissues, cTPI was present but barely detectable in immature

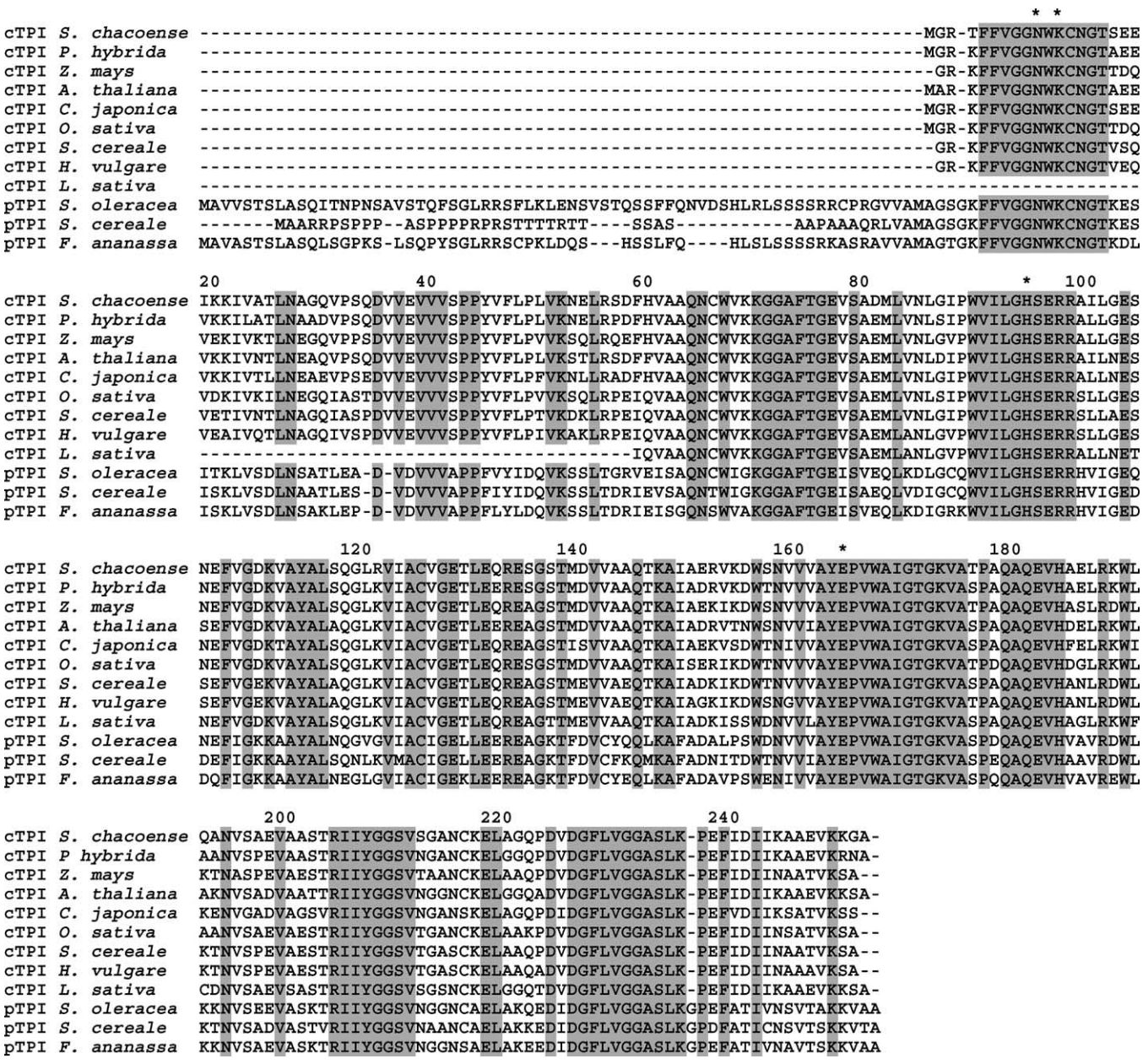

Fig. 1. Sequence analysis of *S. chacoense* cTPI. The derived amino acid sequence of the *S. chacoense* cTPI cDNA was compared to those of other plant cTPIs and pTPIs using CLUSTAL W (1.74). Gaps were introduced to achieve maximum homology between sequences. Conserved amino acid residues are shaded and residue numbering refers to the *S. chacoense* sequence. Residues that have been identified as part of the TPI active site are marked with an asterisk. The accession numbers of the sequences used are as follows for cTPIs: *S. chacoense* (AY438596), *P. hybrida* (P48495), *Z. mays* (P12863), *A. thaliana* (P48491), *C. japonica* (P21820), *O. sativa* (P48494), *S. secale* (P46226), *H. vulgare* (P34937), *L. sativa* (P48493) and for pTPIs: *S. oleracea* (P48496), *S. secale* (P46225), *F. ananassa* (Q9M4S8).

ovaries, sepals and petals whereas cTPI levels were clearly detectable in immature anthers.

