Starch synthesis-, and tuber storage protein genes are differently expressed in *Solanum tuberosum* and in *Solanum brevidens***

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Abstract Studying in vitro stem cuttings of *Solanum tuberosum* induced for tuberization and those of a non-tuberizing *Solanum* species, differences both in morphology and in gene expression were detected. Stolon formation essentially depended on light while tuberization was triggered by the elevated level of sucrose in the medium. Genes involved in starch synthesis were induced by sucrose in both species, however, starch granules were detected only in potato. A new tuber specific cDNA clone, GM7, encoding a putative metallocarboxypeptidase inhibitor and the cDNA of a proline rich cell wall protein with *S. brevidens* specific expression were isolated by differential screening. Sucrose mediated transcription of the tuber storage proteins like patatin and proteinase inhibitors (Kunitz-type, *win*I, GM7) failed in *S. brevidens*.

Key words: Patatin; Proteinase inhibitor; *Solanum brevidens*; *Solanum tuberosum*; Starch synthesis; Tuberization

1. Introduction

Throughout the plant kingdom, specially differentiated or modified cells, groups of cells, or organs have evolved to function as organs of asexual reproduction. The enlarged, fleshy tips of subterranean rhizomes or stolons are known as tubers, examples of which are potatoes.

Four developmental steps are necessary for tuberization under field conditions: stolon induction and initiation; stolon growth; cessation of the longitudinal growth of the stolon and tuber induction and initiation. Stolon formation is favoured by darkness and high relative humidity while tuber development is influenced by daylength, temperature and nitrogen supply. Cytokinins enhance tuber formation whereas giberellic acid have a negative effect on tuber initiation [1].

Genes highly expressed in tubers have been isolated and characterized. The most abundant transcript is patatin which is encoded by the patatin gene family [2]. Patatin is regarded as a storage compound comprising the 40% of the total soluble protein of potato tubers. The other well-characterized genes are the proteinase inhibitors that are thought to play a role in the defense reaction of the plant against microbial and insect attack [3].

Starch accumulation in potato tubers is paralleled by increases in sucrose synthase (Susy), ADP-glucose pyrophosphorylase (AGPaseB and AGPaseS), granule bound starch synthase (GBSS) branching enzyme (BE) and plastidic starch synthase (STP) expressions and activities [4]. Starch is synthesized from sucrose that is assimilated in leaves and transported to the tuber. Translocation of sucrose from mesophyll into the phloem is done via the sucrose transporter (SUT) [5]. Transgenic plants with antisense-RNA inhibition of SUT show a dramatic reduction in tuber yield [6]. In the absence of Susy or AGPase sugar-storing tubers with decreased amount of soluble proteins are formed [4,7].

An alternative approach toward understanding the molecular mechanism of tuber morphogenesis might be to study the evolution of tuberization. *Solanum brevidens* is a close relative of potato (*S. tuberosum*), however, with no tuber-forming ability. Here we report that by isolating single-node stem segments and growing them under various conditions, in vitro, differences both in morphology and in gene expression could be detected between the two species.

2. Materials and methods

2.1. Synchronized tuberization, in vitro

The synchronized in vitro tuberization was developed for the Hungarian *S. tuberosum* cv. Keszthelyi 855. Plants were vegetatively propagated from cuttings in 500-ml Erlenmeyer flasks on MS basal medium [8] supplemented with 2% sucrose at 24°C with a 16 h photoperiod under 5000 lx intensity. Shoots of 2-month-old cultures were harvested and single-node segments with a resting axillary bud were obtained between the second and seventh node. Leaves were removed and the stem segments were explanted in MS medium containing 8% sucrose and 2.5 mg/ml 6-benzylaminopurine (Sigma). Synchronized (98–100%) tuberization was achieved when plants were grown at 24°C either in dark or under a long photoperiod.

For comparative studies the *S. brevidens* stem segments were obtained and cultured in the same way.

2.2. Bacterial strains and plasmids

The cDNA libraries described by Bánfalvi et al. [9] were used for differential screening. The *E. coli* strain XL1-Blue (Stratagene) was transformed by electroporation as described by the manufacturers (BioRad). Cloning vectors pBluescript II SK-and pUC18 were obtained from Stratagene and USB, respectively.

