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One of two different ADP-glucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose

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Summary. The key regulatory step in starch biosynthesis is catalyzed by the tetrameric enzyme ADP-glucose pyrophosphorylase (AGPase). In leaf and storage tissue, the enzyme catalyzes the synthesis of ADP-glucose from glucose-1-phosphate and ATP. Using heterologous probes from maize, two sets (B and S) of cDNA clones encoding potato AGPase were isolated from a tuber-specific cDNA library. Sequence analysis revealed homology to other plant and bacterial sequences. Transcript sizes are 1.9 kb (AGPase B) and 2.1 kb (AGPase S). Northern blot experiments show that the two genes differ in their expression patterns in different organs. Furthermore, one of the genes (AGPase S) is strongly inducible by metabolizable carbohydrates (e.g. sucrose) at the RNA level. The accumulation of AGPase S mRNA was always found to be accompanied by an increase in starch content. This suggests a link between AGPase S expression and the status of a tissue as either a sink for or a source of carbohydrates. By contrast, expression of AGPase B is much less variable under various experimental conditions.

Key words: ADP-glucose pyrophosphorylase – Potato – Starch synthesis – Sucrose induction

Introduction

Carbohydrates are produced during photosynthesis in mature leaves, which act as source tissues (Turgeon 1989). They must then be transported to sites of carbohydrate demand, so-called sink tissues. Two kinds of sink can be distinguished (Ho 1988). In utilization sinks (e.g. meristem tissue and immature leaves) imported assimilates are mainly subjected to respiration and used for biosynthesis of cellular structures. Storage sinks such as tubers, seeds, and fruits use most of the imported assimilates for production of carbohydrates (mainly sucrose and starch) which accumulate and thereby serve storage functions.

In potato plants, developing tubers represent major sink organs, accumulating high levels of starch in their amyloplasts. The sink status of a tuber is reversible. Upon sprouting, the tuber starts to degrade starch, leading to the synthesis of sucrose which is finally directed to the newly forming shoots. The mechanism underlying this sink/source transition is largely unknown. Molecular identification of genes involved in carbohydrate and starch metabolism provides a tool with which to analyze this process in more detail.

ADP-glucose pyrophosphorylase (ATP: α-glucose-1-phosphate adenyl transferase, EC 2.7.7.27) plays a vital role in biosynthesis of α-1,4-glucans in both bacteria and plants (Preiss 1978, 1982a). The enzyme catalyzes the following reversible reaction: ATP + glucose-1-phosphate $\rightleftharpoons$ ADP-glucose + PP_i.

In plants, ADP-glucose is converted to starch by the concerted action of different starch synthases (producing α-1,4-glucan linkages) and branching enzyme (forming α-1,6-glucan branchpoints; Preiss 1982b). ADP-glucose pyrophosphorylases present in leaves of higher plants, in algae, cyanobacteria, and non-chlorophyllic reserve tissue are activated by 3-phospho-glycerate and inhibited by inorganic phosphate (see Preiss 1982b and references therein).

Despite the fact that the native tetrameric AGPases of both bacteria and plants have similar sizes of about 200–240 kDa, different subunit compositions have been reported. In *Escherichia coli* and *Salmonella typhimurium* the AGPases are homotetramers consisting of four identical subunits of about 50 kDa each (Haugen et al. 1976; Lehmann and Preiss 1980). The *E. coli* enzyme is encoded by a single gene (Baecker et al. 1983). On the other hand, several plant enzymes have been reported to be heterotetramers with subunits belonging to two similar, but different, size classes. In spinach leaf extracts, two subunits of 51 kDa and 54 kDa, each

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showing AGPase activity, were identified (Morell et al. 1987a). These subunits were shown to be dissimilar by N-terminal sequencing, tryptic peptide maps and immunological characterization (Morell et al. 1987a, b), and are therefore believed to be encoded by different genes (Morell et al. 1988). Using polyclonal antibodies directed against the spinach leaf holoenzyme or the purified subunits, a similar polypeptide composition was observed in leaf tissue of *Arabidopsis thaliana* (Lin et al. 1988a, b), wheat and rice (Krishnan et al. 1986). Electrophoresis of purified potato tuber AGPase under native conditions revealed two closely associated protein bands that coincided with enzyme activity (Sowokinos and Preiss 1982). Only one major protein of 50 kDa was observed upon electrophoresis in SDS polyacrylamide gels, suggesting a homotetrameric structure for the active AGPase.

Recently, cDNA clones encoding AGPase polypeptides of wheat leaf and endosperm (Olive et al. 1989), of rice seeds (Anderson et al. 1989), and of maize endosperm (Bhave et al. 1990; Bae et al. 1990) have been isolated. Here we report the isolation of potato cDNA clones representing at least two different genes (called S and B) coding for ADP-glucose pyrophosphorylase. In addition, a detailed analysis of RNA expression is described for both clones in different tissues that serve as either sinks or sources for carbohydrates, and under various physiological conditions.

Materials and methods

Materials. Solanum tuberosum cv. Désirée was obtained from Vereinigte Saatzuchten eG (3112 Ebstorf; FRG). Plants were grown in a greenhouse under a light/dark regime of 16 h light (22° C) and 8 h dark (15° C).

Recombinant DNA techniques. Standard procedures were used for recombinant DNA work (Maniatis et al. 1982). DNA sequences were determined by the dideoxy method (Sanger et al. 1977). Either commercial sequencing primers (Pharmacia) or specifically synthesized oligonucleotides (Applied Biosystems DNA Synthesizer 380A) were used for supercoiled plasmid sequencing with T7 DNA polymerase (Pharmacia).

cDNA library screening. A cDNA library prepared from potato tuber poly(A)$^+$ RNA in λ gt11 was provided by Dr. U. Schmitz (Institut für Genbiologische Forschung Berlin GmbH, Berlin). The library was screened with radioactively labeled cDNA probes encoding the maize brittle-2 and shrunken-2 AGPases (Bae et al. 1990; Bhave et al. 1990). Screening was performed at 42° C in PEG buffer (Amasino 1986) containing 25% formamide. Filters were washed for 30 min in 1.5 × SSC, 0.5% SDS at 50° C.

Isolation and analysis of nucleic acids. Total RNA isolation from plants was done according to Logemann et al. (1987). RNA was denatured in 50% formamide and size-fractionated (50 µg/lane) in 1.5% agarose gels containing formaldehyde, blotted onto nylon membranes (Hybond N, Amersham, UK), and hybridized as described (Amasino 1986). DNA probes were radioactively labeled using a multiprime labeling kit (Amersham, UK). Filters were washed twice in 1.5 × SSC, 0.5% SDS for 30 min at 68° C, and once in 0.1 × SSC, 0.5% SDS for 30 min at 68° C.

