Molecular Characterization of Potato Fumarate Hydratase and Functional Expression in *Escherichia coli*

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The tricarboxylic acid cycle enzyme fumarase (fumarate hydratase; EC 4.2.1.2) catalyzes the reversible hydration of fumarate to L-malate. We report the molecular cloning of a cDNA (*SfFum-1*) that encodes fumarase from potato (*Solanum tuberosum* L.). RNA blot analysis demonstrated that *SfFum-1* is most strongly expressed in flowers, immature leaves, and tubers. The deduced protein contains a typical mitochondrial targeting peptide and has a calculated molecular mass of 50.1 kD (processed form). Potato fumarase complemented a fumarase-deficient *Escherichia coli* mutation for growth on minimal medium that contains acetate or fumarate as the sole carbon source, indicating that functional plant protein was produced in the bacterium. Antiserum raised against the recombinant plant enzyme recognized a 50-kD protein in wild-type but not in *SfFum-1* antisense plants, indicating specificity of the immunoreaction. A protein of identical size was also detected in isolated potato tuber mitochondria. Although elevated activity of fumarase was previously reported for guard cells (as compared with mesophyll cells), additional screening and genomic hybridization data reported here do not support the hypothesis that a second fumarase gene is expressed in potato guard cells.

Mitochondrial metabolism is important for a variety of different processes in higher plants, including ATP production, photosynthesis, and the formation of precursors for amino acid synthesis (Moore and Beechy, 1987; Douce and Neuburger, 1989; ap Rees, 1990; Raghavendra et al., 1994). The TCA cycle represents one of several pathways that operate in mitochondria. Although the presence of the TCA cycle in higher plant cells is well accepted, the precise functional roles of the cycle for plant metabolism, growth, and development are still a matter of discussion. Our laboratory has recently isolated cDNA clones that encode mitochondrial citrate synthase from various plant species, including potato (*Solanum tuberosum* L.; Landschütze et al., 1995a), tobacco, sugar beet, and poplar tree (U. La Cognata, V. Landschütze, L. Willmitzer, and B. Müller-Röber, unpublished data). During these studies we have been able to achieve a substantial reduction of mitochondrial citrate synthase activity in transgenic potato plants using an antisense RNA approach (Landschütze et al., 1995b).

prisingly, shoot growth as well as tuber yield appeared to be largely unaffected in the transgenic plants, even when mitochondrial citrate synthase activity was reduced to less than 15% of the wild-type activity. In contrast, flower formation was impaired in the transgenic plants, indicating that mitochondrial citrate synthase (and hence TCA cycle activity) serve an important role during flowering in potato plants (Landschütze et al., 1995b).

To further investigate the role of the TCA cycle in higher plant cells we are analyzing mitochondrial fumarase (fumarate hydratase; EC 4.2.1.2), which catalyzes the reversible hydration of fumarate into L-malate. Because fumarase is often used as a marker enzyme for mitochondria, its presence has been demonstrated in many plants, including monocotyledonous and dicotyledonous species (see various reports in Lambers and van der Plas, 1992).

Two distinct classes of fumarases have been identified. Class I fumarases are dimeric, iron-sulfur-containing enzymes composed of two identical subunits of approximately 57 to 60 kD (Woods et al., 1988b; Ueda et al., 1991; Reaney et al., 1993). Class I fumarases have been shown to occur in *Escherichia coli* (*fumA* and *fumB* gene products; Miles and Guest, 1984; Bell et al., 1989; Woods et al., 1988b), *Bradyrhizobium japonicum* (Acuna et al., 1991), and *Bacillus stearothermophilus* (*FumA* protein; Reaney et al., 1993). Immunological and biochemical data indicate that a class I enzyme is also present in mitochondria of the eukaryotic alga *Euglena gracilis* (Shibata et al., 1985; Woods et al., 1988b). Class II fumarases are tetrameric enzymes composed of identical subunits of approximately 48 to 50 kD each. Their presence has been demonstrated in several bacteria, including *E. coli* (*fumC* gene product; Woods et al., 1986, 1988b; Ueda et al., 1991), *Bacillus subtilis* (*CitG* protein; Miles and Guest, 1985), *B. japonicum* (*FumC* protein; Acuna et al., 1991), and the thermophilic archaeabacterium *Sulfolobus solfataricus* (*FumC* protein; Colombo et al., 1994). The fumarase proteins from *Saccharomyces cerevisiae*, mammals, and higher plants are also class II enzymes (Wu and Tzagoloff, 1987; Sacchettiini et al., 1988; Suzuki et al., 1989). Class I and class II fumarases exhibit a low degree of sequence homology (less than 20% identity on the amino acid level; Reaney et al., 1993; Colombo et al., 1994). It is interesting that class II fumarase shows significant homologies to aspartase (e.g., 38% amino acid identity between *E. coli* FumA and FumC aspartase; Woods et al., 1986), indicating