A number of primary metabolism enzymes are subject to diurnal regulation [27]. We investigated if such a regulatory pattern existed for TPI activity in potato leaves. Expanding and mature leaves were sampled at different time points over the course of a 24 h light/dark period. Data show that the levels of TPI activity in both types of leaves do not change significantly during the course of the day and that TPI activity in expanding leaves is significantly higher than in mature leaves (Fig. 4). These results corroborate the immunoblot data presented in Fig. 3B, suggesting that TPI could be subject to developmental regulation in leaves.

3.3. TPI activity levels in leaves during the development of potato plants

Since extractable TPI activity seemed to evolve with leaf age, levels of extractable TPI activity and cTPI protein were investigated in leaves of actively growing potato plants

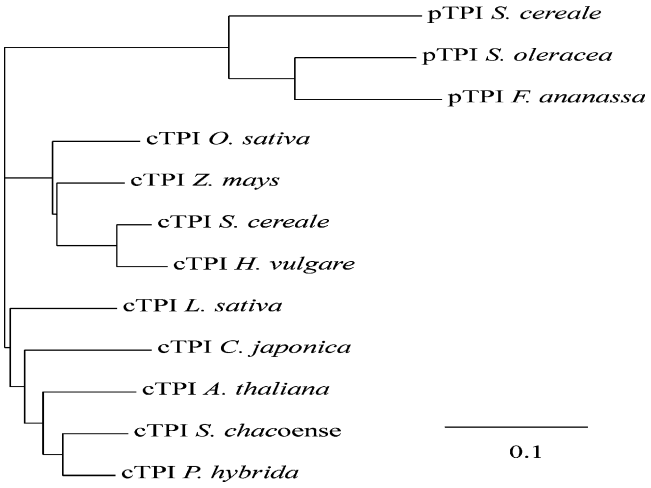

Fig. 2. Phylogenetic tree of plant cTPI and pTPI sequences. Sequences were aligned as in Fig. 1. The TreeView 1.6.6 software was used to visualize the tree. The scale bar at the bottom of the figure represents 0.1 amino acid substitutions per site.