Petiole experiments. Petioles with leaves attached were harvested from 3–5-week-old potato plants at the end of the dark period. Petioles were cut submerged under water to avoid xylem embolism. In each experiment 2–4 leaves from at least two plants were randomly selected and incubated together in the incubation medium. Incubation was done under constant light or in darkness at 25° C. In 'EDTA' experiments leaves were incubated in different media supplemented in addition with 5 mM EDTA (pH 6.0).

Starch measurement. Leaf tissue (80–120 mg) was extracted and homogenized as described (Lin et al. 1988a). Starch was determined using a commercial kit (Boehringer, Mannheim, FRG) according to the manufacturer's instructions.

Results

Isolation of cDNA clones encoding ADP-glucose pyrophosphorylase from potato

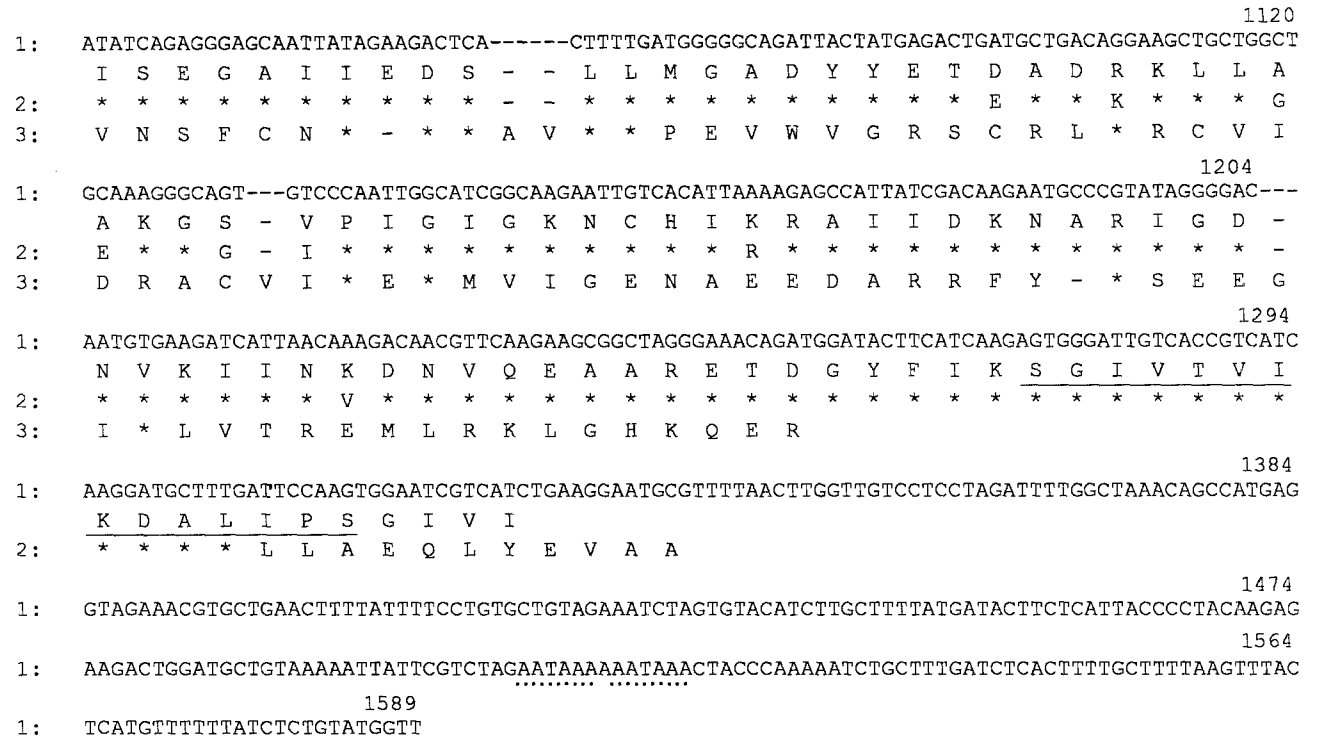
Two cDNA clones, encoding the maize shrunken-2 and brittle-2 ADP-glucose pyrophosphorylases, were used to screen a tuber cDNA library. Screening approximately 1.5×10^5 recombinant λ phages resulted in 78 clones which hybridized to the shrunken-2 probe (S clones) and 54 which hybridized to the brittle-2 probe (B clones). No cross-hybridization between S and B clones was observed under the conditions used (see Materials and methods). This is in agreement with the result of a genomic Southern blot analysis using the potato S and B clones as probes (data not shown). From each set, 30 positive recombinant phages were randomly selected and plaque purified. The sizes of the cDNA inserts ranged from 450–1750 bp. Several inserts were subcloned into the plasmid vector pUC18 for further characterization. For convenience, the AGPases encoded by the two sets of clones will subsequently be abbreviated to AGPase S (represented by S clones) and AGPase B (represented by B clones).

cDNA sequences of AGPase S and AGPase B

Two plasmid clones (S 25-1 and B 22-1) were chosen for sequence analysis in order to verify their identity. Clone B 22-1 was totally sequenced whereas partial sequence information was obtained for the 5′ end of clone S 25-1. The cDNA sequences and the deduced amino acid sequences are shown in Fig. 1. Clone B 22-1 contains a cDNA insert of 1589 bp, whereas the cDNA insert of clone S 25-1 is approximately 1750 bp in size.

