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Molecular Characterization of the Plastidic Glucose-6-Phosphate Dehydrogenase from Potato in Comparison to its Cytosolic Counterpart[1]

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We report on the cloning of a plastidic glucose-6-phosphate dehydrogenase (EC 1.1.1.49) from higher plants. The complete sequence of the plastidic enzyme was obtained after rapid amplification of cDNA ends and comprises a putative plastidic transit peptide. Sequences amplified from leaf or root poly(A^+) RNA are identical. In contrast to the cytosolic enzyme, the plastidic isoform is subject to redox modulation, i.e. thioredoxin-mediated inactivation by light. But when the plastidic enzyme is compared to a cyanobacterial homolog, none of the cysteine residues is conserved. The recombinant enzyme was used to raise antibodies in rabbits. Gene expression was studied in potato (*Solanum tuberosum* L.), at both the RNA and protein levels, revealing different patterns for the isoforms. The gene encoding the cytosolic enzyme was transcribed in all tissues tested, and the highest transcription was detected in tubers. In contrast, expression of the gene encoding the plastidic enzyme was confined to green tissues. Wounding of leaves resulted in a slight increase in the expression of the gene encoding the cytosolic isoform and a shutdown of the plastidic counterpart. Compared to the situation in soil, elevated transcription of the gene encoding the plastidic enzyme is found in roots of hydroponically grown potato plants, which is in agreement with the postulated role for this isoform in nitrite reduction.

G6PDH (EC 1.1.1.49) catalyzes the rate-limiting step of the OPP, which represents a route for the dissimilation of carbohydrates besides glycolysis (Williams, 1980; Copeland and Turner, 1987). The main function of the enzyme is the generation of NADPH for reductive biosyntheses, which is achieved by the sequential action of two dehydrogenases in the first part of the pathway. In the second, completely reversible part of the pathway, sugar phosphates are generated, among them erythrose-4-phosphate, which constitutes a precursor for the synthesis of secondary plant products. In plants, G6PDH exists at least in two compartments—in the cytosol and in the plastidic stroma (Heber et al., 1967; Schnarrenberger et al., 1973). The cytosolic isoform is regulated by metabolites alone, whereas the chloroplastic counterpart, in addition, is posttranslationally inactivated by covalent redox modification via the Fd/thioredoxin system in the light (Scheibe and Anderson, 1981) to avoid futile cycles with photosynthetic CO_2 fixation (see Buchanan, 1991, for review). Thus, in chloroplasts, the OPP operates only at night. Owing to the mechanism of light-mediated inactivation, there must exist profound structural differences between the two plant G6PDH isoforms. However, sequence information for any plant isoform has not been available until recently (Graeve et al., 1994).

The molecular basis for the mechanism of redox-mediated inactivation of the chloroplastic G6PDH in the light is still unknown. In this respect, this isoform is particularly interesting, since, conversely, other redox-modulated chloroplast enzymes (e.g. phosphoribulokinase, NADP-malate dehydrogenase, Fru-1,6-bisphosphate-phosphatase) are active in their reduced forms. The involvement of Cys residues in mediating enzyme activation upon reduction is well documented (for reviews, see Scheibe, 1990; Buchanan, 1991). Recently, the sequence of a cyanobacterial G6PDH that contains two Cys residues became available (Scanlan et al., 1992). This isoform is known to be subject to redox modulation as well (Cossar et al., 1984). Whereas G6PDHs from heterotrophic organisms have been characterized in great detail, information on the respective plant isoforms is scarce (Miernyk, 1990). To this end, the cytosolic enzyme has been purified from pea shoots (Fickenscher and Scheibe, 1986) and from potato (*Solanum tuberosum* L.) tubers, of which we obtained the cDNA sequence only recently (Graeve et al., 1994). Attempts to purify the redox-modulated chloroplastic G6PDH have been largely unsuccessful due to its tendency to aggregate unspecifically during purification. Therefore, the kinetic and regulatory properties of this isoform have been studied only in stromal extracts (Fickenscher and Scheibe, 1986; Scheibe et al., 1989) or in enriched enzyme preparations from pea chloroplasts (Srivastava and Anderson, 1983). To overcome the purification problems, we applied a molecular approach, i.e. using RT and PCR technology, to obtain sequence information on this elusive plant G6PDH. We identified a

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Abbreviations: G6PDH, Glc-6-P dehydrogenase; GST, glutathione *S*-transferase; OPP, oxidative pentose-phosphate pathway; RACE, rapid amplification of cDNA ends; RT, reverse transcription.

full-length cDNA encoding the plastidic isoform, overexpressed the recombinant enzyme in *Escherichia coli*, raised antibodies against the recombinant protein, and showed differential expression of cytosolic and plastidic G6PDH in potato.

MATERIALS AND METHODS

Preparation of mRNA

Tissue of 3- to 4-week-old *Arabidopsis thaliana* (L.) Heynh. var Columbia or potato (*Solanum tuberosum* L. var Desirée) greenhouse plants was frozen in liquid nitrogen. Total RNA was isolated as described by Logemann et al. (1987) with the modifications described by Geerts et al. (1994). Poly(A^+) RNA was selected from 250 μg of total RNA using the Oligotex mRNA Mini Kit (Qiagen, Chatsworth, CA).

Preparation of Genomic DNA

Genomic DNA was isolated from 0.5 to 1 g of frozen leaf tissue as described by Dellaporta et al. (1983).