The plasmids pAM66, pAM98, pGM7, pLL12 were isolated from the cDNA libraries by differential screening as described in section 3.

2.3. Nucleic acid isolation and analysis

Basic methods (gel electrophoresis, plasmid DNA isolation and cloning, colony hybridization, Southern blotting and hybridization, differential screening) were performed according to Sambrook et al. [10]. Total RNAs were extracted as described by Stiekema et al. [11] and Northern hybridizations were performed using Amasino's method [12]. First strand cDNA probes were prepared from 50 μg of total RNAs. The 5'-cDNA end of GM7 was obtained by the RACE technique from 20 μg of total RNA isolated from 15-day-old tuberous potato shoots using the method of Harvey and Darlison [13]. The PCR reaction was first performed at 52°C, then at 65°C. The GM7 primer used in the reaction is indicated in Fig. 3. Cloning of the PCR fragment was at the *Eco*RV site of the plasmid Bluescript II SK-.

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** The nucleotide sequence data reported will appear in the EMBL Database under the accession numbers U30304 (LL12), U30388 (GM7), U30814 (AM66), U30861 (AM98).

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2.4. DNA sequence analysis

Double stranded cDNA clones in the vector pBluescript II SK-were sequenced using Sequenase version 2.0 kit (USB) and forward and reverse M13 primers, alternatively. Sequencing of the clone pAM66 was completed by subcloning an *Ava*II-*Xho*I fragment into the *Sma*I site of a dephosphorylated pUC18 plasmid DNA. LL12 was subcloned at an internal *Hin*dIII site and the *Bam*HI-*Hin*dIII, *Hin*dIII-*Xho*I fragments used in sequencing reactions.

Comparative sequence analysis was performed with programs from the Genetics Computer Group [14].

2.5. Detection of starch granules

Light microscopy was performed on shoot and tuber sections. Whole micro-tubers and 2 mm shoot segments were fixed overnight in a solution of 5% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer, adjusted to pH 7.2, dehydrated in an ethanol series and embedded in SPI-PON 812 resin (SPI Supplies, USA). Polymerization was performed at 60°C over 48 h. Embedded samples were cut with an ultramicrotome (MT 7000 Ultra RMC, USA) with glass knives. Semithin sections, 1.5 μm thick, were deposited on glass slides and observed by light microscopy after staining with Lugol's solution (2 g KI and 1 g I_2/300 ml).

The relative amount of starch granules in the cells was determined by computer-assisted image analysis (Soft-Imaging-Software GmBH, Germany). The most starch granules were detected in the tuber (10.7 ± 1.6% of the area tested). The middle part of the *S. tuberosum* shoots contained fewer granules (1.4 ± 0.4% or in a few samples less than 0.01%) while the tip was practically free of starch (less than 0.01%).

3. Results

3.1. Morphological changes brought about by various factors on single-node stem segments of S. tuberosum *and* S. brevidens

Morphological differences between a tuberizing (*S. tuberosum*) and a non-tuberizing *Solanum* species (*S. brevidens*) were studied in vitro. Single-node stem segments without leaves were isolated from both species and were cultured futher under various conditions.

In agreement with the result of previous studies [15] etiolated shoots with reduced leaf growth developed from the axillary buds of *S. tuberosum* in dark on MS medium containing 8% sucrose. Visible swellings (tubers) appeared around day 10 and further elongation of the shoot stopped. By day 15 all the plants possessed a tuber. When the level of sucrose in the medium was reduced to 2%, even after 1 month no tubers were formed on any of the plants tested (Fig. 1, left panel). The absence of BAP in the medium or the elevation of temperature, however, only diminished the synchronized manner of tuberization. After 2 weeks, though, the majority of the plants carried a small tuber (data not shown). Under long-day (LD) conditions (16 h light, 8 h dark) in the presence of 8% sucrose, brown-colored sessile tubers were formed while at 2% sucrose green leafy shoots developed (Fig. 2, left panel). These results suggest that tuber formation is basically dependent on sucrose concentration and confirms the conclusion drawn by Garner and Blake [16] that microtuber production can be obtained without any addition of growth regulators in the culture medium.