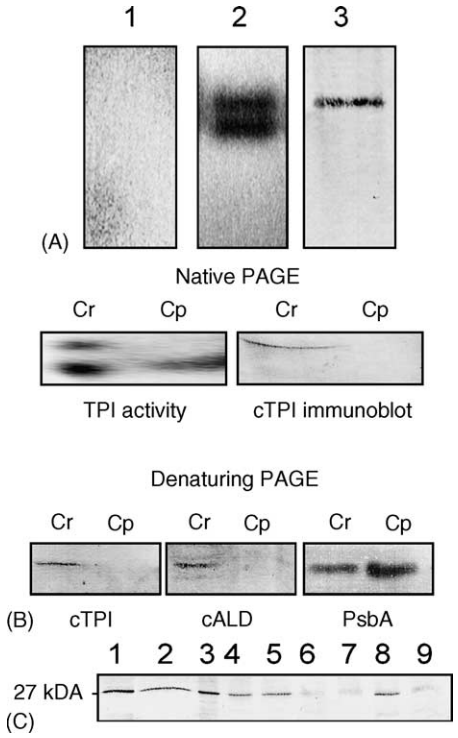

Fig. 3. Electrophoretic and immunoblot analysis of potato cTPI in various extracts. (A) Native gel and immunoblot analysis of potato leaf TPI. Mature leaf samples (2 mg FW) were separated by native PAGE. After electrophoresis, the gel was incubated in TPI activity development buffer in the absence (lane 1) or presence (lane 2) of GAP. When viewed over a UV transilluminator, TPI activity bands appear dark over a fluorescent background. In lane 3, the native gel was transferred to PVDF membrane and the membrane was probed with the anti-cTPI immune-serum. (B) Analysis of anti-cTPI immune-serum specificity on crude (Cr) and Percoll-purified potato chloroplasts (Cp) extracts. Extracts prepared as described in Section 2 were analyzed by native PAGE (top) or denaturing PAGE (bottom). Native gels were stained for TPI activity (left) or subjected to immunoblot analysis with the anti-cTPI immune-serum (right). Denaturing gels were subjected to immunoblot analysis with the anti-cTPI immune-serum (cTPI), anti-cytosolic aldolase affinity-purified IgGs (cALD, cytosolic marker) or an anti-PsbA immune-serum (PsbA, chloroplastic marker). (C) Immunoblot analysis of potato cTPI in different tissues. Fifteen μg proteins from tuber (lane 1), root (lane 2), expanding leaves (lane 3), mature leaves (lane 4), senescing leaves (lane 5), sepals (lane 6), petals (lane 7), anthers (lane 8) and ovaries (lane 9) were separated by SDS/PAGE followed and transferred onto a nitrocellulose membrane. Tubers used in this experiment were stored in the dark at 4 °C and 98% relative humidity for 12 months after harvest. The other plant tissues were collected on 68-day-old potato plants.

(Fig. 5). There was a clear gradient of leaf TPI activity from the bottom to the top of the plants, with the youngest leaves having the highest amounts of extractable activity (Fig. 5A). Immunoblot analysis of leaf extracts (Fig. 5B) showed that levels of cTPI protein followed the same trend as the total extractable TPI activity.

Since the level of TPI activity varied with leaf age, the spatial distribution and possible temporal modulation of leaf TPI activity was assessed during growth and development. TPI activity was monitored in leaves of potato plants over a 3-months period covering the entire development of potato plants in growth chambers. Measurement of aerial parts FW over time (Fig. 6A) was used to estimate plant growth. Plants typically started to emerge 7–10 days after planting. The first data points were collected from 17-day-old plants, which had only expanding leaves. At day 24, the first mature leaves could be collected and 25% of plants exhibited stolon initiation. At 34 day, stolon initiation was apparent in 75% of the plants. After day 44, the amount of expanding leaves per plant started to decline and microtubers were apparent in 75% of the plants. The first signs of senescence appeared on the oldest leaves of the stem after 60 days of growth, at which time plants had maturing tubers. After 80 days of growth there was a significant slowing down of overall plant FW gain. At 91 and 100 days, senescing leaves and mature leaves FW increased whereas no new leaves were formed. The final tuber yield (g per plant ± S.E.) was 166 ± 31 (n = 9). Leaf TPI activity was followed during the overall development of these plants (Fig. 6B). At all time points, TPI activity expressed on a FW basis was higher in expanding leaves than in mature and senescing leaves. This trend was also observed when the data were expressed on a protein basis, although, the differences were much lower (not shown). An interesting pattern of expression also emerged when examining the TPI activity per g FW in each category of leaf and throughout development. During the early part of potato plant growth, TPI activity of expanding and mature leaves increased with plant age. This was more apparent in expanding leaves. In both types of leaves, extractable TPI activity doubled between 24 and 66 days. During this period, plants initiated stolons which developed into tubers. Towards the end of the development (day 66 onwards), activity in expanding and mature leaves declined dramatically whereas in senescing leaves, TPI remained relatively unchanged. Thus, high levels of TPI activity were observed in young, growing tissues and during periods of active growth in potato plants.

