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A second L-type isozyme of potato glucan phosphorylase: cloning, antisense inhibition and expression analysis

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Abstract

In potato tubers two starch phosphorylase isozymes, types L and H, have been described and are believed to be responsible for the complete starch breakdown in this tissue. Type L has been localized in amyloplasts, whereas type H is located within the cytosol. In order to investigate whether the same isozymes are also present in potato leaf tissue a cDNA expression library from potato leaves was screened using a monoclonal antibody recognizing both isozyme forms. Besides the already described tuber L-type isozyme a cDNA clone encoding a second L-type isozyme was isolated. The 3171 nucleotide long cDNA clone contains an uninterrupted open reading frame of 2922 nucleotides which encodes a polypeptide of 974 amino acids. Sequence comparison between both L-type isozymes on the amino acid level showed that the polypeptides are highly homologous to each other, reaching 81–84% identity over most parts of the polypeptide. However the regions containing the transit peptide (amino acids 1–81) and the insertion sequence (amino acids 463–570) are highly diverse, reaching identities of only 22.0% and 29.0% respectively.

Northern analysis revealed that both forms are differentially expressed. The steady-state mRNA levels of the tuber L-type isozyme accumulates strongly in potato tubers and only weakly in leaf tissues, whereas the mRNA of the leaf L-type isozyme accumulates in both tissues to the same extent. Constitutive expression of an antisense RNA specific for the leaf L-type gene resulted in a strong reduction of starch phosphorylase L-type activity in leaf tissue, but had only sparse effects in potato tuber tissues. Determination of the leaf starch content revealed that antisense repression of the starch phosphorylase activity has no significant influence on starch accumulation in leaves of transgenic potato plants. This result indicated that different L-type genes are responsible for the starch phosphorylase activity in different tissues, but the function of the different enzymes remains unclear.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X73684.

Introduction

The principal pathway of starch biosynthesis in higher plants has been demonstrated to proceed via the ADP-glucose pyrophosphorylase pathway in leaves and storage tissues [8, 14, 15, 20, 31]. The knowledge about starch degradation is much more rudimentary and restricted to the identification and characterization of starch-degrading enzymes. Among those starch phosphorylases and amylases are believed to be the most important enzymes. The presence of different isoforms in different compartments complicates the interpretation of the role of individual enzymes. Although starch metabolism follows the same scheme in all cell types the regulation must be adapted to the specific demand. In leaf mesophyll cells starch metabolism follows a diurnal rhythm. During the light CO_2 fixation exceeds the demand for photoassimilates in metabolic processes which leads to the deposition of excess photoassimilates into starch in mesophyll cells which is degraded during the dark period. Starch turnover in guard cells is under environmental control and associated with stomatal opening and closure. In storage tissues such as seeds or tubers starch is stored for a prolonged time in amyloplasts in order to supply the next generation with energy, and hence is under developmental control. Irrespective of the obvious differences not much is known about the underlying processes.

We are analysing the role of different starch phosphorylase isozymes during leaf or storage starch breakdown. Starch phosphorylases are known to exist in two different forms (types L and H) which differ not only in their localization but also in their affinities for glucans. The L-type isozyme is localized within plastids [28] and has a low affinity to oligoglucans [26], whereas the H-type isozyme is located within the cytosol [6] and has a high affinity for oligoglucans [26]. cDNA clones have been isolated for starch phosphorylase type L from potato tuber [4, 22] and sweet potato roots [16] and starch phosphorylase type H from potato tuber [19]. The presence of a cytosolic starch phosphorylase remains to be an enigma. Yang and Steup [33] identified a cytosolic polysaccharide fraction which strongly interacts with the cytosolic starch phosphorylase from spinach and pea. This finding opens the possibility that the cytosolic isoform has a different function than starch breakdown. In order to shed some light on the role of starch phosphorylase isozymes we are using transgenic plants expressing antisense RNA to the respective starch phosphorylase isoform.