The response of *S. brevidens* to the various factors was fairly similar to that detected in the case of *S. tuberosum* except that no tubers were formed on this species. Etiolated plantlets with reduced leaf growth developed in dark while green leafy shoots were formed under long-day conditions. High sucrose concentration reduced shoot elongation (Figs. 1 and 2, right panels). Thus, we have concluded that *S. brevidens*, like potato, is able to sense the enviromental and nutritional factors tested in our experiments.

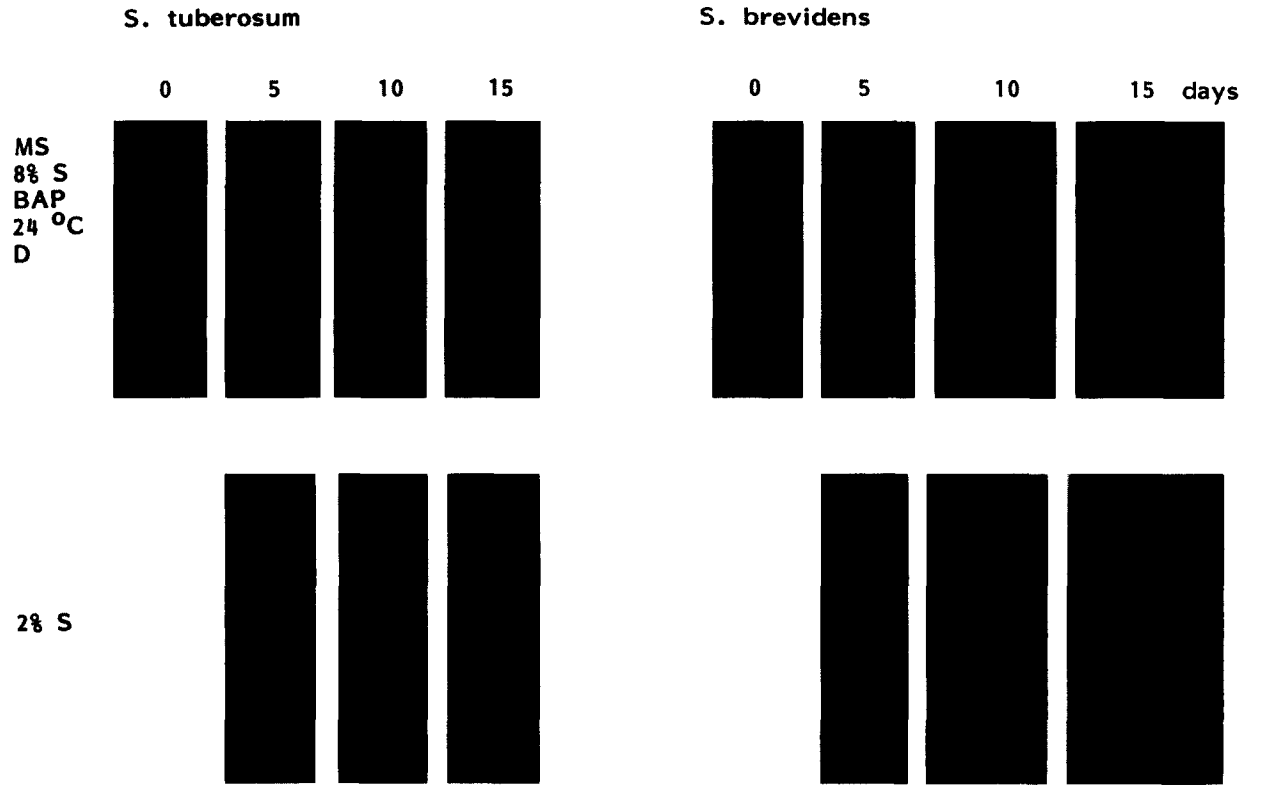

Fig. 1. Development of axillary buds from stem cuttings of *S. tuberosum* and *S. brevidens* in dark. Growth conditions: MS+8% sucrose+BAP and MS+2% sucrose+BAP, respectively.