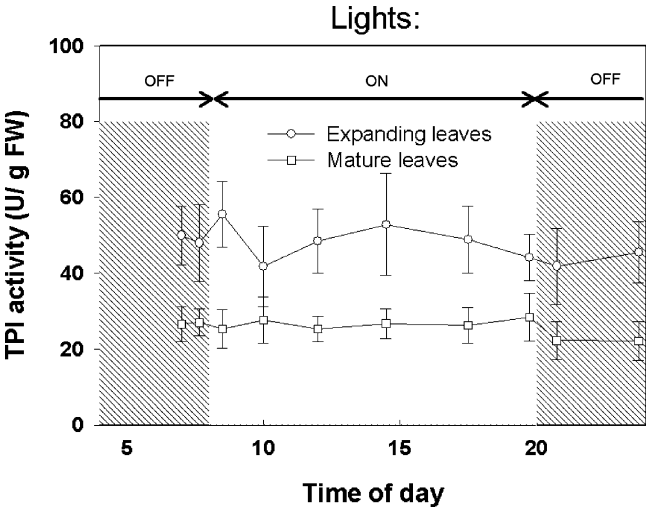

Fig. 4. TPI extractable activity is not subject to diel regulation. TPI activity was followed in expanding and mature leaves from 25-day-old plants during the course of diurnal light/dark period. Leaves were sampled at various times during the course of the day, weighed and frozen in liquid nitrogen. TPI activity was measured using a coupled enzyme spectrophotometric assay. Each data point represents the mean ± S.D. of assays carried out on three plants.

3.4. Analytical purification of TPI isoforms in various potato tissues

In order to determine whether the relative proportion of cTPI and pTPI isoforms varied in the 3 categories of leaves and in different potato tissues, leaf protein extracts were analyzed by anion exchange chromatography (Fig. 7A–E). Two peaks of activity were resolved in all extracts. To identify the cTPI peak in these activity profiles, fractions were subjected to immunoblot analysis with the anti-cTPI immune-serum. All the results were similar to those obtained with the mature leaf extract (Fig. 7F) and clearly indicated that only the first activity peak was recognized by our anti-cTPI immune-serum. Moreover, the elution profile of the 27 kDa cTPI polypeptide followed exactly the activity profile associated with the first peak. Native PAGE analysis of fractions encompassing the two TPI activity peaks eluting from the anion exchange column (Fig. 7G) showed that they matched the two TPI isoforms previously resolved by native PAGE. These results strongly support the view that the first DEAE peak corresponds to the cytosolic isoform whereas the second DEAE peak corresponds to the plastidic TPI isoform. Integration of TPI activity present in the two peaks allowed the quantification of absolute levels of cTPI activity in the various leaf extracts. cTPI was more abundant in

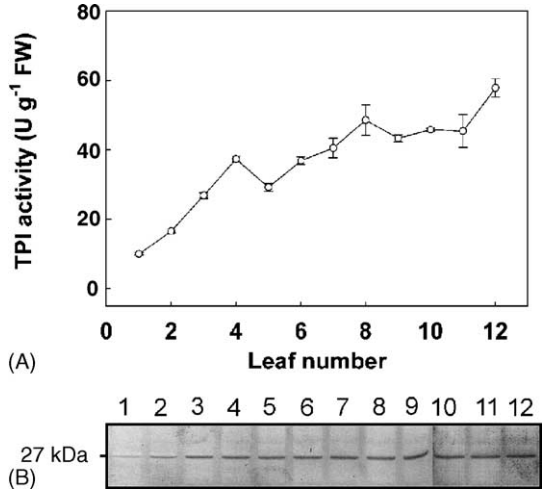

Fig. 5. Gradient of TPI activity in leaves of different age in potato plants. (A) Extractable TPI activity as a function of leaf age in potato plants. Different leaves of a 42-day-old plant were harvested separately. Leaves were numbered according to their position along the stem with number 1 corresponding to the oldest leaf. TPI activity was extracted and assayed as in Fig. 4. Data points represent mean ± S.D. for leaf samples from a single potato plant. Similar data were obtained in 8 different experiments carried out with 34 or 42-day-old plants. (B) Immunoblot analysis of extracts used in panel A. Proteins contained in volumes of extracts corresponding to 2 mg FW were separated by SDS/PAGE and subjected to immunoblot analysis.