Oligonucleotides and Primers

Degenerate oligonucleotides were designed according to highly conserved regions in the G6PDH sequences from potato and *Synechococcus* (Graeve et al., 1994) and synthesized on a GeneAssembler apparatus (Pharmacia) with the appropriate chemicals.

PFL037: 29-mer, "sense"-primer based on region $^{172/162}$TR(T/L)VVEKPFG in the cytosolic/cyanobacterial sequence. 5′-ACI (C/A)GI (A/C/T)(T/C)I GTI GTN GA(A/G) AA(A/G) CCN TT(C/T) GG-3′

PFL038: 24-mer, "antisense"-primer based on region $^{206/196}$IDHYLGKE in the cytosolic/cyanobacterial sequence. 5′-CTC (T/C)TT (G/A)CC CA(G/A) GTA (G/A)TG GTC (G/A)-AT-3′

PFL046: 33-mer, a specific "antisense"-primer for the plastidic *g6pdh*-gene, based on region ^{373}GHSNGAKSYPA, a portion missing in the cytosolic sequence (see Fig. 3). 5′-GC TGG ATA TGA TTT AGC ACC ATT GCT ATG ACC C-3′

PFL007/8/9: Equimolar mixture of 36-mers, used to prime first-strand cDNA-syntheses. 5′-$T_{(35)}$A-3′; 5′-$T_{(35)}$C-3′; and 5′-$T_{(35)}$G-3′

cDNA Synthesis

RTs were performed as described by Graeve et al. (1994) with the following modifications: poly(A^+) RNA (1 μg) and 50 ng of oligo$(dT)_{35}$ (PFL007/8/9, equimolar primer mixture) were used in the initial heat-denaturing step. Subsequent PCRs were primed with 5 μL of the 20-μL RT reaction.

PCR

Standard PCR reactions were essentially as described by Graeve et al. (1994), using 30 to 60 pmol of each primer and 2 units of *Taq* DNA Polymerase (MBI Fermentas, St. Leon-Rot, Germany) in the supplied buffer and a final volume of 50 μL. Template amounts were in the following ranges: plasmid DNA, 1 to 10 ng; genomic DNA, approximately 1 μg; 5′-RACE, 10^{-2} dilution of the anchor-ligation reaction.

5′-RACE Procedure

Amplification of the 5′ ends of plastidic *g6pdh* sequences from both leaf and root poly(A^+) RNA was conducted using the 5′-AmpliFINDER RACE Kit (Clontech, Palo Alto, CA). PCR conditions were as described above.

Cloning of PCR Products

Gel-purified PCR products were repaired using the DOUBLE GENECLEAN procedure as recommended in the GENECLEAN I kit instructions (BIO 101, La Jolla, CA) using 10 units of each T4 polynucleotide kinase (GIBCO BRL) and DNA polymerase I (MBI Fermentas) in appropriate buffer for 1 h at 37°C. DNA fragments, either blunt ended or cut with specific restriction enzymes, were ligated to the *Eco*RV site or to compatible restriction sites in the cloning vector pBluescript SK (Stratagene) by standard procedures (Sambrook et al., 1989) and introduced into RbCl-treated competent (Hanahan, 1983) *E. coli* XL1-Blue cells (Stratagene).

Isolation of Plastidic *g6pdh* cDNA Clones

Nonradioactive labeling of probes using digoxigenin DNA Labeling Mix (Boehringer) for PCR was essentially as described by Graeve et al. (1994). Subsequent screening of a potato leaf cDNA library constructed in λ ZAP II (Stratagene) and bacterial colony lifts were performed according to standard procedures (Sambrook et al., 1989). In vivo excision of λ ZAP II clones followed the Stratagene cDNA-cloning kit instruction manual.

Nucleotide-Sequence Analysis

Sequence analysis was as described by Graeve et al. (1994). Internal restriction sites in the plastidic *g6pdh* cDNA sequence (*Bst*EII, *Hin*dIII, *Kpn*I, and *Sca*I) were used for the generation of additional subclones.

Radioactive Labeling of *g6pdh* Probes

For Southern and northern analyses, *g6pdh*-cDNA fragments were radioactively labeled using the ReadyToGo DNA Labeling Kit (Pharmacia) and [α-^{32}P]dCTP (≥3000 Ci $mmol^{-1}$, Amersham). Nonincorporated nucleotides were separated with NAP-5 columns (Pharmacia) in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 0.5% (w/v) SDS.

Genomic Southern Analysis

About 10 μg of genomic DNA were digested with restriction enzymes. DNA fragments were separated in 0.8% agarose gels, denatured, and neutralized according to standard procedures (Sambrook et al., 1989). After transfer to Nytran membranes (Schleicher & Schuell), DNA fragments

were fixed to the nylon filters by optimal UV cross-link (254 nm, 120 mJ cm^{-2}) in a Spectrolinker (Spectronics, Westbury, NY). Blots were hybridized overnight to radioactively labeled *Not*I fragments of the respective *g6pdh* cDNA clones after prehybridization for at least 2 h at 42°C in hybridization buffer according to Church and Gilbert (1984). Filters were washed three times for 30 min at 68°C with 3× SSC containing 0.1% (w/v) SDS.

Illumination and Stress Conditions

Tubers were placed in soil and grown for 4 to 6 weeks in growth chambers in the dark (16 h at 25°C, 8 h at 22°C, 85% RH). Controls were harvested in darkness and etiolated potato shoots were transferred to constant light (about 150 μmol quanta m^{-2} s^{-1}) at room temperature. After various periods of time in the light, shoot tips were cut below the first leaf primordia (about 2 cm in length) and frozen in liquid nitrogen. Each sample was harvested from a new plant to avoid wound effects.