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Abbreviations: EST, expressed sequence tag; IPTG, isopropyl-β-D-thiogalactopyranoside; TCA cycle, tricarboxylic acid cycle.
ing close structural and evolutionary relationships (Woods et al., 1988a). Although the primary structures are known for a few eukaryotic fumarases, including those from mammals (pig, Sacchettini et al., 1988; rat, Suzuki et al., 1989; human, Kinsella and Doornan, 1986) and fungi (S. cerevisiae FUM1 protein, Wu and Tzagoloff, 1987; Rhizopus oryzae FumR protein, Friedberg et al., 1995), to our knowledge, no primary structures have been described for plant fumarases, and no plant mutants deficient in fumarase activity are known. Antisense RNA inhibition of fumarase in transgenic plants would allow us to study the role of fumarase in higher plants in more detail. To achieve this goal we initiated a molecular approach on plant fumarases. Here we report the molecular cloning of potato fumarase and its functional expression in E. coli. We also provide evidence that the guard-cell-localized fumarase is identical to the fumarase that is expressed in other cell types of the potato leaf.

MATERIALS AND METHODS

Enzymes and Chemicals

Enzymes for DNA restriction and modification were obtained from New England Biolabs (Beverly, MA) and Boehringer Mannheim. Reagents for PAGE were obtained from Bio-Rad, Roth (Karlsruhe, Germany), and Sigma. Other chemicals were from Boehringer Mannheim, Sigma, or Merck (Darmstadt, Germany).

Bacteria and Plants

Escherichia coli was cultivated at 37°C in YT medium supplemented with appropriate antibiotics using standard methods (Sambrook et al., 1989). E. coli strain XL-1 Blue (Stratagene) was used for screening procedures using λZAP II. E. coli strains DH5α (BRL) and JM109 (Clontech, Palo Alto, CA) were used for DNA-cloning experiments. E. coli mutant EJ1353 (Guest and Roberts, 1983) was obtained from the E. coli Genetic Stock Center (Yale University, New Haven, CT).

Agrobacterium tumefaciens strain C58Cl:pGV2260 (Deblaere et al., 1985), transformed with a binary plasmid harboring the chimeric StFum-1 antisense gene (see below), was cultured at 28°C in YEB medium (Vervliet et al., 1975), supplemented with rifampicin (100 μg/mL) and kanamycin (50 μg/mL).

Solanum tuberosum L. cv Désirée was obtained from Saat- zucht Fritz Lange (Bad Schwartau, Germany). Plants in soil were grown from tubers in a greenhouse with an approximately 16-h light (22°C) and 8-h dark (15°C) period and were grown in individual pots and watered daily.

DNA Manipulations and Sequence Analysis

Standard DNA manipulation procedures were as described by Sambrook et al. (1989). The dideoxy chain termination method (Sanger et al., 1977) was used to determine DNA sequences using the T7 sequencing kit from Pharmacia. Commercial sequencing primers (Pharmacia) or specifically synthesized oligonucleotides (Tib Molbiol, Berlin, Germany) were used for sequencing. Sequence analysis was performed with the help of the programs of the Wisconsin Genetics Computer Group (GGG Package, version 8.1; Devereux et al., 1984). The Fasta (Pearson and Lipman, 1988) and Blast (Altschul et al., 1990) search programs were used for sequence comparisons of the cDNA and amino acid sequences in the GenBank, EMBL, dbEST, and SwissProt databases. Optimal alignments between sequences (Table I) were determined using the program Best-fit, and sequence alignments (Fig. 1) were performed using default parameters of the programs Pileup and Prettybox.