Here we report the isolation of a second cDNA encoding starch phosphorylase type L from potato leaves which is different from the already published tuber cDNA. We present evidence for a differential expression of both L-type genes and demonstrate that the second L-type isoform is the dominant form in potato leaves using the antisense technology. This finding indicates that, besides the known metabolic regulation of starch metabolism (for review see [23]), a differential expression of respective enzymes might be responsible for the adaption of the demand in different organs and cell types.

Materials and methods

Plants, bacterial strains and media

Potato plants (*Solanum tuberosum* cv. Désirée) were obtained through 'Vereinigte Saatzuchten eG', 3112 Ebstorf, FRG. Plants in tissue culture were grown under a 16 h light/8 h dark regime on Murashige and Skoog medium [21] containing 2% sucrose. Plants used for biochemical analysis were grown in a 16 h light/8 h dark cycle (light 200–250 μmol m^{-2} s^{-1}) in individual pots (diameter 16 cm, depth 12 cm) under greenhouse conditions. Sink leaves (< 2 cm in length) and source leaves (> 15 cm in length) were taken from 4- and 10-week old plants as indicated in the text. Growing potato tubers were harvested from non-senescing plants (10 weeks old). *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) and XL-1 Blue [5] were cultivated using standard techniques [18]. *Agrobacterium tumefaciens* strain C58C1 containing pGV2260 [7] was cultivated in YEB medium [32].

Reagents

DNA restriction and modification enzymes were obtained from Boehringer Mannheim (Ingelheim, FRG) and New England Biolabs (Danvers, MA). Reagents for SDS-PAGE were purchased from BioRad (St Louis, MO). Chemicals were obtained through Sigma (St Louis, MO) or Merck (Darmstadt, FRG). Radiochemicals were obtained through Amersham Buchler (Braunschweig, FRG).

Plant transformation

Plant transformation using the *Agrobacterium*-mediated gene transfer for potato was done according to Rocha-Sosa *et al.* [27].

Construction of cDNA library and screening

The potato leaf cDNA in λZAP II was as described by Kossmann *et al.* [13]. The cDNA library was screened using the monoclonal antibody J82-5-C2 [11] following standard protocols. Positive signals were detected using the peroxidase system from Amersham Buchler (Braunschweig, FRG).

Expression of starch phosphorylase in E. coli

Following *in vivo* excision of positive λZAP II clones (Stratagene, Heidelberg), plasmids (pBlue-Script SK–) encoding the full-size starch phosphorylase polypeptides (including the transit peptide) were used for the expression of the proteins in *E. coli*. Transformed XL-1 blue cells were cultivated at 37 °C in the presence of 200 μg/ml ampicillin and 10 μg/ml tetracycline with shaking until the value A_{600} reached 0.3. After addition of IPTG (f.c. 1 mM) and pyridoxine (f.c. 10 mM) the cultivation was continued overnight at the indicated temperatures (Fig. 2). After incubation, cells were harvested by centrifugation and disrupted by sonication on ice in a buffer containing 25 mM Tris-HCl pH 7,5, 1 mM EDTA and 5 mM DTT [19]. Debris was removed by centrifugation and the protein extract used for the determination of starch phosphorylase activity in polyacrylamide gels (40 μg protein/lane). The protein concentration was determined according to Bradford [3].

Construction of chimeric plasmids

The complete cDNA insert of clone STP-27, belonging to the STP-1 family, was isolated with the restriction enzymes *Eco* RV and *Sma* I and was cloned in the antisense orientation between the 35S cauliflower mosaic virus promoter [9] and the octopine synthase polyadenylation signal [10]. For plant transformation the final construct was cloned into the binary vector Bin 19 [2] and the plasmid pBin-STP-2 was used for the direct transformation of *Agrobacterium* strain C58C1: pGV2260 [12].

Northern analysis

Total RNA isolation and electrophoretic separation was done according to Logemann *et al.* [17]. RNA blot hybridization was performed as described by Amasino [1]. Membranes (Hybond N. Amersham, UK) were hybridized in buffers containing polyethylene glycol (7% w/v) and formamide (50% v/v). Radioactive labelling of DNA probes were performed using a multiprime labelling kit (Amersham, UK). Full-size cDNA clones STP-1 and STP-23 were used for the labelling reaction. After hybridization at 42 °C, filters were washed twice in 1 × SSC, 0.5% SDS for 30 min and once in 0.1 × SSC, 0.5% SDS for 30 min at 68 °C.