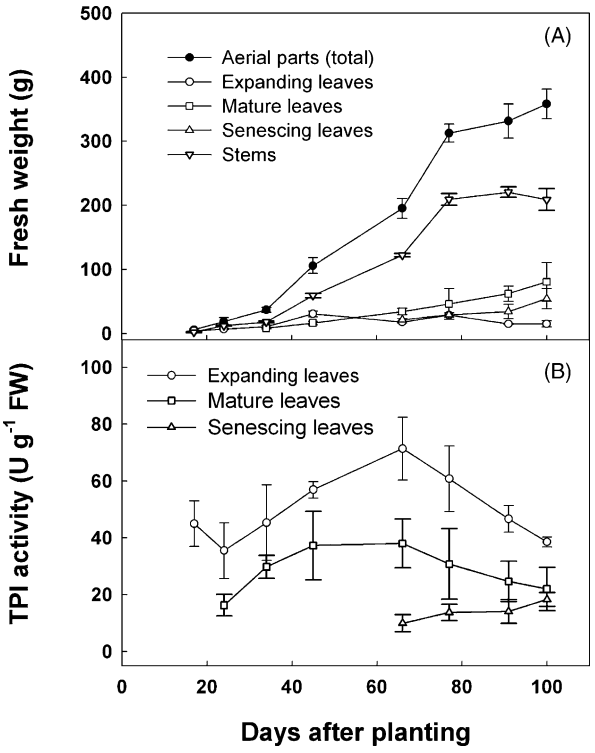

Fig. 6. Evolution of TPI extractable activity in different leaves during the development of potato plants. (A) Fresh weight accumulation in aerial parts. At different time points during the course of potato plants development, 3–5 plants were harvested, defoliated and the different tissues were weighted. Mature leaves were fully expanded and senescing leaves showed clear signs of senescence (e.g. loss of chlorophyll). The fresh weight in stems also accounts for petioles. (B) Extractable TPI activity in different leaves. TPI activity was extracted and assayed as in Fig. 4. Three samples (2.5–3.5 g FW) from each leaf category were used and data points represent mean TPI activity ± S.D. for different extracts. Expanding leaves samples represent tissue from the two youngest leaves of sampled plants. Mature leaves samples were taken from the most recently fully expanded leaves of sampled plants. Senescing leaves were taken from the leaves number 1 or 2 on the stem and showed clear signs of senescence.