a AGPase B (B 22-1):

```
                                                                                                                    70
1: AGPase B  ACAGACATGTCTAGACCCAGATGCTAGCCGGAGTGTT------------TTGGGAATTATTCTTGGAGGTGGAGCTGGGACC
                Q  T  C  L  D  P  D  A  S  R  S  V  -  -  -  -  L  G  I  I  L  G  G  G  A  G  T
2: RSc-6        N  K  N  *  *  -  -  -  -  *  *  *  D  E  S  V  *  *  *  *  *  *  *  *  *  *
3: Ec glg c     M  L  A  R  Q  -  -  -  -  L  P  L  K  -  S  V  A  L  *  L  A  -  *  *  R  *  *

                                                                                                                   160
1:    CGACTTTATCCTCTAACTAAAAAAAGAGCAAAACCAGCCGTTCCACTTGGAGCAAATTATCGTCTGATTGACATTCCCGTAAGCAATTGC
       R  L  Y  P  L  T  K  K  R  A  K  P  A  V  P  L  G  A  N  Y  R  L  I  D  I  P  V  S  N  C
2:     *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
3:     *  *  K  D  *  *  N  *  *  *  *  *  *  *  H  F  *  G  K  F  *  I  *  *  F  A  L  *  *  *

                                                                                                                   250
1:    TTGAACAGTAACATATCCAAGATCTATGTTCTCACACAATTCAACTCTGCCTCCCTGAATCGCCACCTTTCACGGGCATATGCTAGCAAT
       L  N  S  N  I  S  K  I  Y  V  L  T  Q  F  N  S  A  S  L  N  R  H  L  S  R  A  Y  A  S  N
2:     *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  P  *  *  *  *  *  *  *  G  N  *
3:     I  *  *  G  *  R  R  M  G  *  I  *  *  Y  Q  *  H  T  *  V  Q  *  I  Q  *  G  W  S  F  F

                                                                                                                   340
1:    ATGGGAGGATACAAAAACGAGGGCTTTGTGGAAGTTCTTGCGGCTCAACAAAGTCCAGAGAACCCCGATTGGTTCCAGGGCACGGCTGAT
       M  G  G  Y  K  N  E  G  F  V  E  V  L  A  A  Q  Q  S  P  E  N  P  D  W  F  Q  G  T  A  D
2:     I  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  D  *  *  N  *  *  *  *  *  *
3:     N  E  E  M  -  *  *  -  *  *  D  L  *  P  *  *  *  R  M  K  G  E  N  *  Y  R  *  *  *  *

                                                                                                                   430
1:    GCTGTTAGACAATATCTGTGGTTGTTTGAGGAGCATACTGTTCTTGAATACCTTATACTTGCTGGAGATCATCTGTATCGAATGGATTAT
       A  V  R  Q  Y  L  W  L  F  E  E  H  T  V  L  E  Y  L  I  L  A  G  D  H  L  Y  R  M  D  Y
2:     *  *  *  *  *  *  *  *  *  *  *  *  N  *  M  *  F  *  *  *  *  *  *  *  *  *  *  *  *
3:     *  *  T  *  N  *  D  I  I  R  R  Y  K  A  E  Y  V  V  *  *  *  *  *  *  I  *  K  Q  *  *

                                                                                                                   520
1:    GAAAAGTTTATTCAAGCCCACAGAGAAACAGATGCTGATATTACCGTTGCCGCACTGCCAATGGACGAGAAGCGTGCCACTGCATTCGGT
       E  K  F  I  Q  A  H  R  E  T  D  A  D  I  T  V  A  A  L  P  M  D  E  K  R  A  T  A  F  G
2:     *  *  *  *  *  *  *  *  *  *  *  S  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
3:     S  R  M  L  I  D  *  V  *  K  G  V  R  C  *  *  V  C  M  *  V  P  I  E  E  *  S  *  *  *

                                                                                                                   610
1:    CTCATGAAGATTGACGAAGAAGGACGCATTATTGAATTTGCAGAGAAACCGCAAGGAGAGCAATTGCAAGCAATGAAAGTGGATACTACC
       L  M  K  I  D  E  E  G  R  I  I  E  F  A  E  K  P  Q  G  E  Q  L  Q  A  M  K  V  D  T  T
2:     *  *  *  *  *  *  *  *  *  *  *  V  *  *  *  *  *  *  K  *  *  *  *  K  *  *  M  *  *  *  *
3:     V  *  A  V  *  *  N  D  K  T  *  *  *  V  *  *  *  A  N  P  P  S  M  P  N  D  P  S  K  S

                                                                                                                   700
1:    ATTTTAGGTCTTGATGACAAGAGAGCTAAAGAAATGCCTTTCATTGCTAGTATGGGTATATATGTCATTAGCAAAGACGTGATGTTAAAC
       I  L  G  L  D  D  K  R  A  K  E  M  P  F  I  A  S  M  G  I  Y  V  I  S  K  D  V  M  L  N
2:     *  *  *  *  *  *  V  *  *  *  *  *  *  *  Y  *  *  *  *  *  *  *  *  *  *  *  N  *  *  *  Q
3:     L  A  S  M  G  I  Y  V  F  D  A  D  Y  L  Y  E  L  L  E  E  D  D  R  D  E  N  S  S  H  D

                                                                                                                   790
1:    CTACTTCGTGACAAGTTCCCTGGGGCCAATGATTTTGGTAGTGAAGTTATTCCTGGTGCAACTTCACTTGGGATGAGAGTGCAAGCTTAT
       L  L  R  D  K  F  P  G  A  N  D  F  G  S  E  V  I  P  G  A  T  S  L  G  M  R  V  Q  A  Y
2:     *  *  *  E  Q  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  N  I  *  *  *  *  *  *  *
3:     F  G  K  *  L  I  *  K  I  T  E  A  *  L  A  Y  A  H  P  F  P  -  *  S  C  -  *  *  S  D

                                                                                                                   874
1:    TTATATGATGGG------TACTGGGAAGATATTGGTACCATTGAAGCTTTCTACAATGCCAATTTGGGCATTACAAAAAAGCCGGTGCCA
       L  Y  D  G  -  -  Y  W  E  D  I  G  T  I  E  A  F  Y  N  A  N  L  G  I  T  K  K  P  V  P
2:     *  *  *  *  -  -  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
3:     P  -  *  A  E  P  *  *  R  *  V  *  *  L  *  *  Y  W  K  *  *  *  D  L  -  A  S  V  *  *

                                                                                                                   949
1:    GATTTTAGCTTTTATGACCGATCAGCCCCAATCTACACCCAACCTCGATATCTACCACCATCAAAA---ATGCTTGAT------------
       D  F  S  F  Y  D  R  S  A  P  I  Y  T  Q  P  R  Y  L  P  P  S  K  -  M  L  D  -  -  -  -
2:     *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  H  *  *  *  *  *  -  V  *  *  -  -  -  -
3:     K  L  D  M  *  *  *  N  W  *  *  R  *  Y  N  E  S  *  *  *  A  *  F  V  Q  *  R  S  G  S

                                                                                                                  1036
1:    ---GCTGATGTCACAGATAGTGTCATTGGTGAAGGTTGTGTGATCAAGAACTGTAAGATTCACCATTCCGTGGTTGGGCTCAGATCATGC
       -  A  D  V  T  D  S  V  I  G  E  G  C  V  I  K  N  C  K  I  H  H  S  V  V  G  L  R  S  C
2:     -  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
3:     H  G  M  T  L  N  *  L  V  S  G  *  *  *  *  S  G  S  V  V  V  Q  *  *  L  F  S  *  V  R
```



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b AGPase S (S 25-1):