3.2. Differential screening

In order to find molecular markers characteristic for the differences between *S. tuberosum* and *S. brevidens*, cDNA libraries were established from 5-day-old shoots grown in dark from axillary buds under tuber inducing conditions. These were then screened in colony hybridization with radioactive cDNA from 5-day-old induced *S. tuberosum* and *S. brevidens* shoots. From a total of 1000 colonies of the potato cDNA library replicated to Hybond-N filters 40 clones were found to be differentially expressed between the two species. Based on hybridization with the insert of the plasmid pTu58 [2] all of them were patatin cDNA. When radioactive probes from 15-day-old *S. tuberosum* and *S. brevidens* shoots (tuberous shoots in the case of potato) were used, however, two additional clones (pAM66, pAM98) with different expression could be recognized.

We have attempted to increase the sensitivity of the differential screening by isolating plasmid DNA in groups of 10 from clones showing no signal in colony hybridization with probes prepared from 5-day-old shoots and retesting them after digestion, electrophoresis and blotting to Hybond-N filters. In this way one additional clone (pGM7) with *S. tuberosum* specific expression was isolated.

Morphological differences may arise not only from expression, but repression of certain genes or gene families. Therefore, 700 colonies of the cDNA library established from *S. brevidens* were tested with the same probes. One difference (pLL12) was detected showing hybridization to *S. brevidens* but not to the potato probe.

3.3. DNA sequence analysis of AM66, AM98, GM7 and LL12 cDNAs

The genes differentially expressed in *S. tuberum*/*S. brevidens* have been identified by DNA sequence analysis. AM66 (GenBank accession no. U30814) showed 99% homology to the Kunitz-type proteinase inhibitor described by Ishikawa et al. [17] (accession no. M17329). AM98 (accession no. U30861) was 98% identical to the wound inducible gene *win*I [18] (accession no. M17108).

GM7, at the nucleic acid level, showed 86% identity to the 'entirely fruit-specific' tomato cDNA clone 2A11 (Fig. 3A) that is expressed steadily in increasing levels from anthesis to breaker, and accounts for approx. 1% of the mRNA in mature tomato fruit [19]. Blast analysis revealed partial similarities to the metallocarboxypeptidase inhibitor proteins of potato and tomato [20,21] (Fig. 3B). Thus, GM7 may represent a new type of proteinase inhibitors, the cDNA of which has not yet been isolated from potato.

The *S. brevidens* specific clone pLL12 (accesion no. U30304) represents a proline-rich protein 95% homologous to the cDNA isolated by Salts et al. [22] (accession no. X57076) as a copy of a gene highly expressed in young tomato fruit. Thus, LL12, like the other proteins characterized by high proline or hydroxyproline content, may contribute to the cell wall architecture of *S. brevidens* shoots.

3.4. Factor(s) affecting the transcription of the genes differentially expressed in S. tuberosum and S. brevidens

The result of the differential screening was verified and the temporal expression of the patatin gene and that of the proteinase inhibitors were studied by Northern hybridization. Probes were prepared from the corresponding cDNAs (Tu58, GM7, AM66, AM98) and hybridized to the filters containing RNAs isolated from axillary buds and from 5-, 10- and 15-day-old shoots developed under inducing conditions in the dark both from *S. tuberosum* and from *S. brevi-*