For stress experiments, discs of 1 cm diameter were cut from leaf and tuber (4 to 5 mm thick) "source" tissue and incubated separately in medium (20 mM Tris-maleate, 0.4 mM EDTA, pH 8.0). To avoid anaerobiosis, a constant stream of atmospheric air was applied to the medium throughout the incubation period (Ricardo and ap Rees, 1972). As a variation, the terminal leaf was crushed with a dialysis clamp (Peña-Cortes et al., 1988). Discs were harvested from the neighboring leaves prior to and periodically after clamp application.

Northern Analysis

Total RNA for northern analysis was prepared as described by Logemann et al. (1987) with the following modifications. The final pellet was resuspended in water, adjusted to 2 M LiCl, and incubated for at least 10 h at 4°C. After centrifugation, the precipitate was washed once with 70% ethanol, dried, and resuspended in water. Separation of RNA was in formaldehyde gels according to Lehrach et al. (1977) and Sambrook et al. (1989). Transfer to nylon membranes and development of the filters was as described under "Genomic Southern Analysis." Standard filters for estimating the detection levels of both *g6pdh* isoforms were routinely hybridized and developed along with the blots. Serial dilutions of the respective *g6pdh* cDNA clones in sheared herring sperm DNA (2.5 mg/mL) were boiled for 10 min, cooled on ice, dotted (2 μL) onto nylon membranes, dried, and fixed by UV cross-linking.

Preparation of GST/G6PDH Fusion Proteins

For high-level expression and affinity purification of recombinant G6PDH isoforms, both the cytosolic and the plastidic cDNAs, respectively, were inserted into the *Eco*RI site of vector pGEX-3X (GST Gene Fusion System, Pharma-

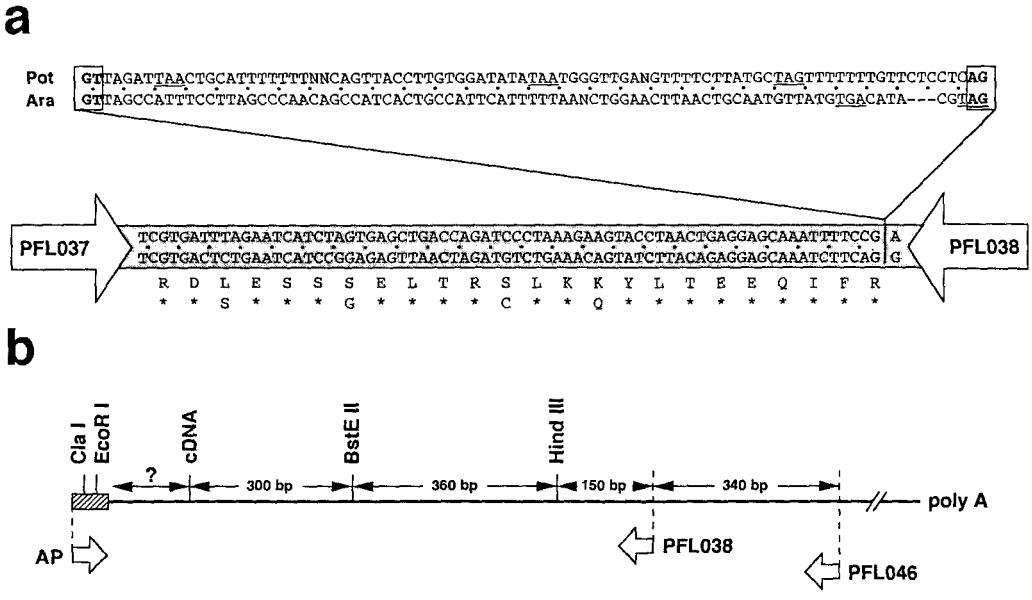

Figure 1. Graphic representation of the cloning strategy. a, Comparison of the new intron-containing *g6pdh* sequences amplified from genomic potato (Pot) and Arabidopsis (Ara) DNA. The intron is shown as an insert in the cDNA sequence (shaded), and bases at the intron/exon borders are boxed and marked in bold. The reading frame is indicated by dots, and stop codons are underlined. Amino acids are given in one-letter code underneath the cDNA sequence, and identical residues in the Arabidopsis sequence are indicated by stars. Oligonucleotide primers used for PCR are shown as open arrows. b, Schematic depiction of the 5′-RACE procedure using *g6pdh*-specific oligonucleotide primers and poly(A^+) RNA from potato leaf and root tissue. Restriction sites are indicated on the sequence. cDNA, 5′ border of the longest plastidic cDNA clone isolated from a potato leaf cDNA library. Primer positions are indicated by open arrows. AP, Anchor primer. PFL038 and PFL046, *g6pdh*-specific oligonucleotides. The anchor sequence is represented by a hatched box. Borders of the resulting PCR fragments are shown as dashed lines. ?, Full-length 5′-*g6pdh* sequence unknown prior to 5′-RACE.