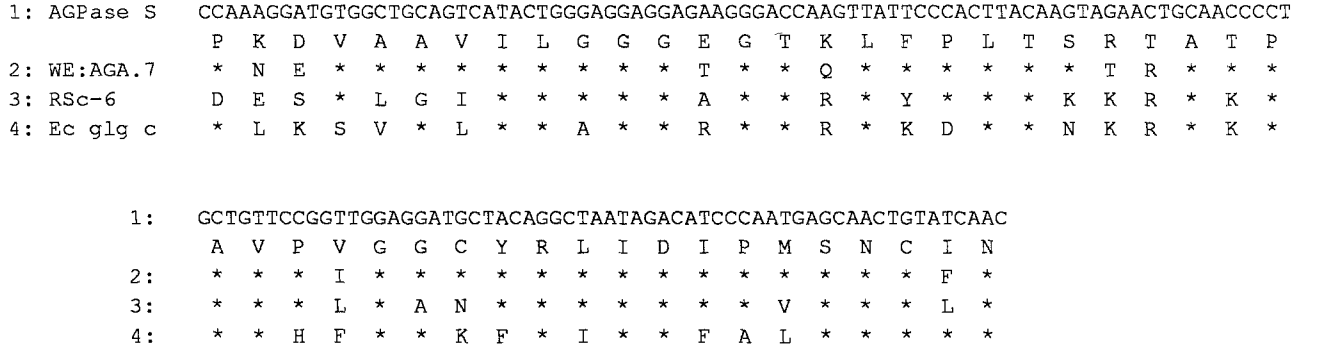

Fig. 1a and b. Comparison of deduced amino acid sequences of clones B 22-1 and S 25-1 with ADP-glucose pyrophosphorylase (AGPase) sequences from other organisms. **a** Alignment of clone B 22-1 (line 1, cDNA and deduced amino acid sequence) with rice endosperm (line 2, clone RSc-6) and *Escherichia coli* (line 3, glg c gene) AGPases. **b** Alignment of N-terminal S 25-1 sequence (line 1, cDNA and amino acid sequence) with corresponding regions of wheat endosperm (line 2, clone WE: AGA.7), rice endosperm (line 3, clone RSc-6) and *E. coli* (line 4, glg c gene) AGPases. Amino acids identical to AGPase B (**a**) or AGPase S (**b**), respectively, are indicated by *stars*. *Horizontal bars* indicate nucleotides/amino acids not present in either clone. The putative allosteric activator site of AGPase B (**a**) is *underlined*. Putative polyadenylation signals of AGPase B are marked by *dotted lines*

An open reading frame of 442 amino acids is observed for clone B 22-1. In the 3′ untranslated region, two neighboring sequences homologous to the eukaryotic polyadenylation signal (AATAAA) (Proudfoot and Brownlee 1976; Proudfoot 1984) are found around position 1513 after the stop codon of clone B 22-1. A comparison of the deduced amino acid sequence of clone B 22-1 with published amino acid sequences of AGPases from other organisms (Baecker et al. 1983; Anderson et al. 1989; Olive et al. 1989) shows a remarkably high homology, e.g. 88% homology is found between the rice endosperm clone RSc-6 (Anderson et al. 1989) and the potato AGPase clone B 22-1 (see Fig. 1a). The homology at the nucleotide level is still 75%. A lower degree of homology is found to the wheat endosperm AGPase clone WE: AGA.7 (48% at the amino acid level; Olive et al. 1989). By affinity-labeling techniques, Morell et al. (1988) identified the putative allosteric activator site of the 51 kDa subunit of spinach AGPase. A tryptic peptide with the sequence SGIVTVIKDALIPS was found

to bind to pyridoxal phosphate, an activator of several plant AGPases (Preiss et al. 1987). Exactly the same sequence is located at the C-terminus of the deduced AGPase B polypeptide sequence.

The deduced N-terminal amino acid sequence of the second potato clone, S 25-1, was compared with homologous regions of the wheat, rice and *E. coli* AGPases. The highest degree of homology was found to the wheat endosperm clone WE: AGA.7 (83% homology). The homology to the rice and *E. coli* sequences are 62% and 53%, respectively.

The amino acid sequence homology in this region between the two potato clones B 22-1 and S 25-1 is 62% (compare Fig. 1a, line 1 with Fig. 1b, line 1). Thus the homology between the two potato AGPases is much less than that of AGPase B to the rice protein (88%) and of AGPase S to the wheat endosperm polypeptide (83%).

AGPase S and AGPase B differ in their expression levels in different potato organs

In contrast to the vast amount of information available concerning control of the enzymatic activity of AGPase from photosynthetic and non-photosynthetic tissues by different metabolites (Sowokinos and Preiss 1982; Plaxton and Preiss 1987; Preiss et al. 1987), very little is known about the mechanisms that regulate transcription of the genes coding for AGPase. As a first step towards the analysis of the expression of both AGPase B and S genes in potato, total RNA was extracted from different organs of the potato plant (see legend to Fig. 2). All tissues analysed originate from the same (8-week-old) plant except stored tubers and sprouting tubers.

Hybridization with clones S 25-1 and B 22-1 revealed transcripts with sizes of 2.1 and 1.9 kb, respectively. A comparison of the hybridization data obtained (see Fig. 2) shows that in flowers, leaves of different developmental stages, and in the middle part of the stem, the level of expression of AGPase S is significantly lower than that of AGPase B (lanes Flo, uLe, mLe, lLe, and mSt). By contrast, the expression levels for both clones in the lower stem and roots (lanes lSt and Roo) are similar. No signal was detected in the seed tuber (lane MTu). For both clones, large amounts of RNA are seen in developing tubers of different sizes (lanes Tu 1–Tu 4), except for very small ones (below 1 g fresh weight; lane Tu 5). This observation is interesting in the light of the fact that Hawker et al. (1979) described a much higher activity of starch phosphorylase in very small tubers compared to the activity of ADP-glucose pyrophosphorylase in the same tissue. This was interpreted as evidence for the involvement of starch phosphorylase in starch biosynthesis in very small tubers (Hawker et al. 1979).

Interestingly, AGPase S consistently shows a higher steady state mRNA level than the AGPase B in developing tubers (lanes Tu 1–Tu 4). In stored tubers and sprouting (i.e. source) tubers the expression is similar for the two clones and clearly weaker in comparison to developing tubers.