Detection of starch phosphorylase activity in polyacrylamide gels

Plant material was homogenized in 50 mM sodium phosphate, 2 mM EDTA pH 7.2, 10% (v/v)

glycerol and 0.4 mM phenylmethylsulphonyl fluoride. The proteins (40 μg/lane) were separated in anionic acrylamide gels containing 2% glycogen according to Steup [29]. After electrophoresis the gel was equilibrated with 100 mM citrate-NaOH pH 6.0. Glucan phosphorylase activity was determined by incubating the gel in 100 mM citrate-NaOH pH 6.0 containing 0.05% soluble (w/v) starch and 20 mM glucose-1-phosphate. After the incubation (2 h, 37 °C) the gel was washed with 100 mM citrate-NaOH pH 6.0. Upon iodine staining (10 mM I_2 and 14 mM KI) bands of phosphorylase activity appeared as blue zones.

Determination of starch

Leaf discs (taken at the times indicated) were extracted in 80% ethanol (10 mM HEPES-KOH, pH 7.4) at 80 °C for 1 h. The supernatant containing soluble sugars was removed and the remaining leaf discs were extracted a second time, washed in water and dried. Determination of starch was done according to Stitt *et al.* [30].

Results and discussion

Cloning and sequencing of a second L-type starch phosphorylase isozyme from potato leaves

To isolated cDNA clones encoding starch phosphorylase isozymes from potato leaves a λZap expression library was screened using the monoclonal antibody J82-5-C2. This monoclonal antibody recognizes both starch phosphorylase isoform types L and H in western blot experiments [11]. From about 10^5 plaques 45 positive transformants were isolated. These transformants were grouped into two families based on restriction analysis and their cross-reactivity in Southern blot experiments (data not shown). No cross-hybridization between the two families was observed under the conditions used. Further analysis was done on two representatives of the two families named STP-23 and STP-1. Sequencing one member of each family revealed that one of the families (STP-23) was identical to the already published L-type isozyme [22]. The members of the second family (STP-1), however, were different from the published L-type isozyme and the complete nucleotide sequence was determined. The 3171 nucleotide long cDNA clone contains a 2922 nucleotide long open reading frame which encodes a polypeptide of 974 amino acids. The translational start site can be predicted to be at the first methionine codon after the last stop codon found in the 5′-untranslated region. In the 3′-untranslated region the sequence (AATAAA) was found which is homologous to the eucaryotic polyadenylation signal [24, 25]. At the 3′ end of the cDNA clone a poly(A) tail is present. Based on the nucleotide sequence the amino acid sequence of clone STP-1 was deduced and compared to the already published L-type isozyme (Fig. 1A). On the amino acid level the polypeptides can be divided into four regions of homologies to different degrees (Fig. 1B). Region 1 (amino acids (aa) 1-81) shows only 22% identity; this part contains the transit peptide of the STP-23 isozyme (aa 1–50). Region II (aa 82–463) shows 81% identity, region III (aa 464–570) is 29% identical and comprises the insertion sequence characteristic for L-type isoforms. Region IV (aa 571–974) shows 84% identity.

Due to their different affinities to glycogen type H (high affinity) and type L (low affinity) starch phosphorylase isoforms can be separated in glycogen-containing polyacrylamide gels.

To test whether the newly isolated clone STP-1 had the properties of the L-type isozyme, the cDNA clone, containing the complete starch

Fig. 1. Amino acid comparison between the two starch phosphorylase L-type isoforms from potato. A. Amino acid alignment, upper sequence: STP-1 (newly isolated leaf form); lower sequence STP-23 (identical to the published sequences [4, 22]). Sequence aligment was done using the program MacMolly version 3.5.1. on a MacIntosh computer. Dots indicate identical amino acids; dashes indicate amino acid deletions. B. Subdivision of the starch phosphorylase polypeptides in regions of variable homologies. Region I, containing the transit peptide; region III, containing the starch phosphorylase L-type specific insertion sequence.