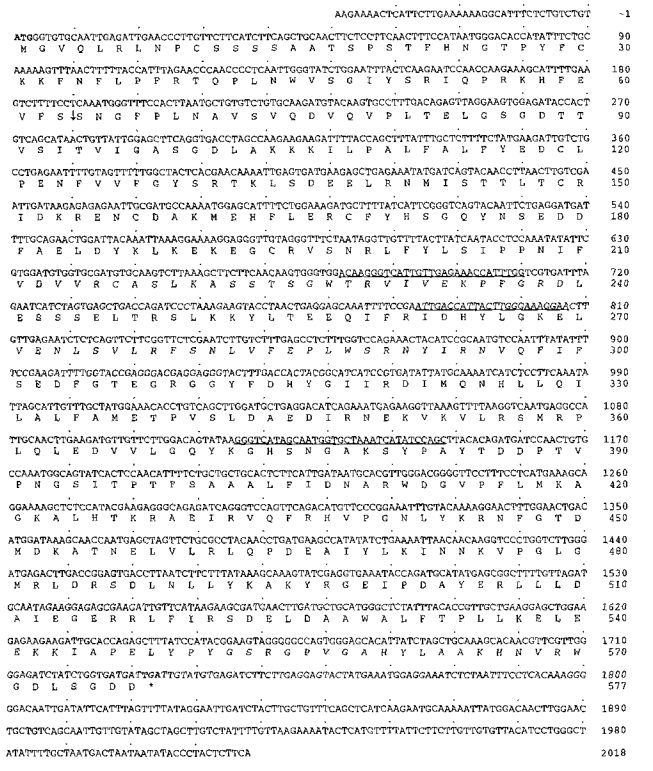

Figure 2. Nucleotide and deduced amino-acid sequence of the full-length plastidic *g6pdh* cDNA. The complete sequence was obtained by analysis of the longest cDNA clone isolated from a potato leaf cDNA library and several 5′-RACE clones [four from leaf and two from root poly(A$^+$) RNA]. The positions of the oligonucleotides for priming PCR are underlined. The missing 5′ portion in the longest cDNA clone comprises about 50 bp of the 5′ untranslated region plus the first seven amino acids of the transit peptide.

cia). For the cytosolic G6PDH construct, the 1.6-kb *Sfa*NI/*Not*I fragment of cDNA clone K4 (Graeve et al., 1994) was inserted via blunt ends. For the plastidic G6PDH construct, the entire *Eco*RI cDNA insert of one of the plastidic cDNA clones (2.8) was used.

Isopropylthio-β-galactoside-induced overexpression in *E. coli* XL1-Blue cells (Stratagene) and subsequent purification of the GST/G6PDH fusion proteins was according to Frangioni and Neel (1993), using 4% (v/v) Triton X-100 and glutathione agarose from Sigma.

Production of Isoform-Specific G6PDH Antisera

Antisera were raised in New Zealand White rabbits using 300 to 500 μg of affinity-purified GST fusion protein per subcutaneous injection. After collection of preimmune serum, the first injection was given in complete Freund's adjuvant, whereas all further boosts were done with incomplete Freund's adjuvant in 4-week intervals. Collection and processing of serum was as described by Harlow and Lane (1988).

Preparation of Protein Samples for Immunoblot Analysis

Leaf or tuber material was powdered in liquid nitrogen and suspended in protein-extraction buffer (50 mM Hepes-KOH, pH 7.0–7.4, 2 mM sodium bisulfite [$Na_2S_2O_5$], 0.1% [w/v] SDS). Insoluble proteins and membranes were pelleted by centrifugation at 14,000 rpm in a table-top centrifuge at 4°C for 5 min. Chloroplast preparations from potato leaf tissue were virtually devoid of cytosolic contamination (Quick et al., 1995). Prior to SDS-PAGE, chloroplast proteins were acetone precipitated, collected by centrifugation, and washed with 80% (v/v) ethanol. The dried pellet was resuspended in protein-extraction buffer. Estimation of protein concentration, SDS-PAGE, and subsequent immunoblot analysis were essentially as described by Graeve et al. (1994).

RESULTS

Obtaining a Probe for a Further G6PDH Isoform

Degenerate oligonucleotide primers PFL037 and PFL038 based on two highly conserved regions in the amino acid alignment of the cytosolic G6PDH sequence from potato and from *Synechococcus* were generated. We suspected that different plant *g6pdh* genes might carry introns of various lengths in the region between the two PCR primers. Thus, new G6PDH isoforms should simply be recognized by size fractionation following PCR of genomic DNA. This strategy yielded two products, using both Arabidopsis and potato genomic DNA as a template, one being of the same size as PCR fragments obtained from the cytosolic cDNA clone (120 bp), and a second one 100 bp larger in size (220 bp). Both PCR products were subcloned and sequenced, showing that the smaller fragments stem from the respective cytosolic *g6pdh* genes in both Arabidopsis and potato. Sequence analysis of the larger fragments revealed that a different *g6pdh* gene fragment containing a class II intron of about 90 bp (88 bp in potato and 85 bp in Arabidopsis) was obtained (Fig. 1a).