Screening of a Potato Leaf cDNA Library

A rice (Oryza sativa) EST (GenBank accession no. D39538), showing strong similarity (Blast probability score better than 10^-20) to fumarase-coding sequences from bacterial and animal sources, was used as a hybridization probe to isolate potato fumarase cDNA clones. The 0.3-kb-long EST fragment was amplified via PCR using 20 ng of first-strand rice cDNA as template. Rice cDNA was established from 5 μg of rice leaf poly(A)+ RNA using a cDNA synthesis kit (Pharmacia) according to the manufacturer’s instructions. Oligonucleotides RFum-1 (5'-AAAAAGTCGCCCGATAGGTAATG-3') and RFum-2 (5'-GAAATGTATCATTTGAGGACTGTGATCTG-3'), corresponding to the two ends of the EST sequence, were used as amplification primers during the PCR (temperature profile: 40 s at 94°C, 30 s at 50°C, 1 min at 72°C, 40 cycles). Approximately 5 × 10⁵ plaque-forming units of a λZAP II cDNA library established from potato (S. tuberosum L. cv Désirée) whole leaves (Kossmann et al., 1992) was screened using the amplified rice DNA fragment as a hybridization probe. Radioactive labeling of DNA fragments with [α-³²P]dCTP (Amersham) was performed using a random prime labeling kit from Boehringer Mannheim. Filters were hybridized overnight at 42°C in 250 mM sodium phosphate buffer (pH 7.2) containing 7% SDS, 1% BSA, and 1 mM EDTA. Filters were washed at 42°C in 5× SSC, 0.5% SDS, 5% formamide (20 min) and in 3× SSC, 0.5% SDS (10 min). Plaque-purified phages were converted to pBluescript SK derivatives by in vivo excision, using the helper phage Exasslist, according to the protocol given by the manufacturer (Stratagene). Rescued phagemids were investigated by restriction analysis and DNA sequencing.

Screening of a Potato Epidermal Fragment cDNA Library

We have recently established a λZAP II cDNA library from potato leaf epidermal fragments. In a typical epidermal fragment preparation, more than 90% of the RNA-containing cells are stomatal guard cells (J. Kopka, N. Provart, and B. Müller-Röber, unpublished data). The cDNA library was screened with the potato StFum-1 cDNA (Amp718/BamHI insert of phagemid pFum-1; see “Results and Discussion”) under reduced stringency (same conditions as described above for the screening with the rice EST fragment). Phage clones hybridizing to the StFum-1 cDNA were processed as described above.

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Nucleic Acid Analysis

Potato tissues used for RNA extraction were frozen in liquid nitrogen and extracted according to the method of Logemann et al. (1987). RNA was denatured in 40% formamide, subjected to agarose gel electrophoresis (1.5% agarose, 15% formaldehyde; 50 µg of total RNA/lane), and blotted in 20× SSC onto nylon membranes (Hybond N, Amersham). The radioactively labeled Asp718/BamHI cDNA insert of phagemid clone pFUM-1 (see "Results and Discussion") was used as a hybridization probe. For control hybridizations a cDNA representing potato 25S RNA (N. Provart, L. Willmitzer, and B. Müller-Röber, GenBank accession no. R28706) was used. Membranes were hybridized at 60°C in 250 mM sodium phosphate buffer (pH 7.2) containing 7% SDS, 1% BSA, and 1 mM EDTA. Filters were washed at 60°C in 3× SSC, 0.5% SDS (20 min) and in 0.2× SSC, 0.5% SDS (20 min) and subjected to autoradiography (X-Omat AR film, Kodak) between intensifying screens for 1 to 2 d at −70°C. Phosphor imaging analysis for quantification of expression levels was performed using ImageQuant 3.3 software (Molecular Dynamics, Krefeld, Germany). Each experiment was repeated twice with RNA isolated from different sets of plants.

Genomic DNA was extracted from potato leaves according to the method of Rogers and Bendich (1985). DNA blot analysis was performed as described previously (Fieuw et al., 1995). The complete StFum-I cDNA was used as a radioactively labeled hybridization probe, and after hybridization membranes were washed as indicated in the legend to Figure 7.