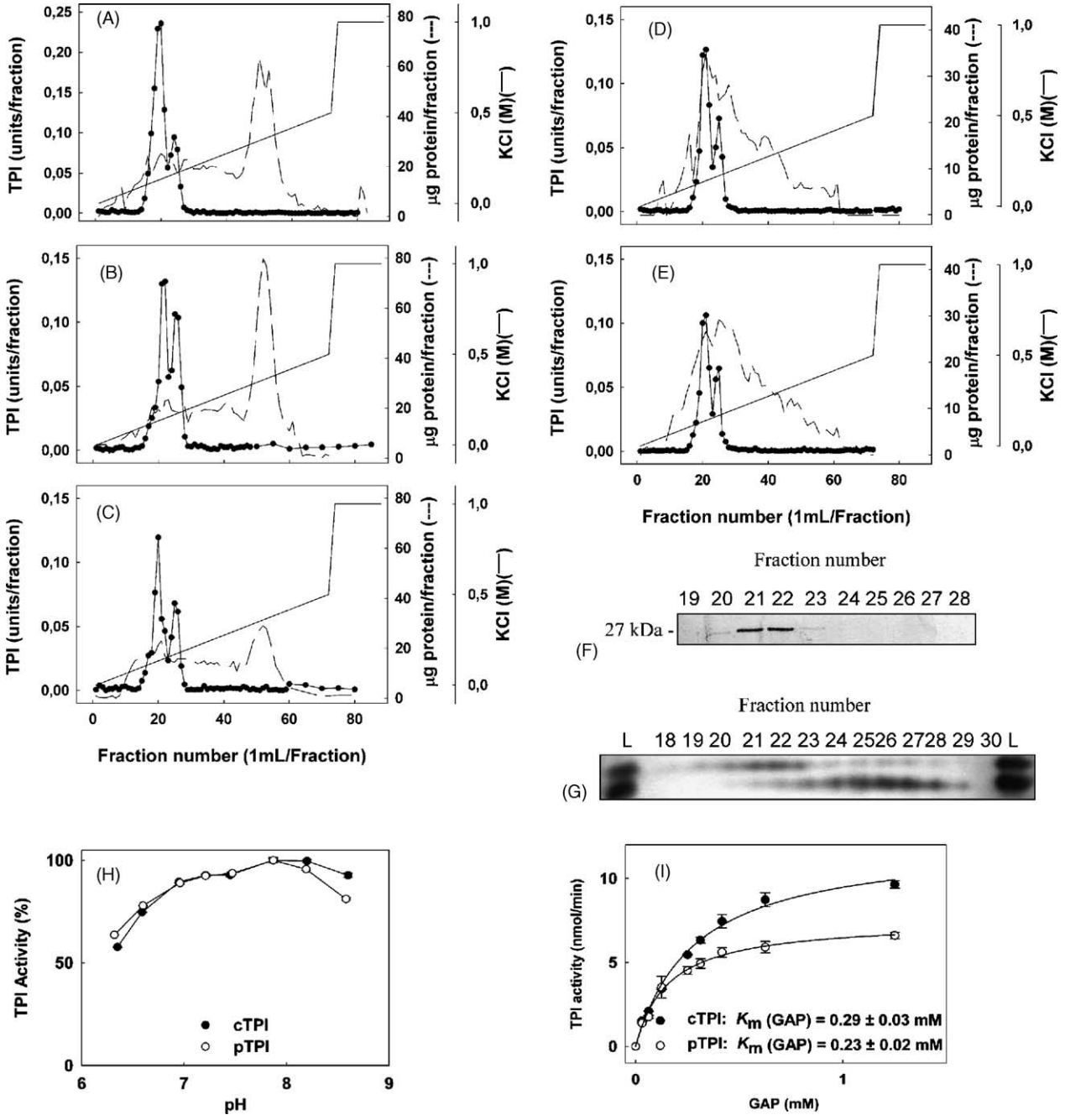

Fig. 7. Relative abundance of cTPI and pTPI in various potato extracts. Potato extracts were subjected to analytical purification of TPI isoforms using anion exchange chromatography. Extracts were prepared from frozen tissue and desalted on PD-10 columns. For each analysis, approximately 2 mg soluble proteins were loaded on a 6 mL DEAE Fractogel column. Proteins were eluted using a KCl gradient. The elution profile of TPI activity (●) is plotted as a function of fraction number. The panels represent elution profiles of extracts from: (A) expanding leaf, (B) mature leaf, (C) senescing leaf, (D) root tip, (E) potato cell culture, (F) representative immunoblot analysis of fractions containing TPI activity. An aliquot of each fraction from (B) (mature leaf extract) was analyzed by SDS/PAGE and immunoblot with the anti-cTPI immune-serum, (G) representative native gel analysis of fractions containing TPI activity from mature leaf extract (B). An aliquot of the mature leaf sample loaded on the column (L) and aliquots of fractions 18–30 (numbers above the gel) were analyzed by native PAGE and the gel stained for TPI activity, (H) Effect of pH on partially purified TPI isoforms. TPI isoforms were partially purified from mature leaf extracts. Kinetic analysis were done on fractions 21–22 for cTPI and fractions 26–27 for pTPI. Activity was determined using the buffer mixture described in Section 2, (I) effect of GAP concentration on activity of partially purified TPI isoforms. TPI isoforms were obtained as described in panel I. Activity was determined using standard assay conditions described in Section 2 and varying concentrations of GAP.

expanding leaves (0.99 U) compared to older leaves: 2.2-fold more than in mature leaves (0.44 U) and 2.6-fold compared to senescing leaves (0.37 U). These data also allowed us to calculate the relative proportions of cTPI/pTPI activities in various potato tissues within a plant: in the data presented, proportions were 77/23 for expanding leaves (Fig. 7A), 57/43 for mature leaves (Fig. 7B), 65/35 for senescing leaves (Fig. 7C). For non-photosynthetic tissues,

the ratios were very similar: 70/30 for root tips (Fig. 7D) and 71/29 for cell cultures (Fig. 7E). Hence, cTPI was relatively more abundant than pTPI in expanding leaves than in all the other tissues surveyed, including non-photosynthetic tissues (roots and cell cultures). Accurate quantification of TPI isoforms depends on the fact that optimal assay conditions are used for cTPI and pTPI. Optimal pH and affinity for GAP were determined on DEAE Fractogel purified TPI isoforms. Both isoforms exhibited a pH optimum between 7 and 8 (Fig. 7I). cTPI and pTPI had respective K_m values for GAP of 0.29 and 0.23 mM (Fig. 7H). Hence, optimal assay conditions were used for isoform analysis.