Determination of the Complete cDNA Sequence Encoding a New G6PDH Isoform

First, we wanted to verify that the new *g6pdh* gene is expressed in potato. Using leaf poly(A$^+$) RNA, first-strand cDNA was synthesized and used as a template for PCR with *g6pdh* primer pair PFL037/PFL038. The resulting fragments were cloned in *E. coli*. A negative hybridization screen eliminated clones encoding the cytosolic isoform. Sequence analysis of the remaining clones revealed that most contained the new *g6pdh* cDNA fragment. Subsequently, a digoxigenin-labeled probe was prepared by PCR and used to analyze a potato leaf cDNA library. Of the 10 cDNA clones carrying the new isoform, none was full length. The sequence of the longest clone was determined (Fig. 2) and the deduced amino acid sequence aligned with known G6PDHs. Figure 3 shows that the new isoform is longer at its N-terminal end. This extra sequence is rich in hydroxylated residues, interspersed with positively charged or hydrophobic amino acids, but almost devoid of negatively charged residues. These features are typical for transit sequences of nuclear-encoded proteins that are post-

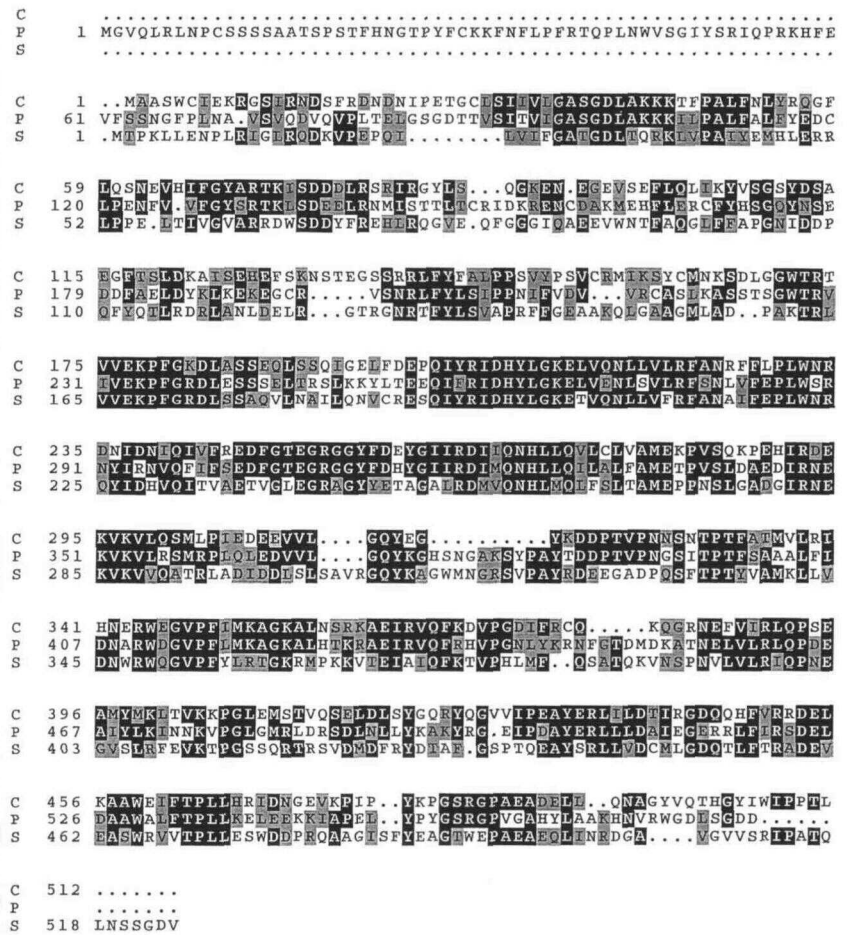

Figure 3. Alignment of G6PDH amino acid sequences from photoautotrophic organisms. The alignment was done using the Genetics Computer Group software package (Devereux et al., 1984) implemented on a DEC μVAX 3800 using the VMS operating system. The putative plastidic *g6pdh* sequence presented in this paper (P) was compared to the cytosolic isoform (C) from potato (Graeve et al., 1994) and from *Synechococcus* (S) PCC 7942 (Scanlan et al., 1992). Identical amino acid residues are marked by a black background; similar residues are indicated by a gray background. Gaps resulting from the attempt to obtain high homology during the alignment procedure appear as dots. The two plant sequences show the highest similarity (65%). Lower values are found when comparing both plant G6PDH sequences with the one from *Synechococcus* (plastidic 56%, cytosolic 54%).

translationally imported into plastids (Gavel and von Heijne, 1990; de Boer and Weisbeek, 1991). Thus, the new G6PDH isoform will be referred to as plastidic.

To obtain the missing part of the plastidic sequence, 5′-RACE was performed. A stretch that is missing in the cytosolic G6PDH sequence (Fig. 3) was chosen to synthesize an isoform-specific antisense primer (PFL046). This oligonucleotide was used to prime first-strand cDNA synthesis on potato leaf or root poly(A^+) RNA. Subsequently, 5′-RACE was done with a commercial kit and oligonucleotide PFL038 as a nested, gene-specific primer (compare with Fig. 1b). Fragments of the expected size (≥800 bp) were checked by restriction analysis with enzymes known to cut specifically in the plastidic cDNA sequence (*Hin*dIII, *Bst*EII) and subcloned. Positive clones were selected by colony hybridization and checked by restriction nuclease digests to determine if they contain larger 5′ ends than the longest cDNA clone. Sequence analysis revealed that even the 5′ untranslated ends amplified from potato leaf and root poly(A^+) RNA are identical. This indicates that in both photosynthetic and heterotrophic plant tissues the plastidic G6PDH originates from the same gene.