1A

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1   MATFAVSGLN SISSISSFNN NFRSKNSNIL LSRRRILLFS FRRRRRSFSV SSVASDQKQK  60
1   M..ANGAH.F NHY.SN.RFI H.T.R.TSSK .FLTKTSH.R RPK.CFHVNN TLSEKIHHPI  60

61  TKDSSSDEGF TLDVFQPDST SVLSSIKYHA EFTPSFSPEK FELPKAYYAT AESVRDTLII 120
61  .EQGG---ES D.SS.A.DAA .IT..... ....V....R ......FF.. .Q....S.L. 117

121 NWNATYEFYE KMNVKQAYYL SMEFLQGRAL LNAIGNLGLT GPYADALTKL GYSLEDVARQ 180
118 ......DI.. .L.M..... ..........  .......E.. .AF.E..KN. .HN..N..S. 177

181 EPDAALG-NG GLGRLASCFL DSMATLNYPA WGYGLRYQYG LFKQLITKDG QEEVAENWLE 239
178 .......N.. ..........  ..L.......  .......K.. ....R..... ......D... 237

240 MGNPWEIVRN DISYPVKFYG KVIEGADGRK EWAGGEDITA VAYDVPIPGY KTKTTINLRL 299
238 I.S...V... .V...I.... ..ST.S..KR Y.I.....K. ..........  ..R...S... 297

300 WTTKLAAEAF DLYAFNNGDH AKAYEAQKKA EKICYVLYPG DESLEGKTLR LKQQYTLCSA 359
298 .S.QVPSAD. ..S...A.E. T..C...AN. .....I.... ...E...I.. .......... 357

360 SLQDIIARFE KRSGNAVNWD QFPEKVAVQM NDTHPTLCIP ELLRILMDVK GLSWKQAWEI 419
358 ......S... R...DRIK.E E.........  ..........  ..M...I.L. ..N.NE..N. 417

420 TQRTVAYTNH TVLPEALEKW SFTLLGELLP RHVEIIAMID EELLHTILAE YGTEDLDLLQ 479
418 ..........  ..........  .YE.MQK...  ......EA..  ...VHE.VLK ..SM..NK.E 477

480 EKLNQMRILD NVEIPSSVLE LLIKAEESAA DVEKAADEEQ EEEGKDDSKD EETEAVKAET 539
478 ...TT....E .FDL....A. .F..P.I.VD .DTETVEVHD KVEAS.KVVT NDEDDTGKKT 537

540 TNEEEETEVK KVEVEDSQAK IKRIFGPHPN KPQVVHMANL CVVSGHAVNG VAEIHSEIVK 599
538 SVKI.AAAE. DIDKKTPVS- ----PE.AVI P.KK.R.... ...G......  .......... 592

600 DEVFNEFYKL WPEKFQNKTN GVTPRRWLSF CNPELSEIIT KWTGSDDWLV NTEKLAELRK 659
593 E....D..E. ..........  .......IR. ...P..A...  ....TE..VL K.......Q. 652

660 FADNEELQSE WRKAKGNNKM KIVSLIKEKT GYVVSPDAMF DVQIKRIHEY KRQLLNIFGI 719
653 .....D..N. ..EA.RS..I .V..FL.... ..S.......  .I.V......  .......... 712

720 VYRYKKMKEM SPEERKEKFV PRVCIFGGKA FATYVQAKRI VKFITDVGET VNHDPEIGDL 779
713 ..........  TAA...TN..  ..........  ..........  ........A. I......... 772

780 LKVVFVPDYN VSVAEVLIPG SELSQHISTA GMEASGTSNM KFSMNGCLLI GTLDGANVEI 839
773 ..........  .....L...A .D..E.....  ..........  ..A.....Q. .......... 832

840 REEVGEDNFF LFGAQAHEIA GLRKERAEGK FVPDPRFEEV KAFIRTGVFG TYNYEELMGS 899
833 ......E...  ..........  .......D..  ....E.....  ...V.S.A.. S...DD.I.. 892