4. Discussion

4.1. cTPI is highly conserved and ubiquitous but not expressed at the same level in all potato tissues

Sequence analysis data presented in Figs. 1 and 2 indicate that the TPI cDNA isolated in this study encodes for a highly conserved cytosolic isoform of this enzyme. Such high degree of conservation was expected since (i) TPI has a central function in the glycolytic pathway by catalyzing the isomerization of triose phosphates and (ii) several authors consider that the catalytic reaction of TPI has reached the end of its evolutionary development [1,28]. According to a current model for the catalytic mechanism of TPI [15,28], His96 and Asn10 would be involved in forming H bonds with the substrate, Glu166 would serve to generate a reaction intermediate and Lys12 would be essential for binding and catalysis. This reaction is limited only by substrate/product diffusion rates [15]. The recent discovery that several TPIs, including *Arabidopsis* cTPI [16], are glutathionylated suggests that this enzyme, which is poorly characterized in plants, may be subject to an elaborate regulatory mechanism. In this respect, the investigation of the role of all the conserved Cys residues in the protein (C13, C127 or C67 which is conserved only in cTPIs) should be undertaken since one of them is probably involved in this regulatory mechanism.

We raised a polyclonal immune-serum against recombinant cTPI purified by affinity chromatography. The anti-cTPI immune-serum recognized only one of the two TPI isoforms separated by native gel electrophoresis (Fig. 3A). Together with the data in Figs. 3B, 7F and G, this demonstrates that our immune-serum is specific for cTPI. Using this serum, we were able to show that cTPI is present at high levels in both autotrophic and heterotrophic tissues (Figs. 3 and 7). Quantification of TPI isoforms after separation by anion exchange chromatography (Fig. 7) allowed us to demonstrate that cTPI was the major TPI isoform in all tissues surveyed. The steady-state levels of cTPI protein, however, varied widely (Fig. 3C). These data suggest that potato cTPI may be subject to tight regulation of gene expression depending on tissue type. For example, in reproductive tissues cTPI protein levels were extremely low, with the exception of anthers. Contrastingly, expanding leaves had very high levels of cTPI. Taking into account that cTPI gene expression has been shown to be developmentally and hormonally regulated in petunia corollas [8], further work will be required to investigate the various developmental or physiological factors that may be involved in the complex modulation of cTPI expression in different plant tissues. Our results may nevertheless reconcile previous apparently conflicting findings [2,3] concerning the levels of cTPI mRNAs in leaves and roots. It appears that, at least when considering cTPI protein levels, it is important to take into account tissue age in addition to the type of organ when making comparisons.