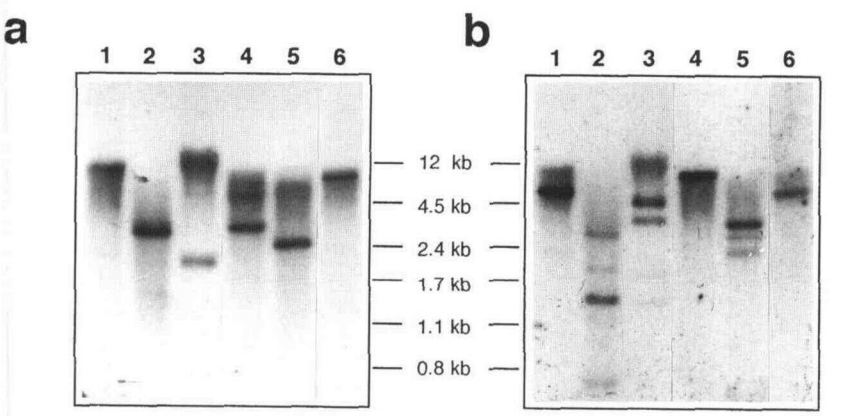

Figure 4. Genomic Southern analysis of potato DNA. Nuclear DNA of *S. tuberosum* L. var Desirée was digested with *Bam*HI (lanes 1), *Hin*cII (lanes 2), *Pst*I (lanes 3), *Xba*I (lanes 4), *Hin*dIII (lanes 5), *Eco*RV (lanes 6), and subjected to Southern analysis. *Not*I fragments, comprising the entire *g6pdh* cDNAs (1.7 kb for the cytosolic and 1.9 kb for the plastidic gene) were radioactively labeled and used as hybridization probes. a, Plastidic probe; b, cytosolic probe. The sizes of DNA molecular mass standards are indicated.

Table I. *Estimation of mRNA amounts that encode the G6PDH isozymes in various potato tissues*

Results represent data of three independent experiments. The signal strengths on the dot-blot standard filters were correlated with the intensities of the respective *g6pdh* signals in northern blots: −, Below detection limit; +, 0.7–7 pg; ++, 7–70 pg; +++, 70–250 pg; ++++, 250–700 pg.

Tissue	*g6pdh* Probes	
	Cytosolic	Plastidic
Leaf		
Sink	+	+
Source	++	+
Tuber		
Sink	++++	−
Source	++++	−
Stolons	+++	+[a]/−[b]
Roots	+++	+[c]/−[b]
Flower buds	+++	−

[a] Harvested from tissue growing above ground. [b] Harvested from tissue growing in soil. [c] Harvested from tissue growing hydroponically.

Southern and Northern Blot Analyses

Genomic Southern analysis using the cytosolic and plastidic *g6pdh* cDNA fragments as probes revealed that the plastidic enzyme is encoded by a single gene family and the cytosolic isoform is encoded by a low copy number gene family in potato (Fig. 4).

To compare the transcription patterns for both *g6pdh* genes, RNA was isolated from different potato tissues and subjected to northern analysis. Using the cytosolic *g6pdh* probe, hybridization was observed predominantly with RNA from heterotrophic tissues (i.e. tubers, stolons, roots, and flower buds), whereas the plastidic *g6pdh* probe selected RNA from leaves, stolons growing above ground, and roots of hydroponically grown potato plants. The results of several experiments are summarized in Table I. In addition, Figures 5, a and b, and 6, a and b, further document that the two isoforms exhibit tissue-specific transcription patterns in potato.

Influence of Greening and Wound Stress Conditions on *g6pdh* Expression

To check whether the expression of *g6pdh* genes in potato is influenced during the transition of etiolated to green tissue, potato tubers were placed in soil and grown in the dark. After 4 to 6 weeks, the tips of etiolated potato shoots were first harvested in darkness and then at various times during a period of 5 d after transfer to constant light. Total RNA was isolated and subjected to northern analysis. Figure 5b shows that the transcription of the plastidic G6PDH isoform is stimulated in the light concomitant with the development of green leaves. Hybridization of the same blot with the cytosolic *g6pdh* probe shows that transcription of this isoform declines over time. It is interesting that in this actively growing, etiolated tissue and within 5 d of illumination (Fig. 5a, lanes 1–5), the cytosolic gene is expressed at much higher levels than in fully developed, mature green leaves (Fig. 5a, lanes 6 and 7).

Earlier reports describe an increased flux through the OPP concomitant with elevated G6PDH activity in sliced tissue as "aging" or "wound respiration" (Muto et al., 1969; Ricardo and ap Rees, 1972). To determine whether this observation reflects an induction at the transcriptional level, we tested various stress conditions. Discs were cut from potato leaf and tuber source tissue, sliced, and incubated under aerobic conditions. Total RNA was isolated and subjected to northern analysis. Figure 6 shows that under these conditions the transcription levels of the cytosolic isoform remain unaltered in leaf and tuber tissue,