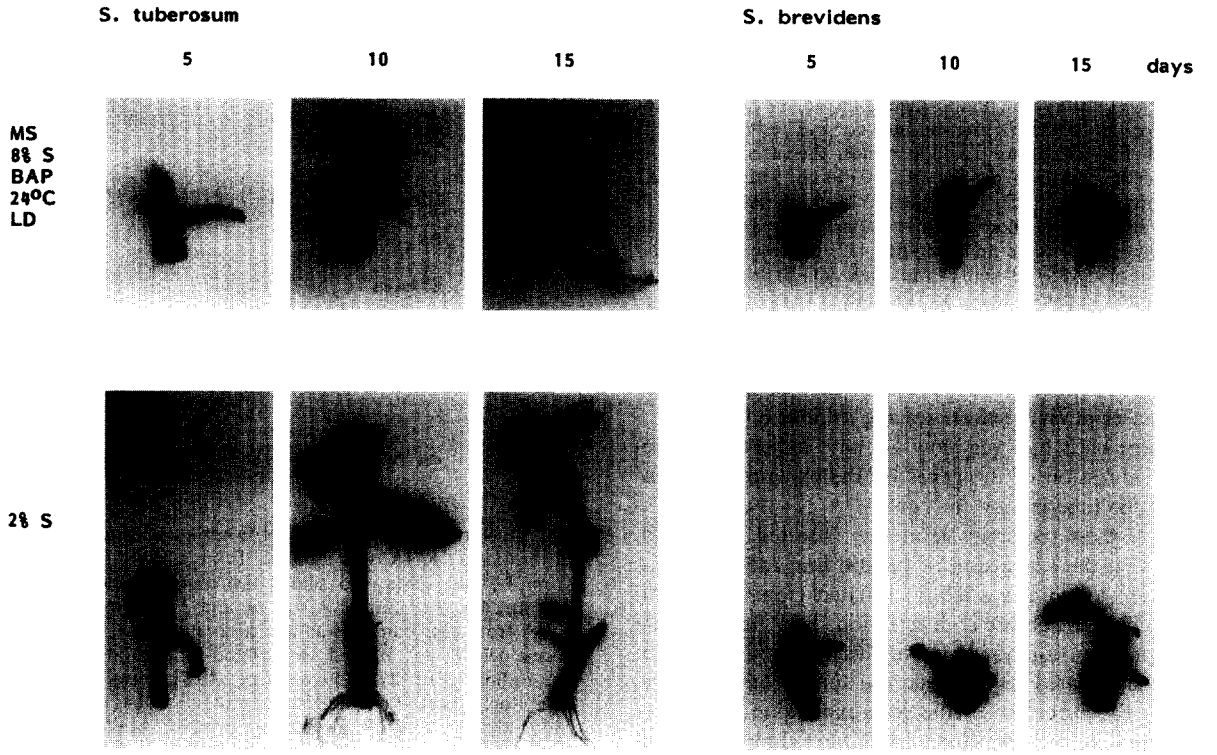

Fig. 2. Development of axillary buds from stem cuttings of *S. tuberosum* and *S. brevidens* under long-day conditions. Growth conditions: MS+8% sucrose+BAP and MS+2% sucrose+BAP, respectively.

A

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1    CTGTTCATCAATTAGCAATTAATCCAAAACCATAATGGCTGCCAAAAATT
                                    M  A  A  K  N  S

51   CAGAGATGAAGTTTGCTATCTTCTTCGTTGTTCTCTTGACGACCACTTTA
      E  M  K  F  A  I  F  P  V  V  L  L  T  T  T  L

101  GTTAATATGCAAGTGATGGCTCTTCGAGACATGCCGCCACAAGAAACATT
     V  N  M  Q  V  M  A  L  R  D  M  P  P  Q  E  T  L

151  GCTGAAAATGAAGCTATTTTCCTCAAATGTTTTGGGAACTTGTAACGATT
      L  K  M  K  L  F  S  S  N  V  L  G  T  C  N  D  Y

201  ATTGCAACACAAACGCCGATTGCTTCGGAATTACCCTCTGCCCGTGGTGT
       C  N  T  N  A  D  C  F  G  I  T  L  C  P  W  C

251  AAGCTGAAGAAGTCCTCCAGTGGTTTCACATACAGTGAATGCTCCCTGTT
     K  L  K  K  S  S  S  G  F  T  Y  S  E  C  S  L  L

301  GCCTTGAACAATATCTACGATCTATCTATTATCTATCTATCTCTACGTGT
      P  *

351  GTTTATGTGCGTGCGCGTACTTTTTCTTTTTTTTTTTAATTTTCGTCTTT

401  GTACCTTTTCCAATCAACACTACTATGTTTAGTGTTGTCTTTGTATGTCT

451  TTACCCTTTGGTGTGAAGAATATGAATAAAGGGATATGTATCTAGATATA

501  TTCTAGGTAATGTCCTATTGTTTAT
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B

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                          1  1     1     1  1
MCPI S.t.     QQHADPICNKPCKTH DDCSGAWFCQ ACWNSARTCG PYVG