900 LEGNEGYGRA DYFLVGKDFP DYIECQDKVD EAYRDQKKWT KMSILNTAGS FKFSSDRTIH 859
893 ......F...  ..........  S.....E...  .......R..  T.........  Y......... 952

960 QYARDIWRIE PVELP                                                   974
953 E..K...N.. A..IA                                                     967
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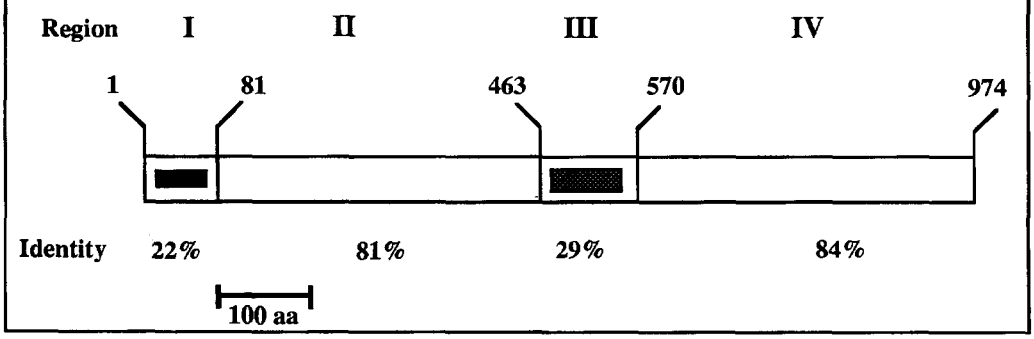
1B



phosphorylase-coding region, was expressed in *E. coli* at different temperatures (20 °C, 28 °C and 37 °C) overnight. After induction, protein extracts were separated on polyacrylamide gels containing glycogen. Starch phosphorylase activity was visualized after incubation by iodine staining of the synthesized starch (see Materials and methods). The result of such an experiment is shown in Fig. 2. Starch phosphorylase activity which was not retarded by glycogen could be found at 20 °C (Fig. 2, panel 2) and 28 °C (Fig. 2, panel 3) but not at 37 °C (Fig. 2, panel 4). In control extracts (Fig. 2, panels 5 to 7) no starch phosphorylase activity could be detected. The migration behaviour of the starch phosphorylase produced in *E. coli* was identical to the L-type starch phosphorylase isoform present in leaf protein extracts from potato (Fig. 2, lanes 1 and 8; lower band). The temperature dependence is in agreement with the recently published data obtained for starch phosphorylase type H expressed in *E. coli* [19]. The cDNA clone STP-1 contains stop codons in front of the ATG and encodes the precursor starch phosphorylase polypeptide. The observation that a polypeptide of the predicted migration behaviour is synthesized can be explained, if an alternative translational start codon is used or if some structural features of the polypeptide obscure the migration behaviour of the protein in native polyacrylamide gels.

Expression analysis of starch phosphorylase L-type isozymes

To elucidate the possible function of the different starch phosphorylase L-type isoforms, the tissue-specific expression of the two isolated classes (STP-23 and STP-1) was investigated. Total RNA from different potato tissues (Fig. 3) was isolated from 10-week old plants grown in the greenhouse and probed for the presence of the specific mRNAs using the full-size cDNA clones STP-1 and STP-23 as probes. This analysis (Fig. 3) revealed that STP-23-specific transcripts accumulate strongly in growing potato tubers (> 10 g fresh weight) but only weakly in stolons, roots, flowers and stem node segments. In sink (length < 2 cm) and source (length > 15 cm) leaves harvested after 8 h illumination STP-23 transcript was hardly detectable. In contrast to the low abundance of STP-23 specific transcripts