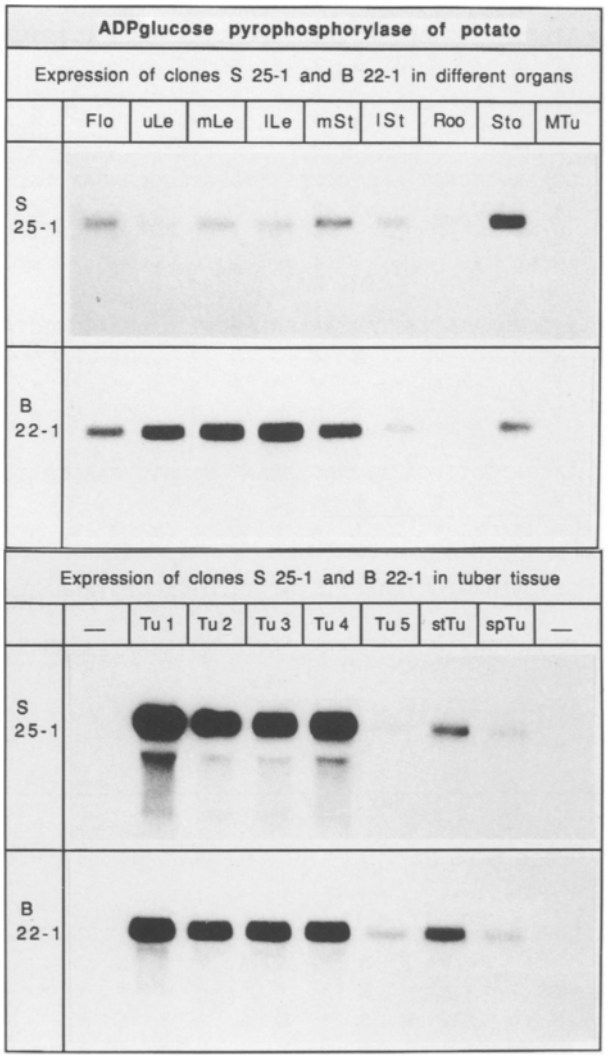

Fig. 2. Northern blot analysis of AGPase S (S 25-1) and AGPase B (B 22-1) in different tissues. An equal amount (50 µg) of total RNA from each sample was loaded in each lane. The blot was probed with ^{32}P-labeled cDNA inserts of clones S 25-1 and B 22-1. RNA was extracted from total flowers (Flo), small leaves of the upper part of the plant (uLe), leaves from the middle part of the plant (mLe), leaves from the lower part of the plant (lLo), middle part of the stem (mSt), lower part of the stem (from the air/soil interface; lSt), roots (Roo), stolons (Sto), the seed tuber used for planting (MTu), developing tubers of different fresh weights (Tu 1, 10 g; Tu 2, 8.7 g; Tu 3, 7.5 g; Tu 4, 3.2 g; Tu 5, 0.8 g), tubers stored for 7 months (stTu), and sprouting tubers (spTu)

Expression of AGPase S but not AGPase B is strongly inducible in detached leaves kept under constant light and can be further stimulated by addition of sucrose

When tubers are the receiving organs, sucrose is mainly converted into starch after unloading from the phloem. Synthesis of starch is accompanied by an increase in the enzymatic activity of AGPase (Sowokinos 1976). The high level of expression observed for clone S 25-1 in tuber tissue and its relatively low expression in leaves could indicate that AGPase S is preferentially involved

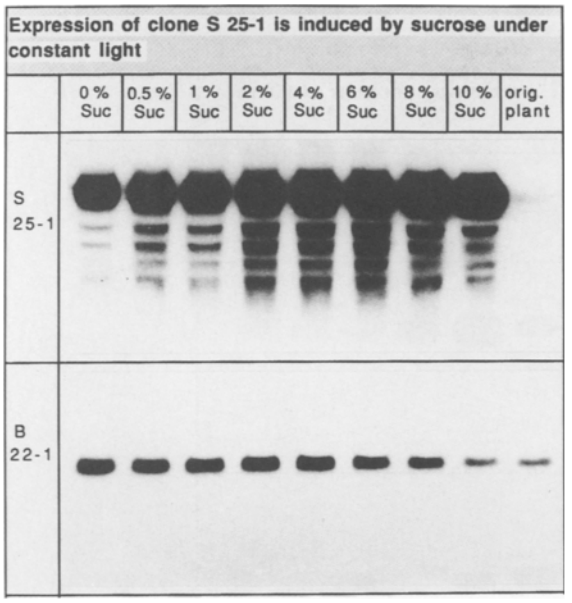

Fig. 3. Expression of AGPase S and AGPase B in detached leaves. Total RNA (50 µg each) extracted from leaves of untreated plants at the end of the dark period (lane 9, orig. plant), and from detached leaves incubated for 24 h in water (lane 1, 0% Suc) or increasing amounts of sucrose (lanes 2–8, 0.5% Suc to 10% Suc), was probed with cDNA inserts of clones S 25-1 and B 22-1. The multiple lower molecular weight bands seen upon probing with the S 25-1 clone show specific hybridization only with this clone and therefore most likely represent degradation products. Approximately 50 ng of DNA were used for the labeling reaction. Incorporation of radioactivity was similar for the two clones

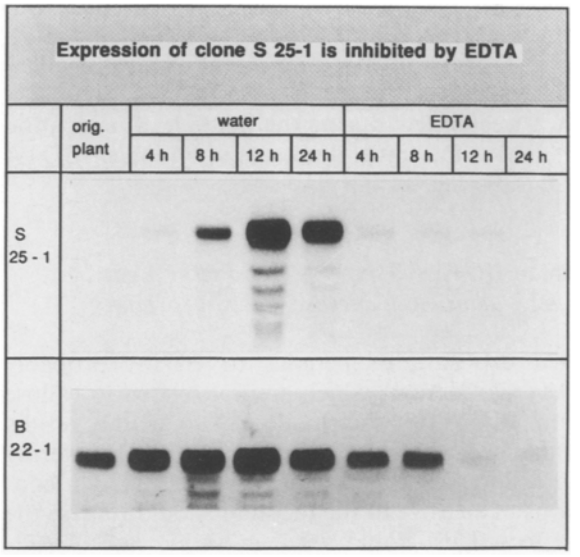

Fig. 4. Inhibition of AGPase S expression by EDTA treatment. Total RNA (50 µg each) extracted from untreated leaves at the end of the dark period (lane 1, orig. plant), and from detached leaves incubated for 4, 8, 12, and 24 h in water (lanes 2–5), and in water containing EDTA (lanes 6–9) was hybridized to cDNA inserts of clones S 25-1 and B 22-1

in starch synthesis in the tuber or, more generally, in storage sinks. This could either be related to the sink status of the tissue and/or high levels of carbohydrates (e.g. sucrose). In order to test whether or not expression of AGPase S might be modulated by high concentrations of the metabolizable carbohydrate sucrose in tissues other than tubers, the following experiment was performed. Potato leaves were detached from the plant at the end of the dark period and incubated in water, or water supplemented with increasing amounts of sucrose (0.5%–10%). Control leaves were directly frozen in liquid nitrogen. After incubation for 24 h under constant light, total RNA was extracted from the leaves, and from leaves of the untreated (original) plants, and analysed for accumulation of AGPase S and B RNAs. The results are shown in Fig. 3. A very faint AGPase S-specific signal is detectable in leaves of the untreated plant (lane orig. plant). Incubation in water supplemented with sucrose results in a dramatic increase in transcript levels, irrespective of the amount of exogenous sucrose (lanes 0.5% Suc–10% Suc). The same holds true for incubation in water without addition of sucrose (lane 0% Suc). Despite the fact that incubation with and without sucrose leads to strong induction of AGPase S, sucrose has a stimulatory effect on the expression, which can be seen on a shorter exposure of the same autoradiogram (data not shown). AGPase B behaves differently. Firstly, a clear signal is detectable in the untreated plant (lane orig. plant). Secondly, incubation in water has only a slight effect on the steady-state transcript level (lane 0% Suc). Thirdly, very high levels of sucrose inhibit expression of AGPase B, which is also seen, though to a lesser extent, for the AGPase S.