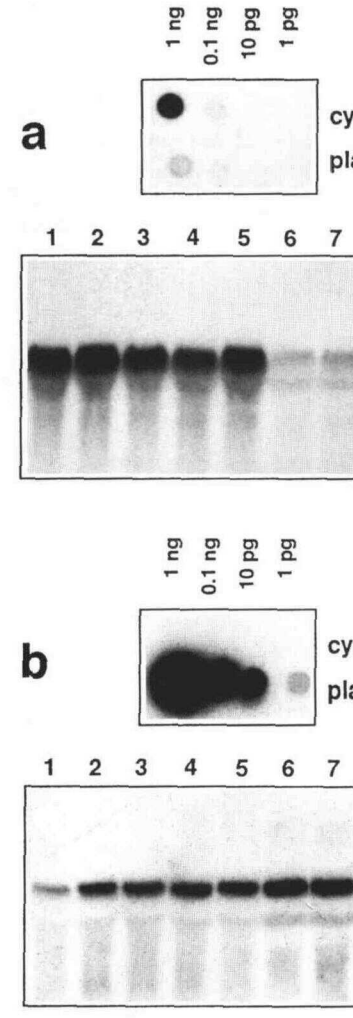

Figure 5. Time course of steady-state *g6pdh* mRNA levels in etiolated potato shoots upon illumination. Total RNA (30 μg) prepared from etiolated shoot tips (2 cm) was subjected to northern analysis. Radioactively labeled hybridization probes were the same as those used for Southern analysis. a, Cytosolic probe; b, plastidic probe. Lanes 1, Dark control; lanes 2, 7 h after transfer to light; lanes 3, 22 h after transfer to light; lanes 4, 48 h after transfer to light; lanes 5, 120 h after transfer to light; lanes 6 and 7, mature leaf control. For estimation of *g6pdh* mRNA levels, standard filters with dotted serial dilutions of the respective cDNA clones (cyt/pla) were developed with the blots.

whereas incubation of leaf discs in aerated medium selectively shuts off transcription of the plastidic isoform (Fig. 6, lanes 2). Systemic wounding of potato leaves did not result in any significant change of *g6pdh* gene expression (Fig. 6, lanes 3).

Immunoblot Analysis

Isoform-specific antisera were obtained after immunization of rabbits with the respective recombinant GST fusion proteins. Tissue-specific expression of the two G6PDH isoforms was analyzed by western blotting and immunoprinting after SDS-PAGE of protein extracts from green and nongreen potato tissue (Fig. 7). The cytosolic G6PDH antiserum shows a signal with potato tuber extracts, a slightly weaker signal with total leaf extracts, and no signal with isolated chloroplasts. In contrast, the plastidic G6PDH antiserum shows a signal with isolated chloroplasts, a weaker signal with total leaf extracts, and no reaction with tuber extracts.

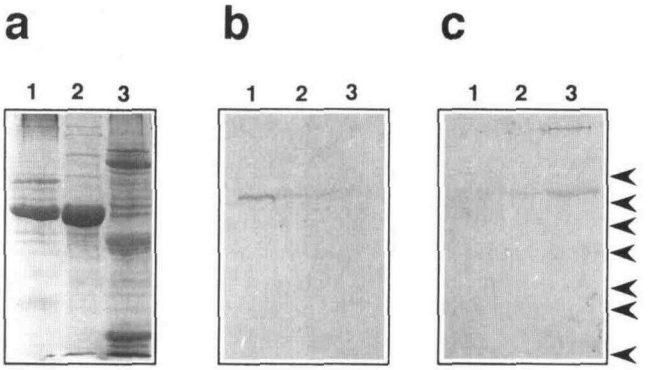

Figure 7. Detection of G6PDH isoforms on western blots. Protein extracts were prepared and subjected to immunoblot analysis (50 μg). a, Total proteins visualized by Coomassie blue staining of the SDS gel. Immunoblots were developed with plastidic G6PDH antiserum (b), and cytosolic G6PDH antiserum (c). Lanes 1, Potato chloroplast extract; lanes 2, potato leaf extract; lanes 3, potato tuber extract. Arrowheads indicate molecular mass markers (Dalton Mark VII-L, Sigma) of 66, 45, 36, 29, 24, 20, and 14.2 kD, respectively.

DISCUSSION

In plants, G6PDH is located in at least two compartments, the cytosol and the plastidic stroma, where both enzymes catalyze the rate-limiting step of the OPP. In contrast to the cytosolic isoform, in green tissues the plastidic counterpart is regulated by redox inactivation during photosynthesis, and thus operates only at night. We have previously determined a full-length cDNA sequence encoding the cytosolic enzyme from potato (Graeve et al., 1994). Using PCR technology, we now report on the complete cDNA sequence of the corresponding plastidic G6PDH isoform, shown by the alignment of the deduced amino acid sequence with two G6PDHs from potato and *Synechococcus* (Fig. 3). The significantly longer 5′ region of the new isoform most likely encodes a transit peptide. The putative cleavage site is suspected to lie between Ser^{63} and Ser^{64} [V-F-S ↓ S], following the consensus motif [(V/I)-X-(A/C) ↓ A] as defined by Gavel and von Heijne (1990). The weakness of this consensus is documented by the cleavage sites reported for various NADP-malate dehydrogenases [(V/I)-X-C ↓ S], i.e. from *Sorghum* (Crétin et al., 1990), maize (Agostino et al., 1992), pea (Reng et al., 1993), spinach, and *Selaginella* (J. Harnecker and O. Ocheretina, personal communication). Taking this into account, the start of the mature plastidic protein would coincide with the first amino acid in the cytosolic G6PDH sequence from potato and the one from *Synechococcus* (Fig. 3).