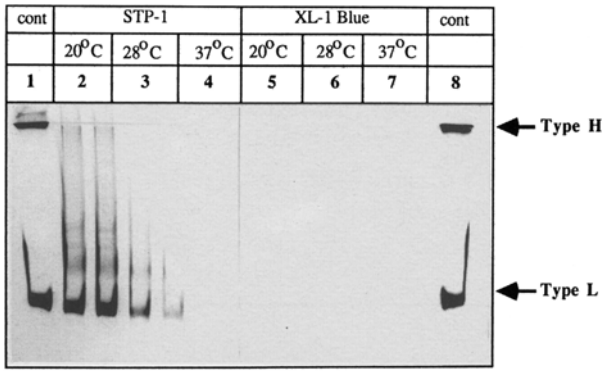


Fig. 2. Temperature-dependent starch phosphorylase activity in transformed *E. coli* cells. Protein extracts (40 μg/lane) from untransformed (XL-1-Blue) and transformed (STP-1) *E. coli* cells were analysed for starch phosphorylase activity on polyacrylamide gels containing 2% glycogen (see Materials and methods). Following induction of growing *E. coli* with IPTG the cultivation was continued at 20 °C, 28 °C and 37 °C overnight. As controls (cont; lanes, 1 and 8) 40 μg total potato leaf protein extracts were included. H-type and L-type isoforms are marked by arrows.

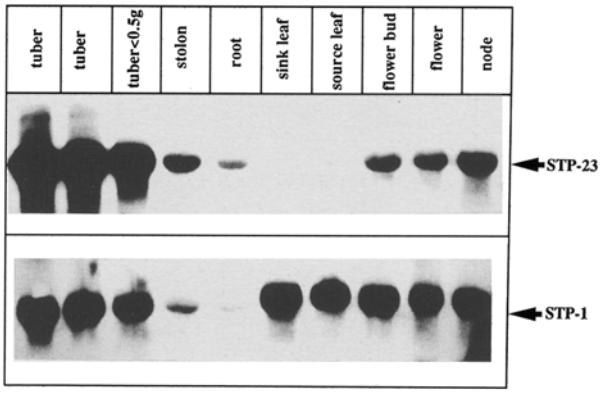


Fig. 3. Tissue-specific expression of the two starch phosphorylase L-type isoforms from potato. Total RNA was extracted from different potato tissues of 10-week old plants. Equal amounts of RNA (50 μg/lane) were separated in formaldehyde-containing 1.5% agarose gels, blotted onto a nylon membrane (Hybond N) and probed for the presence of the STP-1 and STP-23 specific transcripts using ^{32}P-labelled full-size cDNA fragments. The tubers (fresh weight > 10 g) used were harvested from growing potato plants.

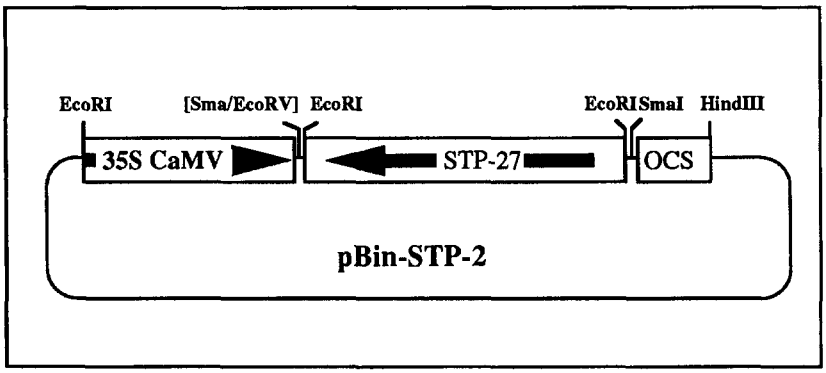


Fig. 4. Construction of a chimeric gene expressing STP-1 antisense RNA. 35S CaMV, 540 bp promotor fragment of the cauliflower mosaic virus 35S transcript; STP-27, *Sma* I/*Eco* RV cDNA fragment of clone 27 belonging to the STP-1 family; OCS, 214 bp polyadenylation signal of the octopine synthase gene; vector, Bin19.

in potato leaves, accumulation of STP-1 mRNA was found to be high in sink and source leaves (Fig. 3). In all other tissues the STP-1 specific transcripts accumulated to the same extent (Fig. 3) with the exception of roots and stolons where the STP-1-specific mRNA was only very weakly expressed. These data indicate that STP-1 might be the dominant isoform in potato leaves, possibly being involved in the turnover of photosynthetically synthesized starch.