Antisense Inhibition of Fumarase in Transgenic Potato Plants

To construct the chimeric fumarase antisense gene, the complete StFum-I cDNA of phagemid pFUM-1 (see "Results and Discussion") was inserted as an Asp718/BamHI fragment into the corresponding restriction sites of the binary vector pBinAR (Höfgén and Willmitzer, 1990). This cloning step placed the StFum-I cDNA in reverse orientation behind the near-constitutive cauliflower mosaic virus 35S promoter. Transgenic potato plants were obtained by A. tumefaciens-mediated leaf disc transformation as described previously (Rocha-Sosa et al., 1989). Plants with reduced expression of fumarase were selected by RNA blot analysis on leaf tissue using the StFum-I cDNA as a radioactively labeled hybridization probe (data not shown). Two independent transgenic lines, T15 and T56, were used for immunoblot analysis (Fig. 6B).

Expression of Potato Fumarase in E. coli Mutant EJ1535

To express potato fumarase in E. coli, the presumptive mitochondrial targeting sequence of the potato enzyme (see "Results and Discussion") was removed by PCR, and the modified cDNA fragment was cloned into the expression vector pKK388-1 (Clontech). Primers Fum-Nco and Fum-Int were used as forward and reverse primers, respectively, to amplify the 5′ part of the potato fumarase-coding region from template plasmid pFum-1. Primer Fum-Nco (5′-AGGTCCATGGCTAC GTCTTTCAGGAGAAA-GAG-3′; the NcoI site is underlined, the added start codon is bold, and the second codon is in italics) covered the coding region that represented amino acids 1 to 8 of the mature fumarase protein. A new ATG initiation codon was introduced, and the first amino acid of the mature protein (Ser) was changed to an Ala by introducing an NcoI restriction site (in vector pKK388-1 the NcoI site surrounding the ATG start codon follows the ribosome-binding site that is required for efficient expression of proteins in E. coli). Primer Fum-Int (5′-CATAGACTCTATCGATTCC-3′) covered an internal sequence approximately 100 bp downstream of the unique EcoRI site that was at position 681 to 686 of the StFum-1 cDNA fragment. PCR amplification (temperature profile: 1 min at 95°C, 1 min at 50°C, 1 min at 72°C; 40 cycles) was performed according to standard procedures. The amplified DNA fragment was cloned as a 0.6-kb NcoI/EcoRI fragment into plasmid pKK388-1, resulting in plasmid pKK-Fum-6. Correct amplification of the cDNA fragment was confirmed by DNA sequence analysis. Finally, the approximately 1.2-kb-long EcoRI fragment of plasmid pFum-1 was inserted in its correct orientation into the EcoRI site of plasmid pKK-Fum-6, leading to the creation of the truncated potato fumarase-coding region in frame with the ATG initiation codon of the expression vector. The resulting plasmid, pKK-Fum-9, schematically presented in Figure 4, was transformed into the fumarase A-deficient E. coli mutant EJ1535 (genotype: F−, λ−, fumA1, and spoT1; Guest and Roberts, 1983). The empty cloning vector pKK388-1 was used as a control. For growth analyses (see "Results and Discussion") E. coli cells were plated on M9 minimal medium (Sambrook et al., 1989) supplemented with Arg (30 µg/mL) and thiamine (5 µg/mL). Carbon sources (malate, fumarate, or acetate) were added as potassium salts. IPTG (1 mM) was added to induce expression of proteins from the trc promoter of plasmids pKK388-1 and pKK-Fum-9. Growth of cells was at 37°C, and for determination of fumarase activity cells were grown overnight in liquid YT medium (Sambrook et al., 1989), diluted 1:100 into fresh YT medium, and grown to an A600 of 0.3 to 0.4, and expression of proteins from the trc promoter was induced by addition of 1 mM IPTG (final concentration). Cells were harvested 4 h later by centrifugation, and proteins were extracted as described below.