MCPI L.e.      QQYDPVCHKPCSTQ DDCSGGTFCQ ACWRFAGTCG PYV

GM7                  CNDYCNTN ADCFGITLCP WCKLKKSSSG
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Fig. 3. Sequence analysis of GM7 (accession no. U30388). (A) Nucleotide and deduced amino acid sequences of GM7, a cDNA homologous to the tomato clone 2A11 (accession no. X13741). The identical basis and amino acids are indicated by stippling. Black square corresponds to a deletion with 6 amino acids in GM7 compared to 2A11. The original cDNA clone GM7 was 'truncated'. Full-length sequence has been obtained by cloning the corresponding 5′ fragment with the help of RACE (see section 2). The primer used in the PCR reaction is labelled with a line. (B) Aligment of the cysteine-rich region of GM7 to the mature metallocarboxypeptidase inhibitors isolated from potato (accession no. P01075) and tomato (accession no. A01315). Arrows indicate five residues of half-cystine, which may occur as disulfide bonds. Replacement of the sixth characteristic cysteine residue of MCPIs by serine in GM7 could have arisen by a single base change. Implementation of Von Heijne's [36] rules for predicting signal sequence clevage sites and the hydropathy plot of Kyte and Doolittle [37] indicate that the N-terminal region of GM7 like that of the other MCPIs may also contain a pro-domain sequence (data not shown).

dens. Fig. 4 verifies the result of the differential screening that none of the four genes tested were expressed in *S. brevidens* while the transcript level was increasing in potato from day 5 to 15. Axillary buds had very low activity, indicating induction of the expression of the genes by the experimental conditions used for tuberization, in vitro.

Since the highest transcript level was detected at day 15, factors affecting the expression of patatin and the proteinase inhibitors were tested using RNAs isolated from 15-day-old plants. The effect of sucrose concentration, BAP, temperature and light has been analyzed in Northern hybridization. In each case, the elevated level of sucrose turned out to be responsible for the induction of the gene expression (Fig. 5).

The *S. brevidens* specific clone LL12, encoding a proline-rich cell wall protein, showed the highest expression on days 5–10 (Fig. 4). Although no significant amount of this transcript could be detected in axillary buds, none of the factors analyzed in our experiments influenced the expression of LL12 (Fig. 6).

3.5. *Expression of the starch synthesis genes in* S. tuberosum *and in* S. brevidens

Although the expression of the genes involved in starch synthesis is characteristic for potato tuber development [23], no corresponding cDNAs have been isolated from *S. tuberosum* by differential screening using *S. brevidens* as a negative partner. Therefore, we have decided to check the expression of these genes in developing shoots and tubers by Northern hybridization experiment.

Using the AGPase B and S as well as the GBSS cDNA (kindly provided by L. Willmitzer) as hybridization probe, expression of each gene was detected in *S. brevidens* as well as in potato induced for tuberization. Although in potato the steady-state level of mRNA increased in time, the opposite tendency was found in *S. brevidens*. The highest amount of mRNA could be detected 5 days after induction and that was reduced by day 15 (Fig. 4). When factors affecting the expression of the genes were tested sucrose was found to be the