Accumulation of AGPase S RNA is detached leaves incubated in water is inhibited by addition of EDTA

The data shown in Fig. 3 clearly indicate that AGPase S is already strongly induced in the absence of exogenously added sucrose, which might argue against a role of sucrose in modulating the expression of AGPase S. However, detachment of petioles from the intact plant leads to phloem clogging due to callose formation which prevents export of metabolites including sucrose. Thus, the induction of AGPase S mRNA accumulation in water could be related to the accumulation of assimilates produced in the detached leaf.

Callose formation is dependent on Ca^{2+} (Kauss 1987) and can be inhibited by low concentrations of the chelating agent EDTA, which has been used to enhance phloem exudation rates (King and Zeevaart 1974; Tully and Hanson 1979; Groussol et al. 1986; Weibull et al. 1990) and found to be superior to the chelating agent EGTA (Groussol et al. 1986). In order to prevent accumulation of metabolites within the leaf tissue, detached leaves were incubated for 4, 8, 12, and 24 h under light in water or water containing EDTA (5 mM, pH 6.0). Total RNA was extracted from the explants and analysed in Northern blot experiments. The results are shown in Fig. 4. Whereas in the original plants only a very weak signal is detectable (lane 1), incubation in

water results in increasing levels of AGPase S mRNA (lanes 2–5). In the presence of EDTA, however, this induction is almost completely suppressed (lanes 6–9). AGPase B expression is strong in the untreated plant as well as in leaves incubated in water (lanes 1–5). EDTA has only a weak effect during the first 8 h of incubation (lanes 6, 7), but the signal decreases upon longer EDTA treatment (lanes 8, 9).

Inhibition of AGPase S expression by EDTA can be overcome by addition of sucrose, but not sorbitol

Prolonged exposure of petioles to EDTA probably causes several reactions which are not related to callose formation. If, on the other hand, EDTA mainly results in an enhanced phloem exudation rate by interfering with the callose sealing reaction, addition of high concentrations of sucrose to the medium should reverse the EDTA effect. This should result in an increase of AGPase S RNA levels. To test this, leaves were preincubated in EDTA for 4 h, subsequently transferred to an EDTA solution containing 6% (175 mM) sucrose, and incubated for a further 4, 8, or 20 h. In control experiments, leaves were transferred to a solution containing 3.2% sorbitol (175 mM) instead of sucrose. Like sucrose, sorbitol is an osmotically active substance, but is not metabolized by the potato plant. Analysis of total RNA (Fig. 5) shows that addition of sucrose also leads to accumulation of AGPase S transcripts in the presence of EDTA (lanes 1–3), whereas sorbitol has only a very minor effect (lanes 4–6). AGPase B expression is only slightly affected by the sucrose treatment resulting in a distinct signal even after 12 h in solutions containing EDTA (4 h EDTA pretreatment +8 h EDTA/sucrose treatment; compare Fig. 4, lane 8 with Fig. 5, lane 2). Sorbitol on the other hand has no effect (compare Fig. 4, lane 8 with Fig. 5, lane 5).

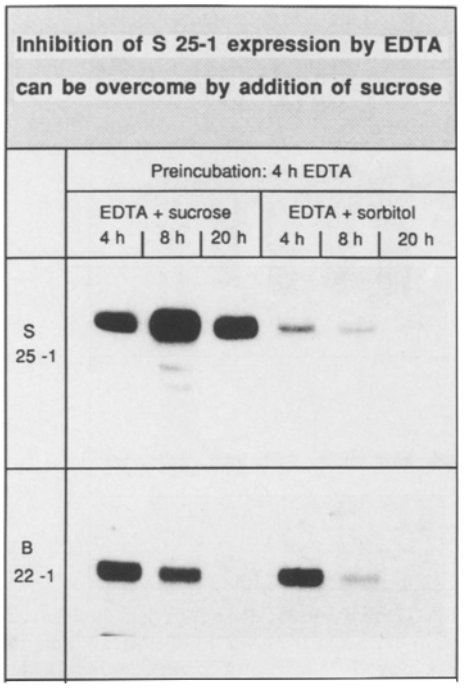

Fig. 5. Effect of sucrose on AGPase expression in detached leaves pre-treated for 4 h in EDTA. Total RNA (50 µg each) was extracted from leaves incubated for another 4, 8, and 20 h in water containing EDTA and sucrose after the initial incubation in EDTA for 4 h (lanes 1–3). In control experiments, sorbitol was used instead of sucrose (lanes 4–6). The blots were probed with cDNA inserts of clones S 25-1 and B 22-1

Carbohydrates, supplied either by endogenous photosynthesis or via exogenous sucrose, are required to induce the accumulation of AGPase S mRNA

The data described above suggest that accumulation of AGPase S mRNA might be triggered by elevated levels of metabolizable carbohydrates, either supplied by sucrose to detached EDTA-treated (i.e. export competent) leaves or via photosynthesis in leaves that are not able to export and thus tend to accumulate assimilates (see previous sections). In order to test this view further, two additional experiments were performed.

In the first, leaves were harvested at the end of the dark period and incubated in water, water supplemented with sucrose (6%), or water supplemented with sorbitol (3.2%) in total darkness for 12 and 24 h. Expression of AGPase S and AGPase B was analysed by Northern blot experiments. The results are shown in Fig. 6. In the untreated plant, AGPase S expression again is nearly undetectable (orig. plant, lane 1). Water alone is unable to induce AGPase S expression in darkness (lanes 4, 5),

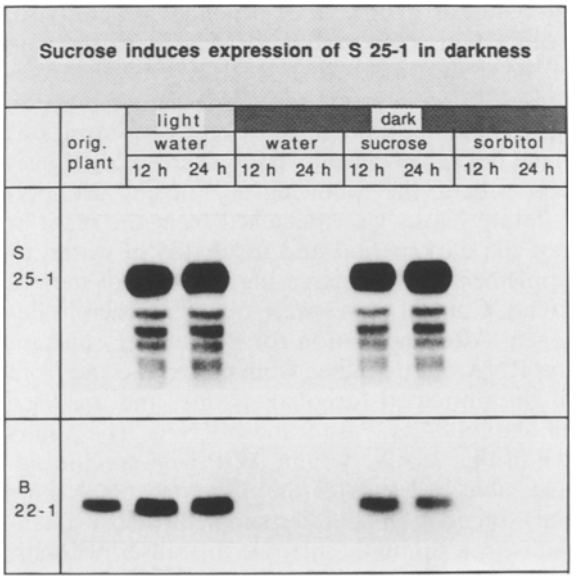

Fig. 6. Expression of AGPases in darkness. Total RNA (50 µg each) was extracted from detached leaves incubated in the dark for 12 and 24 h in water (lanes 4 and 5, dark water), in water supplemented with sucrose (lanes 6 and 7, dark sucrose), and in water supplemented with sorbitol (lanes 8 and 9, dark sorbitol), and hybridized to cDNA inserts of clones S 25-1 and B 22-1. RNA from leaves incubated in water under constant light (lanes 2 and 3, light water) and RNA from leaves of untreated plants (lane 1, orig. plant) were used as control. Approximately 50 ng radioactively labeled DNA was used for each hybridization. Incorporation of radioactivity was comparable for the two clones