Production of Antibodies Directed against Potato Fumarase

The pMal-c2 system (New England Biolabs, Beverly, MA) was used to produce antibodies directed against potato fumarase. The 1.2-kb-long EcoRI/XbaI StFum-1 cDNA fragment (encoding the C-terminal half of the fumarase protein) of plasmid pFum-1 was cloned into the EcoRI/XbaI sites of plasmid pMal-c2, resulting in an in-frame fusion of the E. coli maltose-binding protein and the potato fumarase, as verified by sequence analysis (not shown). Purification of the fusion protein from IPTG-induced E. coli cells was performed according to the manufacturer’s instructions, and immunization of rabbits was performed by Eurogentec (Seraing, Belgium).
SDS-PAGE and Western Blot Analysis

To obtain crude protein extracts, frozen E. coli cells or plant material was homogenized in extraction buffer (50 mM Hepes-KOH, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 0.5 mM PMSF, 10% glycerol, and 0.1% Triton X-100) and centrifuged (for 5 min at 15,000g and 4°C), and the clarified supernatant was used for gel electrophoresis. Proteins were separated on 10% SDS-polyacrylamide gels (Garfin, 1990). Western blot analysis was performed as described previously (Landschütze et al., 1995b). The antiserum raised against potato fumarase (see above) was used at a 1:500 dilution in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20, and 1% BSA. Preimmune serum was used as a control to verify specificity of reactions. Tuber mitochondria were isolated as described previously (Landschütze et al., 1995b). All experiments were repeated at least twice.

Determinition of Fumarase Activity

Proteins were extracted as described above for PAGE. Fumarase activity was assayed spectrophotometrically at 30°C by the procedure of Hill and Bradshaw (1969). Protein concentrations were determined using the Bio-Rad protein assay, with BSA as a standard.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of a cDNA Encoding Potato Mitochondrial Fumarase

To isolate a potato cDNA clone encoding mitochondrial fumarase, we screened a λZAP II cDNA library that was established from potato leaves using a rice EST amplified via PCR (for details see "Materials and Methods"). Phage clones that gave positive signals in the first round of screening were plaque-purified and converted to pBluescript SK derivatives by in vivo excision. Partial DNA sequences were obtained for cDNA inserts of seven independent clones, and homologies to known fumarase sequences from other organisms were identified. All cDNAs appeared to be representatives of a single gene, designated StFum-1, as judged from the complete identity of sequences, including the 3' untranslated regions (not shown). The longest cDNA insert, present in clone pFum-1, was sequenced in its entire length (GenBank accession no. X91615). The cDNA had a length of 1865 bp, with an ATG start codon at position 41 and a TGA stop codon at position 1785. An in-frame stop codon upstream of the ATG indicated that the complete coding region was covered by the cDNA. The 1479-bp-long open reading frame encodes fumarase with a calculated molecular mass of 50.1 kD.

At this stage we do not have firm proof that fumarase encoded by the StFum-1 cDNA represents a mitochondrial enzyme. However, to our knowledge no solid data are available showing the presence of extramitochondrial fumarases in higher plants (in fact, fumarase is often used as a mitochondrial marker enzyme). In addition, the data given below indicate the presence of a mitochondrial targeting peptide in the deduced protein sequence.

Sequence Comparison of Potato Fumarase with Fumarases from Other Organisms

An amino acid sequence alignment of class II fumarases from different organisms is shown in Figure 1. Mitochondrial proteins encoded by the nuclear genome require N-terminal targeting peptides for sorting to the mitochondrial compartment (Hartl et al., 1989; Moore et al., 1994). A 41-amino acid-long targeting peptide was determined for mitochondrial fumarase from rat liver, with ASQNS representing the first amino acids of the mature (i.e. processed) protein (Suzuki et al., 1989; Fig. 1). A similar N terminus (ASQDST) was determined for the purified mitochondrial fumarase from pig liver (Sacchettini et al., 1986; Fig. 1).

To our knowledge no N-terminal sequences are known for mature plant fumarases. However, by adopting the rules obtained by von Heijne et al. (1989), we predict a 29-amino acid-long transit peptide with Phe⁹ at its C-terminal end for potato fumarase. The presumptive targeting sequence has an Arg residue at position −3 and is highly enriched in the amino acids Ala, Leu, Arg, and Ser, which is typical for mitochondrial transit peptides. In the case of the potato fumarase-targeting peptide, 19 of 29 residues are represented by these amino acids (Fig. 1). The processed (i.e. mature) potato fumarase would consist of 464 amino acids with a calculated molecular mass of 50.1 kD.