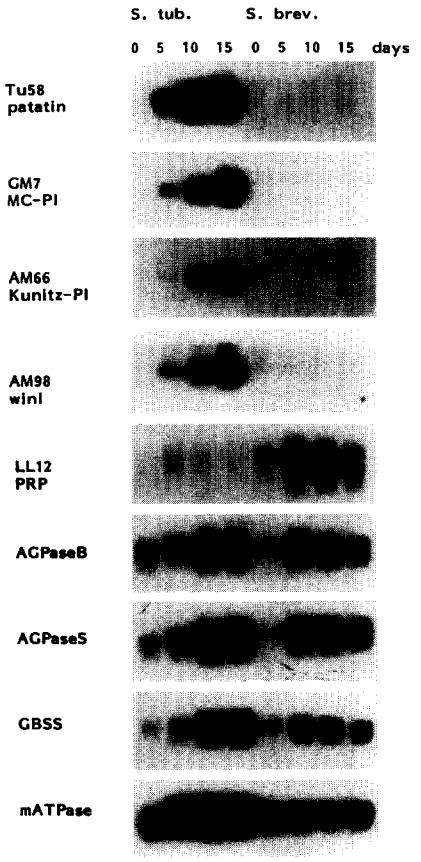

Fig. 4. Northern analysis of RNAs isolated from *S. tuberosum* and *S. brevidens*. RNA was isolated from axillary buds (day 0) and from 5-, 10-, 15-day-old shoots developed in the dark at 24°C in MS medium containing 8% sucrose and 2.5 mg/ml BAP. 20 μg total RNA from each sample was loaded in each lane, separated on a 1% formaldehyde gel, blotted to Hybond-N filter and hybridized with the radiolabelled cDNAs indicated. MCPI, metallocarboxypeptidase inhibitor; Kunitz-PI, Kunitz-type proteinase inhibitor; *win*I, wound-inducible gene I; PRP, proline-rich protein; AGPaseB, ADP-glucose pyrophosphorylase, subunit B; AGPaseS, ADP-glucose pyrophosphorylase, subunit S; GBSS, granule bound starch synthase; mATPase, mitochondrial F_1-ATPase α-subunit.

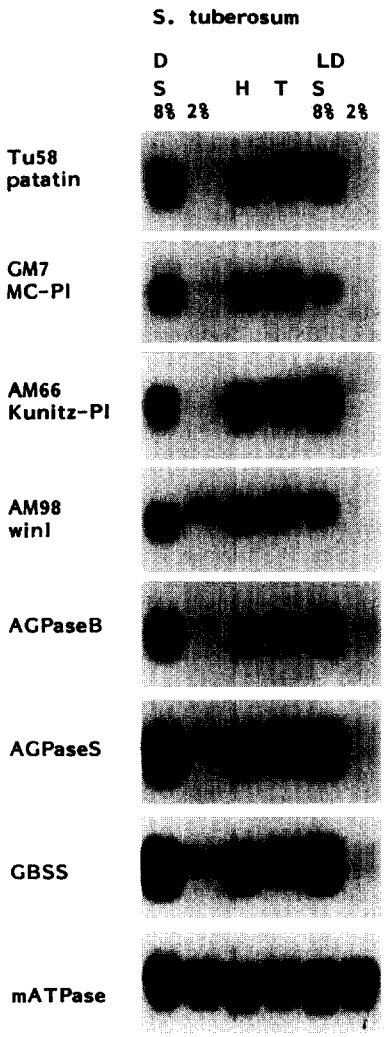

Fig. 5. Identification of factor(s) influencing gene expression in *S. tuberosum*. Northern hybridization was carried out as in Fig. 4 with RNAs isolated from 15-day-old shoots/tuberous shoots developed from axillary buds under various conditions. D, dark; LD, long-day photoperiod; S, sucrose concentration in the medium; H, no BAP in the medium; T, elevated temperature (30°C). Abbreviations of the probes are the same as in Fig. 4.

inducer in both species (Figs. 5 and 6). This finding supports the conclusion of the morphological studies at molecular level: *S. brevidens* like potato is able to sense and respond to the elevated level of sucrose.

Although transcripts derived from the genes involved in starch synthesis could be detected in *S. brevidens* as well as in potato, no starch granules were found in any of the part of the *S. brevidens* shoots tested (see section 2 for details).

4 Discussion

Our results demonstrate that morphological changes leading to tuberization in potato parallel with the sucrose concentration and with the expression of patatin, proteinase inhibitors and that of the genes involved in starch synthesis.