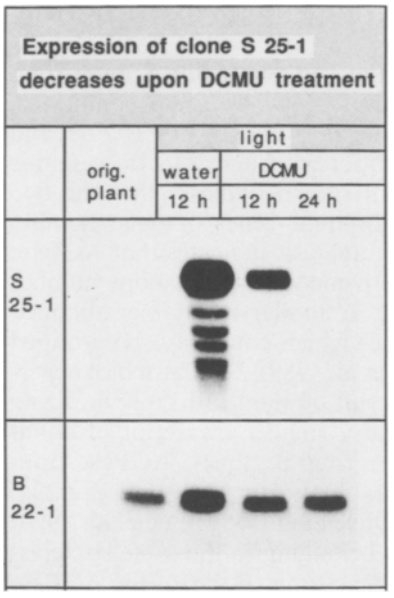

Fig. 7. Reduction of AGPase S mRNA level by inhibition of photosynthesis. Total RNA (50 µg each) extracted from detached leaves incubated for 12 and 24 h in water containing 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was probed with cDNA inserts of clones S 25-1 and B 22-1 (lanes 3 and 4, DCMU). RNA from leaves of the untreated plants (lane 1, orig. plant) and from leaves incubated under light in water for 12 h (lane 2, water) serve as controls

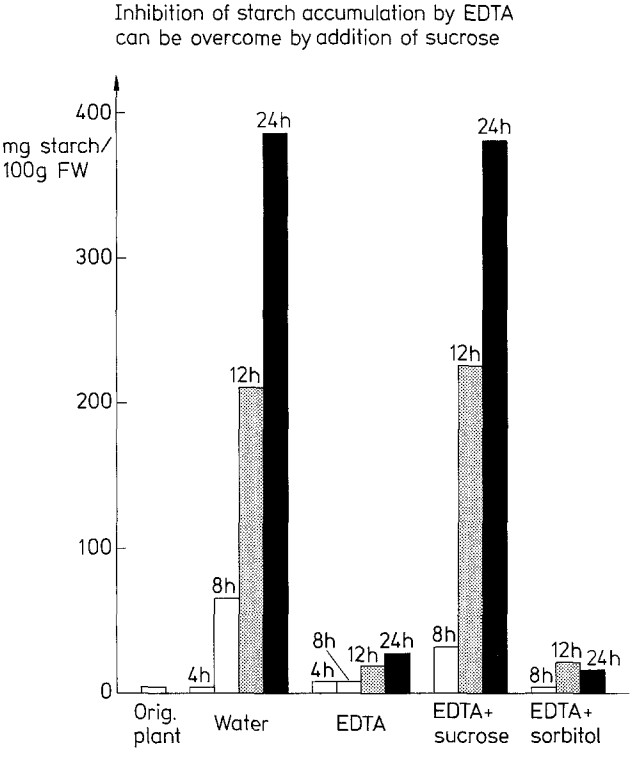

Fig. 8. Accumulation of starch in leaves treated as described in the legends to Figs. 4 and 5. Starch content was determined from the same leaves as used for RNA expression analysis

but addition of sucrose increases the steady-state concentration of transcript (lanes 6, 7) to levels comparable to these observed in water upon incubation in constant light (lanes 2, 3). Sorbitol does not have this effect (lanes 8, 9). AGPase B expression shows a similar pattern under these conditions, except that significant expression is observed in the untreated plant (lane 1) and the mRNA level is lower than that of AGPase S, as has been consistently observed under inducing conditions. These data are therefore in agreement with a model which assumes that high levels of metabolizable carbohydrates are necessary for accumulation of the AGPase S mRNA. In the case of dark-incubated leaves which are unable to perform photosynthesis, this requirement can be supplied in the form of exogenous sucrose. The absence of AGPase B transcripts in the dark could indicate that AGPase B expression might also be related to either carbohydrate levels and/or light per se.

In the second experiment photosynthesis was inhibited in detached illuminated leaves by addition of DCMU (3(3,4-dicklorophenyl)-1,1-dimethylurea; 20 µM) to the medium (Moreland 1980). RNA was analysed after 12 and 24 h. The transcript level of AGPase S is reduced after a 12 h treatment with DCMU and undetectable after 24 h (Fig. 7, lanes 3, 4; compare with water control, lane 2), whereas the expression of AGPase B is not affected by this treatment.

Increased AGPase S expression is always accompanied by increased accumulation of starch in the leaf tissue

As outlined above (see Introduction), AGPase is the key regulatory enzyme in starch biosynthesis. In order to see whether the changes of the AGPase RNA levels are paralleled by changes of the amount of starch, the starch content of the leaves that were used for RNA extraction in all petiole experiments described was determined. The results are shown in Figs. 8 and 9. Leaves at the end of the dark period had very low levels of starch (below 15 mg starch/100 g fresh weight; see lanes orig. plant). Incubation of detached leaves in water under constant light causes accumulation of starch to high levels (up to about 1.5 g starch/100 g fresh weight) whereas in the presence of EDTA only a slight increase in starch content is observed (Fig. 8, lane EDTA). By contrast, addition of sucrose (6%, 175 mM) completely suppresses the EDTA effect, resulting in a starch concentration comparable to the water incubation (Fig. 8, lane EDTA+sucrose). Sorbitol is unable to revert the EDTA mediated inhibition (Fig. 8, lane EDTA+sorbitol). In darkness (see Fig. 9), water alone (i.e. without EDTA) does not lead to an accumulation of starch (lane water). Addition of sucrose enables the leaves to accumulate starch (lane sucrose), whereas addition of sorbitol at the same molar concentration has no effect (lane sorbitol). The amount of starch observed in dark incubation upon sucrose induction is about 50% of that seen in the illuminated water controls. Addition of DCMU in the light leads to drastically reduced starch levels in detached leaves (Fig. 9, lane DCMU).