Table I indicates the homologies of the potato fumarase, as deduced from its corresponding cDNA sequence, with fumarase sequences known from other organisms. The data presented in Table I were deduced from the alignment shown in Figure 1. Potato fumarase showed the greatest similarity to mammalian and fungal fumarases, with a degree of identical amino acids of 67 to 71%. In contrast, the plant fumarase is 40 to 63% identical with the bacterial class II enzymes, with the least similarity found to the fumarase of the thermophilic archaeabacterium Sulfolobus solfataricus. Weak homologies were detected between the bacterial enzymes, and in some cases these similarities were weaker than those between the potato and the bacterial proteins. For instance, the Bradyrhizobium japonicum and the potato fumarases share 62.9% identical amino acids, whereas the enzymes from E. coli and B. japonicum have only 57.4% identical amino acids (Table I). We also compared the potato enzyme with the fumarase sequences deduced from plant ESTs that were deposited in the databases (not included in Table I). The partial fumarase protein (94 amino acids long) deduced from an Arabidopsis thaliana EST (GenBank accession no. T21883) showed 85.1% identity (94.7% similarity) to amino acids 356 to 449 of the potato protein. The partial protein sequence (88 amino acids long) deduced from a rice EST (GenBank accession no. D39558) was 87.5% identical and 94.3% similar to potato fumarase (amino acids 83–170), indicating extensive...
similarity between fumarases from dicotyledonous and monocotyledonous plant species.

Chemical modification of pig heart fumarase by iodoacetate has suggested that a His residue is part of the active site (Bradshaw et al., 1969). The sequence alignment in Figure 1 shows that only two His residues (positions 158 and 217 of the potato protein [unprocessed form]) are conserved in all class II fumarases (indicated by open arrows in Fig. 1). His residues corresponding to potato His217 are also present in the two E. coli class I fumarases (fumarase A and B; Miles and Guest, 1984; Bell et al., 1989; data not shown). This amino acid residue, therefore, might represent the active-site His, as observed by Bradshaw et al. (1969) for the pig fumarase.

**StFum-1 mRNA Expression in Different Tissues of Potato**

To investigate expression of potato fumarase, total RNA was isolated from various tissues of greenhouse-grown potato plants and probed with the *StFum-1* cDNA. The result of a typical RNA blot hybridization experiment is shown in Figure 2. Highest expression was found in flowers of different developmental stages, in sink leaves, and in tubers. High mRNA expression in flowers also was observed for another TCA cycle enzyme from potato, mitochondrial citrate synthase (Landschütze et al., 1995a), which is consistent with the hypothesis that TCA cycle activity plays an important role in flower formation (Landschütze et al., 1995b). When total RNA was isolated from...
leaves at different developmental stages and probed against the StFum-1 cDNA, a steady decrease in expression was observed upon leaf maturation (Fig. 3, top). Based on phosphor imager analysis of representative autoradiograms, we calculated an approximately 1.5- to 2-fold higher mRNA expression in immature potato leaves is reflected in the protein level has to await further analysis. No major differences in StFum-1 mRNA expression were detected when tubers of increasing fresh weight were used as a source of RNA (data not shown). The same pattern of expression was observed when a DNA fragment specific for the 3' untranslated region of the StFum-1 cDNA was used as a hybridization probe (data not shown).

### Functional Expression of Recombinant Potato Fumarase in E. coli

We wanted to know whether the potato StFum-1 cDNA would encode a functional fumarase protein. In an initial experiment E. coli cells harboring plasmid pFum-1 were investigated with respect to fumarase activity. In these experiments no difference was found between E. coli cells carrying plasmid pFum-1 and those containing control plasmid pBluescript SK (data not shown). We attributed this observation to an in-frame stop codon present a few base pairs upstream of the potato fumarase start codon (see above). Alternatively, the fumarase-targeting peptide could have interfered with a functional fumarase protein. To avoid these circumstances, the potato fumarase cDNA (omitting the presumptive targeting sequence) was cloned into plasmid pKK388-1, yielding plasmid pKK-Fum-9 (Fig.