4.2. TPI activity and isoform patterns in leaf development and in non-photosynthetic tissues

Total TPI activity measurements in crude extracts reflect the sum of cTPI and pTPI activity. Since the two isoforms coexist in leaves [5] (Fig. 7), they both contribute to the results presented in Figs. 5 and 6. It was therefore important to determine if the relative proportion of the two isoforms varied during development. This issue was addressed using a combination of techniques. We were able to separate two peaks of activity by anion exchange chromatography (Fig. 7). Peak identification was achieved using immunoblot and native gel analysis. Since TPI activity in crude leaf extracts was very stable, the data presented in Fig. 7 are probably a good assessment of the relative abundance of the two isoforms in vivo. The first activity peak contained a 27 kDa polypeptide recognized by the anti-cTPI immune-serum. This peak therefore corresponds to cTPi whereas the second peak corresponds to pTPI (Fig. 7G). A partial kinetic characterization of the two isoforms was undertaken to insure that cTPI and pTPI were accurately quantified. pH optimum and K_m values determined for potato leaf TPIs are close to values obtained for other plant TPIs [7]. Analysis of chromatograms demonstrates that, in expanding leaves, the relative abundance of cTPI (77% of TPI activity) is the highest of all the tissues that were surveyed in this study (including non-photosynthetic tissues). The absolute amounts of cTPI were also more than two-fold higher in expanding leaves compared to older leaves. This point is also evident when analyzing the results of immunoblot experiments (Figs. 3B and 5B). In mature leaves, the proportion of cTPI decreased and pTPI abundance was maximal (43% of total TPI activity). This is not surprising because of the facts that pTPI is involved in the Calvin cycle and that mature leaves carry out the bulk of photosynthesis. Compared to mature leaves, senescing leaves have less cTPI and even lesser amounts of pTPI. During senescence, a large number of physiological changes occur in the leaf. Senescing leaves undergo a loss of chlorophyll due to a progressive decline in photosynthetic functions together with the breakdown of nucleic acids, proteins and membrane

lipids [29,30]. Stromal enzymes, in particular, are degraded during the early stages of senescence [29]. This targeted degradation may explain why, with the occurrence of leaf senescence, the steady-state level of pTPI decline more rapidly than that of cTPI. This phenomenon may be due to differences in the half-life between the two polypeptides and/or differences in the expression of their genes upon senescence. In relation to these results, it is interesting to note that in different animal models, TPI is developmentally regulated and subject to specific post-translational modifications in degenerating or ageing tissues [31,32]. Our findings also provide a first estimation of the balance between cTPI and pTPI in non-photosynthetic tissues such as roots and cell cultures. pTPI present in these tissues is necessary to carry out plastid glycolysis to support anabolic pathways such as lipid synthesis [33]. Our data suggest that as much as 30% of total TPI activity could be plastid-localized in non-photosynthetic tissues.

4.3. Significance of cTPI activity during leaf development

Leaf TPI activity profiles were investigated in more detail during the course of potato plant development. Our data revealed that TPI expression is more complex than expected. Under the growth conditions used in this study, light regime does not influence total extractable TPI activity in expanding or mature leaves. Leaf age however, does have a significant impact on extractable TPI activity. This was apparent in experiments reported in Figs. 5–7. In particular, examination of the data in Figs. 3B, 5B and 7 demonstrates that cTPI protein level is higher in actively growing leaf tissues. Expanding leaves depend on the supply of sucrose synthesized in mature leaves for growth. The glycolytic pathway in these tissues is therefore very important for the supply of carbon to anabolic pathways. Our data suggest a correlation between sink leaf metabolism and high levels of cTPI expression. These data are reminiscent of the characterization of the expression of primary metabolism enzymes during tobacco leaf ageing [34]. In this study, the youngest leaves had the highest levels of nitrate reductase and cytosolic Gln synthase expression, two enzymes involved in anabolic pathways. The mechanisms responsible for increased levels of cTPI in expanding leaves are unknown but they may involve modulation of gene expression. In the time course experiment presented in Fig. 6, extractable TPI activity increased in leaves when the plants were growing and decreased when growth rate slowed down. It is therefore tempting to speculate that TPI activity, and cTPI in particular, is linked to the anaplerotic function of glycolysis and respiration. In growing tissues, there is a high demand for carbon skeletons needed to synthesize amino acids and proteins (mainly Rubisco). Carbon skeletons are produced by the TCA cycle fed by the glycolytic pathway. High levels of expression of cTPI in these tissues can therefore be interpreted in terms of a possible requirement to participate to the glycolytic and respiratory C flux demand in these growing tissues. This finding can also be related to a previous study on cTPI gene expression in petunia corollas [8]. Indeed, corollas undergo an increase in respiration during tissue enlargement. This increase in O_2 uptake was partly correlated with increased steady-state levels of cTPI mRNA [8]. Analysis of the maize *opaque*-2 mutant [13] linked the deregulation of free aminoacid content in seeds to a locus encoding for cTPI, suggesting a link between cTPI and accumulation of amino acids derived from glycolytic and respiratory pathways. Further investigations will be required to investigate whether cTPI expression levels influence C fluxes in primary metabolism.

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