It should be noted that the maximal amount of starch accumulating in the leaves varies significantly between different experiments (between 350 and 1500 mg starch/100 g fresh weight; compare for example Figs. 8 and

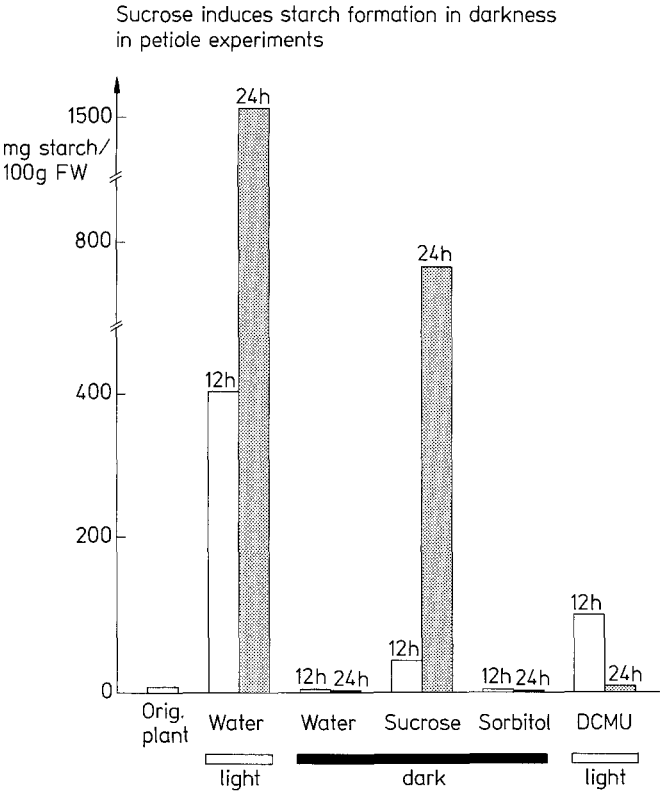

Fig. 9. Starch accumulation in leaves incubated as described in the legends to Figs. 6 and 7. Starch content was determined from the same leaves as used for Northern blot analysis

9, lane water). However, the ratios between the different treatments within one batch of experiment were always clearly reproducible. The different accumulation rates were most probably due to the developmental stages of the leaves.

In conclusion these data clearly show that increased AGPase S transcript levels correlate well with enhanced synthesis and accumulation of starch within the leaf tissue.

Discussion

Screening a cDNA library derived from tuber poly(A)$^+$ RNA resulted in identification of several clones encoding potato AGPase. The clones fall into two groups (S and B) according to their degrees of homology to maize shrunken-2 and brittle-2 cDNAs recently identified (Bae et al. 1990; Bhave et al. 1990). To the best of our knowledge the potato cDNAs represent the first example of AGPase clones isolated from a dicotyledonous plant. When the deduced amino acid sequences of the AGPase B and S clones were compared with published AGPase sequences of monocotyledonous plants (Olive et al. 1989; Anderson et al. 1989; Bae et al. 1990; Bhave et al. 1990) homology levels of between 48% and 88% were found at the amino acid level, indicating strong conservation even between distantly related plant species. Interestingly, the two potato proteins are significantly less homologous to each other than the AGPase B is to the rice subunit, and the AGPase S is to the wheat endosperm polypeptide.

No cross-hybridization between group S and group B clones was observed under low stringency conditions. This observation and the fact that clones B 22-1 and S 25-1 hybridize to different fragments in genomic Southern blots (data not shown) indicate that the two groups represent two different genes or gene families in potato. Southern blot analysis indicates that AGPase S and AGPase B are both encoded by low copy number genes (data not shown). A similar result was obtained with wheat cDNA clones which could also be grouped into two families (Olive et al. 1989). Northern blot experiments revealed a transcript of about 2.1 kb hybridizing with group S clones and a smaller transcript of about 1.9 kb hybridizing with group B clones. AGPase transcript sizes in monocot plants range from 1.7–2.2 kb (Krishnan et al. 1986; Olive et al. 1989; Bae et al. 1990; Bhave et al. 1990), thus belonging to the same size class and in agreement with the estimated size of the AGPase protein monomers (see Introduction).

The expression patterns for the genes represented by the S and B clones are remarkably different, as determined at the steady-state RNA level. The AGPase B shows rather invariant expression in different organs, irrespective of the status of the tissue as a sink (e.g. tuber) or a source (e.g. mature leaf) for carbohydrates. Furthermore, AGPase B RNA levels are not strongly influenced by amounts of carbohydrates produced via photosynthesis, or added exogenously. The exception to this generalization is the loss of expression observed in leaves kept in the dark (Fig. 6). Thus, although it cannot be excluded that AGPase B expression is somehow linked to carbohydrate level, its response to changes in this parameter is much less pronounced. Indeed, its mode of expression can be thought of as constitutive. One possible interpretation is that the AGPase B gene might serve general "house-keeping" purposes with respect to starch biosynthesis.

In contrast the expression pattern of the AGPase S is tissue-specific and dynamic. At the RNA level, it shows significantly higher expression in (sink) tubers compared to (source) leaves. Data obtained using detached leaves as an experimental system show that the amount of carbohydrate produced by the photosynthetic activity of the leaf is sufficient to induce accumulation of AGPase S RNA if export of the sucrose via the phloem is blocked due to callose plugging. That photosynthesis is required for this effect and not light per se is demonstrated by the failure to induce AGPase S RNA accumulation upon incubation with DCMU, a specific inhibitor of photosynthesis, and furthermore by the fact that exogenously added sucrose induces AGPase S mRNA efficiently in the dark. Taken together our data on the accumulation of the AGPase S mRNA are compatible with the assumption that this process is triggered by increased levels of carbohydrate (sucrose). The different expression patterns of the two potato AGPases at the RNA level raises the possibility that a similar regulation might exist in other plant species. However, so far no data have been reported on this paint.

In several plants, two polypeptides of similar sizes have been associated with ADP-glucose pyrophosphorylase activity (see Introduction). Despite the fact that the composition of the enzyme has not been conclusively elucidated, it is believed that, with the exception of the potato tuber AGPase the enzyme exists as a heterotetramer in plants. In potato tubers only one 50 kDa subunit has been so far demonstrated (Sowokinos and Preiss 1982), suggesting that this enzyme is a homotetramer, like the bacterial enzymes (Haugen et al. 1976; Lehmann and Preiss 1980). The isolation of two different groups of AGPase clones (S and B) from a potato tuber cDNA library would seem to contradict this observation. One possible explanation could be that one of the two polypeptides is very unstable in vivo and/or during preparation of protein extracts. An alternative possibility is that only one of the AGPase mRNAs is translated into a functional protein. No final conclusion can yet be drawn regarding the subunit structure of the catalytically active AGPase (i.e, a homotetramer containing either S or B subunits or a heterotetramer containing both B and S subunits). However, whatever final model emerges, it must take account of the different expression patterns of the S and the B gene(s) described in the Results section.

Two maize mutants, shrunken-2 and brittle-2, show reduced levels of AGPase activity in seed endosperm tissue (less than 10% of wild-type level; Tsai and Nelson 1966; Dickinson and Preiss 1969). Mutations in these two loci result in a loss of more than 70% of the wild-type starch content. Recently, mutants of *Arabidopsis thaliana* lacking one (Lin et al. 1988b), or both (Lin et al. 1988a) subunits of ADP-glucose pyrophosphorylase were isolated from a population of mutagenized plants. These mutants have reduced starch levels, demonstrating the role of AGPase for starch production. So far no potato mutants deficient in AGPase have been reported. The isolation of AGPase clones from potato will enable us to study starch biosynthesis and its influence on sink/source transitions in a tuber-bearing plant and on the other hand allow us to study the composition of the active enzyme via, for example, antisense RNA approaches.

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