Previous experiments have already shown that both developmental and metabolic signals activate the promoter of certain tuber storage protein genes [24–26]. Recent studies on patatin promoter have identified tuber-specific and sucrose-inducible *cis* elements [27,28]. Sucrose-mediated expression of genes involved in starch synthesis has also been described before [29–31]. All these genes, however, belong to multigene families, moreover, factors other than sucrose (i.e. anaerobiosis, wounding, light, glutamine, abscisic acid, jasmonates) can also induce their expression [31–33]. In our experiments, leaves were cut off from the stem segments. In this system, we have found that tuber formation was essentially dependent only on sucrose concentration and all types of known genes specific for tuberization were turned on by this factor (Fig. 5). Thus, we have concluded that sucrose is the primary signal of tuberization. Sucrose, however, does not necessarily regulate gene expression directly. It might be only the first signal in a complex signal transduction pathway that may branch at certain points and connect to other regulatory routes (stresses, abscisic acid, jasmonates etc.). Thus, sucrose may induce (or repress) the activities of different genes by different *cis*- and *trans*-acting elements.

Differential screening of *S. tuberosum* and *S. brevidens* resulted in isolation of a new tuber specific gene (GM7) that probably encodes a metallocarboxypeptidase inhibitor. The gene is homologous to the tomato clone 2A11 that is expressed during fruit development.

Southern blot analysis of genomic DNA indicated patatin, and proteinase inhibitor sequences in *S. brevidens* [34]. Hansen and Hannapel [35] showed that the wound-inducible cathepsin D inhibitor and the 22-kDa potato proteinase inhibitor genes are not sucrose inducible in the leaves of non-tuber-bearing species. Our data indicate that the Kunitz-type, *win*I, and GM7 proteinase inhibitors are not turned on by elevated levels of sucrose in *S. brevidens* shoots grown from axillary buds under tuber inducing conditions.

Unlike tuber storage proteins, starch-synthesis genes (AGPaseB, S, and GBSS) were induced by sucrose both in *S. tuberosum* and in *S. brevidens*. No starch granules, however, could be detected in the non-tuberizing species. The transcript

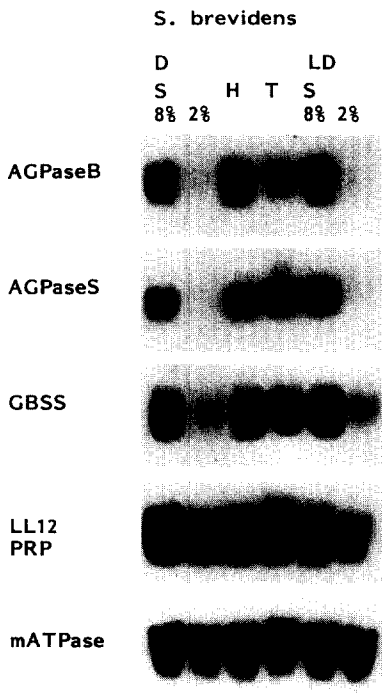

Fig. 6. Identification of factor(s) influencing gene expression in *S. brevidens*. Northern hybridization and abbrevations are the same as in Figs. 4 and 5. RNAs were isolated 10 days after induction.

level of starch-synthesis genes increased in time in potato while decreased in *S. brevidens*. There might be a feed-back mechanism that reduces the amount of mRNAs from the genes involved in starch synthesis in the absence of starch accumulation. In anti-AGPase or anti-Susy potato plants inhibition of starch accumulation decreases the level of patatin and proteinase inhibitor mRNAs [4,7]. We have found that the same set of genes are not induced in *S. brevidens* where starch is not accumulated in the cells. Although both starch-synthesis and tuber storage protein genes are primarily regulated by sucrose, they might be influenced by different factors of a complex signal transduction cascade.

We have isolated a cDNA clone (LL12) of a proline-rich cell wall protein (PRP) gene with *S. brevidens* specific expression. None of the factors tested in our experiments (sucrose, temperature, light, BAP), however, was reponsible for the transcriptional activation of it. Thus, the influence of the PRP on tuberization remains unknown.

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