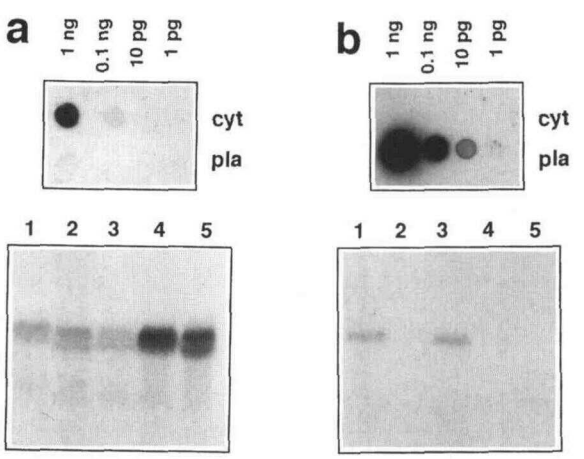

Figure 6. Analysis of mRNA levels in stressed potato leaf and tuber source tissue. Total RNA (30 μg) prepared from stressed potato tissue was subjected to northern analysis. a, Cytosolic probe; b, plastidic probe. Lanes 1, Unstressed leaf control; lanes 2, leaf discs incubated overnight (approximately 16 h) in aerated medium; lanes 3, discs cut from leaf pair next to clamped terminal leaf; lanes 4, tuber control; lanes 5, tuber discs incubated overnight in aerated medium.

Comparing the deduced amino acid sequences of G6PDHs from photoautotrophic organisms shows that higher similarity is shared by the two plant sequences as opposed to either one compared to *Synechococcus* (Fig. 3). Despite highly conserved regions in the N terminus and in the central part of the new G6PDH sequence, comprising the coenzyme binding site ^{97}GASGDLAKKK (Rossmann et al., 1975) and the active site ^{261}RIDHYLGKE (Jeffery et al., 1985), respectively, it was quite unexpected that none of the Cys residues is conserved, not even among the two redox-modulated isoforms. What is striking, however, is that all six Cys residues in the plastidic isoform described here are confined to the N-terminal half of the protein. Cys residues involved in reversible redox modifications can lead to either the formation of intramolecular disulfide bridges (see Buchanan, 1991) or the formation of mixed disulfides (Ocheretina and Scheibe, 1994). Routinely, incubation with reduced DTT is used to mimic redox inactivation of the plastidic G6PDH in vitro (Johnson, 1972) and thus to distinguish between the two plant isoforms. When both recombinant GST fusion proteins were assayed for G6PDH activity (Graeve et al., 1994) in parallel, the new chimeric enzyme responded with a 70% decrease of activity compared to the control without DTT. In contrast, the cytosolic enzyme completely retained its activity under the same assay conditions (data not shown). This further indicates

that the new *g6pdh* cDNA encodes the chloroplastic isoform and will allow for further investigation of the molecular basis of the light/dark modulation.

Antibodies raised against both recombinant enzymes detect proteins of the expected size (56 kD) on western blots. Figure 7 shows that the antiserum against the plastidic form reacts specifically with green tissue, i.e. only with extracts from leaves and chloroplasts, but not from tubers. Conversely, the antiserum against the cytosolic form does not react with proteins of a chloroplast extract, but reacts strongly with a tuber preparation. The observation that the signal for the plastidic isoform is stronger in isolated chloroplasts than in total leaf extracts (based on equal protein amounts) is in agreement with the subcellular location of the redox-modulated isoform. The selectivity of the two antisera further supports the conclusion that the cDNA clones encode two compartment-specific G6PDH isoforms.

Both cDNA sequences were used to determine gene copy number in Southern blots. The plastidic gene seems to be present as a single copy in the potato genome, whereas a small cytosolic gene family is recognized. Our finding that the 5′ cDNA sequences amplified from both potato leaf or root poly(A^+) RNA are identical indicates that higher plants possess only one gene for plastidic G6PDH. Hence, photosynthetic electron flow via the Fd/thioredoxin system would result in the diurnal inactivation of the plastidic isoform in green tissues to avoid futile cycles during CO_2 fixation and ensure permanent activity of this isoform in plastids of heterotrophic tissues, if expressed.

Qualitative and semi-quantitative northern analyses demonstrate a clearly differential expression pattern of both genes in various potato organs. In contrast to the cytosolic isoform, which is transcribed highest in heterotrophic tissues such as tubers, stolons, roots, and flower buds, expression of the new gene is more or less confined to leaves. Under certain conditions, i.e. when stolons growing above ground or roots from hydroponically grown plants are harvested, transcription of the plastidic gene is observed (Table I).

In tubers, where no expression of the plastidic G6PDH is detected, the cytosolic gene reaches its highest levels of expression (Fig. 6; Table I). We estimated that cytosolic *g6pdh* mRNA is around 100 times more abundant in tubers compared to leaves. Both plant isoforms show about comparable expression levels in leaf tissue. These findings parallel our immunoblot results (Fig. 7; data not shown).

Analysis of RNA isolated from greening potato shoots shows an influence of light and/or developmental state on the transcription of both *g6pdh* genes (Fig. 5). Expression of the plastidic isoform increases upon illumination of etiolated potato shoots, whereas the cytosolic counterpart is already expressed at elevated levels in the dark and declines during transformation to mature, green leaves. This was confirmed by immunoblot analysis (data not shown) and indicates a high demand for the products of the OPP in actively growing tissues (Eichhorn and Corbus, 1988).

Conditions resulting in aging or wound respiration (Muto et al., 1969; Ricardo and ap Rees, 1972) of potato leaf and tuber tissue lead only to slight changes of cytosolic *g6pdh* transcription levels. In contrast, the plastidic gene is shut off in leaves under these conditions (Fig. 6). However, wounding of the terminal part of a potato leaf and assaying the neighboring leaf tissue, thus testing the possibility of a systemic wound induction (Peña-Cortes et al., 1988), did not change the transcription levels of either of the plant *g6pdh* isoforms.

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