![Figure 2. StFum-1 mRNA expression in various tissues of potato plants. Total RNA (50 µg/lane) was separated on an agarose gel, blotted onto a nylon membrane, and hybridized to the radioactively labeled StFum-1 cDNA insert of plasmid pFum-1. The transcript detected by the probe had a size of approximately 1.9 kb. RNA was isolated from the following tissues: lane 1, colored flower buds; lane 2, open flowers; lane 3, sink leaves; lane 4, petiole; lane 5, stem (internodes); lane 6, roots; lane 7, stolons; and lane 8, tubers (5-10 g fresh weight).](image)

![Figure 3. StFum-1 mRNA expression in leaves and tubers of different developmental stages. Equal amounts of total RNA (50 µg/lane) were subjected to electrophoresis in an agarose gel, transferred to a nylon membrane, and hybridized to the StFum-1 cDNA. Top, Leaves; bottom, tubers. Leaf lengths (cm) were: lane 1, <1; lane 2, 1 to 2; lane 3, 3 to 4; lane 4, 6 to 8; and lane 5, >12. Tubers had the following fresh weights (g): lane 1, 0.03; lane 2, 0.3; lane 2, 0.3 to 1; lane 5, >12. The probe used was the StFum-1 cDNA, an approximately 1.5- to 2-fold higher expression in immature potato leaves is reflected in the protein level has to await further analysis.](image)
by the StFum-1 cDNA is indeed a mitochondrial isoform. A harvest 4 h after start of IPTG induction) than activities higher (108.0 ± 73.8 nmol mg⁻¹; n = 5; Student's t-test, P = 0.02).

To test the integrity of recombinant potato fumarase expressed in E. coli cells (containing plasmid pKK-Fum-9), an antibody was raised against the potato enzyme (for details see “Materials and Methods”). Protein extracts were prepared from E. coli cells that were harboring the plasmid pKK388-1 or pKK-Fum-9 as well as from potato leaves and isolated potato tuber mitochondria. Immunoblot analysis (Fig. 6A) indicated the presence of a protein of the expected size (approximately 50 kD) in E. coli cells containing plasmid pKK-Fum-9. This protein was not detected when the empty cloning vector was present in E. coli. The protein detected by the antiserum in tuber mitochondria was of the same size as the recombinant potato fumarase expressed in the bacterial background. These data indicated that the plant protein is smaller than calculated from the complete coding sequence of the cloned cDNA. This observation is consistent with the assumption that the fumarase encoded by the StFum-1 cDNA is indeed a mitochondrial isomorph. A faint band of the right size (below the major Rubisco large subunit protein band) was also visible in crude protein extracts prepared from whole leaves.

To prove that the antiserum recognized its cognate protein in plant extracts we used transgenic potato plants transformed with a chimeric fumarase antisense gene (see materials and methods). Transgenic plants with reduced StFum-1 mRNA expression were subjected to immunoblot analysis using the antiserum raised against the recombinant potato fumarase. As is shown in Figure 6B, transgenic plants did not express the 50-kD fumarase protein present in leaves of untransformed control plants.

In conclusion, these data clearly indicate that potato fumarase was functionally expressed in the prokaryotic background and was faithfully detected in plant extracts by the anti-fumarase antiserum.

Figure 5. Complementation of the fumarase A-deficient E. coli mutant. Plasmids pKK388-1 (empty vector) and pKK-Fum-9 (vector harboring the potato fumarase cDNA) were transformed into the E. coli mutant EJ1535 and plated onto M9 minimal medium containing malate, acetate, or fumarate (40 μM each) as the sole carbon source. Plates were incubated overnight at 37°C.

“Materials and Methods”). Transgenic plants with reduced StFum-1 mRNA expression were subjected to immunoblot analysis using the antiserum raised against the recombinant potato fumarase. As is shown in Figure 6B, transgenic plants did not express the 50-kD fumarase protein present in leaves of untransformed control plants.

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Figure 6. Immunodetection of potato fumarase. A, Potato fumarase expressed in the E. coli mutant EJ1535. Proteins (30 μg/lane) were extracted from the T56 (E. coli mutant) and T15 (E. coli mutant harboring the potato fumarase cDNA) as well as from untransformed wild-type (WT) plants. Lines T56 and T15 were preselected on the basis of low StFum-1 mRNA expression. B, Immunodetection of fumarase in potato leaves. Proteins (40 μg/lane) were extracted from transgenic StFum-1 antisense lines T56 and T15 as well as from untransformed wild-type (WT) plants. Lines T56 and T15 were preselected on the basis of low StFum-1 RNA expression (not shown). The major band seen in all preparations corresponds to the Rubisco large subunit (demonstrating equal protein loading in all lanes). Fumarase was, as expected for antisense plants, virtually absent in T56 and T15 (arrow). Two independent protein preparations of each line were run.

Figure 4. Construct used for expression of potato fumarase in E. coli cells. The P<sub>trc</sub> promoter/potato fumarase cDNA region of plasmid pKK-Fum-9 used for complementation of the E. coli mutant EJ1535 is shown. A comparison of the amino acid sequences of the original potato enzyme surrounding the cleavage site (indicated by an arrow) and the modified sequence in pKK-Fum-9 is presented. The NcoI recognition site, covering the ATG start codon (underlined) of the modified protein, is shown in italics. P<sub>trc</sub>, IPTG-inducible trc (trp/lac fusion) promoter.
Fumarase Expressed in Guard Cells Is Probably Encoded by the Same Gene Expressed in Other Leaf Cells

Respiratory reactions represent an essential component of the metabolism and movement of stomatal guard cells (Raghavendra and Vani, 1989; Gautier et al., 1991; Vani and Raghavendra, 1994). Specific activity of fumarase was found to be 3- to 7-fold higher on a chlorophyll basis (4-fold higher on a protein basis) in dissected guard cells than in the mesophyll cells of Vicia faba leaves (Hampp et al., 1982). Similarly, in pea (Pisum sativum) fumarase activity was 2-fold higher in guard cell protoplasts than in mesophyll cell protoplasts (Vani and Raghavendra, 1994). Recently, we showed that another enzyme, i.e. PEP carboxylase of potato guard cells, is encoded by a gene different from the one encoding the mesophyll PEP carboxylase isomerase (U. La Cognata and B. Müller-Röber, unpublished data). We wanted to know whether a similar situation would exist for fumarase expressed in potato guard cells. To evaluate this issue we performed the following two experiments: (a) We screened a cDNA library established from potato leaf epidermal fragments highly enriched for guard cells, using the StFum-1 cDNA as a radioactively labeled probe. Hybridization was performed under reduced stringency to allow fumarase cDNA clones different from the StFum-1 cDNA to be discovered. Several cross-hybridizing phage clones were isolated, and cDNA inserts were investigated by partial sequence analysis from both ends. From the 12 independent clones obtained, all appeared to be representatives of the StFum-1 gene. (b) We performed a low-stringency DNA blot hybridization on potato genomic DNA, using the StFum-1 cDNA as a radioactively labeled probe. As shown in Figure 7, the same fragments were detected under low- and high-stringency washing conditions. Taken together these data indicate that fumarase from guard cells and other potato (leaf) cells is probably encoded by the same gene.

CONCLUDING REMARKS

We report here the molecular cloning and characterization of the first mitochondrial fumarase from a plant species, i.e. potato. Previous biochemical data and the sequence information presented here indicate that plant enzymes belong to the class II fumarases, originally described for the bacterial proteins (Woods et al., 1988b; Ueda et al., 1991). Although a class I fumarase was detected in the mitochondria of the green alga Euglena gracilis (Shibata et al., 1985; Woods et al., 1988b), there are no biochemical or molecular data supporting the view that class I fumarases are present in higher plant tissues.

The availability of the StFum-1 cDNA will allow several interesting experiments to be performed in the future: (a) It should be possible to produce high amounts of fumarase protein by heterologous expression in microbial systems such as E. coli or Pichia pastoris (for review, see Faber et al., 1995). Recombinant protein produced in this way should be useful for biochemical analysis of potato fumarase and should allow crystals to be obtained for structural analyses. (b) Biochemical studies have indicated interactions between several enzymes of the TCA cycle, leading to the metabolon concept of supramolecular organization of TCA cycle components (see Srere, 1987, and refs. therein). We previously cloned the mitochondrial isozymes of citrate synthase (Landschütze et al., 1995a) and malate dehydrogenase (G. Nast and B. Müller-Röber, unpublished data) from potato. It might be possible to identify domains within fumarase, citrate synthase, and malate dehydrogenase involved in these interactions using the yeast-based two-hybrid system, which allows the detection of interactions between proteins in vivo (for a recent review, see Fields and Sternglanz, 1994). (c) Physiological analysis of StFum-1 antisense plants should allow the investigation of the role of the TCA cycle and fumarase for higher plant cells in greater detail.

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