Antisense expression of STP-1 inhibits starch phosphorylase type L-specific activity in transgenic potato plants

Based on the expression data obtained for STP-23 and STP-1 it was postulated that the STP-1 isoform is the major starch phosphorylase type-L isoform in potato leaves. To address the question whether STP-1 is the dominant activity in potato leaves, a chimeric genes was constructed containing the CaMV promoter, the cDNA sequence of clone STP-27 (a member of the STP-1 family) in the antisense orientation and the octopine synthase polyadenylation signal (Fig. 4, pBin-STP-2). Subsequently potato plants were transformed using the *Agrobacterium tumefaciens* system. Regenerated and kanamycin-resistant plants were transferred into the greenhouse. The reduction of STP-1-specific transcripts was analysed in source leaves of four-week old plants. Several plants with an almost complete loss of STP-1 specific mRNA could be detected in northern blot experiments (Fig. 5; plants 5, 6, 8, 40, 49, 55, 56, 66, 68). To investigate whether the loss of STP-1-specific transcripts would reduce the starch phosphory-

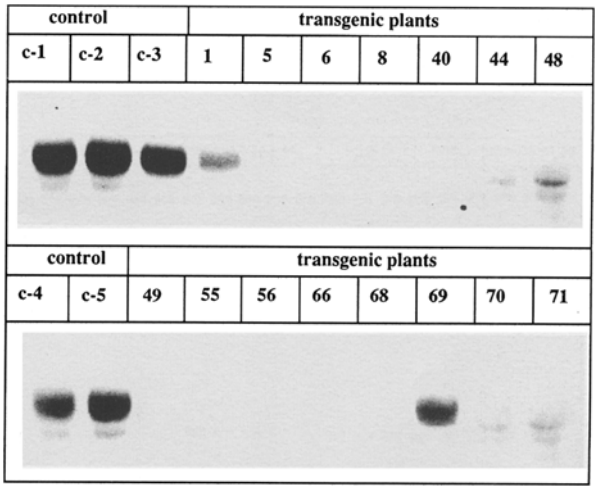


Fig. 5. RNA blot analysis of transgenic potato plants expressing antisense RNA of STP-1. Total potato leaf RNA (50 μg each lane) harvested from 4-week old plants was separated by gel electrophoresis and transferred onto a nylon membrane. Subsequently, the filter was hybridized using the ^{32}P-labelled STP-1 cDNA as a probe. C-1 to C-5, RNA isolated from untransformed control plants; 1, 5, 6, 8, 40, 44, 48, 55, 56, 66, 68, 69, 70, 71: independent transformants, expressing STP-1 antisense RNA showing strongly reduced STP-1 transcript levels.

lase L-type activity in leaves, total leaf protein was extracted from wild-type and transgenic plants and analysed for starch phosphorylase activity after electrophoresis on polyacrylamide gels containing glycogen (see Materials and methods). Analysis of protein extracts demonstrated that in the most highly inhibited plants (8, 40, 55 and 56) the majority of the L-type starch phosphorylase activity was removed from leaf extracts (Fig. 6). To verify that STP-1 antisense transcripts have no effect on the starch phosphorylase activity in growing potato tubers (> 10 g fresh weight), total soluble proteins were extracted from leaves and tubers from 10-week old plants. After electrophoresis starch phosphorylase activity was visualized in polyacrylamide gels. As shown in Fig. 7, no obvious inhibition of starch phosphorylase activity occurs in tubers of transgenic potato plants (STP-2–6, 44, 48, 56, 70 and 71) as compared to wild-type controls (c-1 and c-2). The antisense inhibition was specific for the type-L isoform, since no reduction of the H-type isoform was visible (Figs. 6 and 7, upper band). These data can only be explained assuming that STP-1 is the dominant L-type starch phosphorylase activity in leaves.

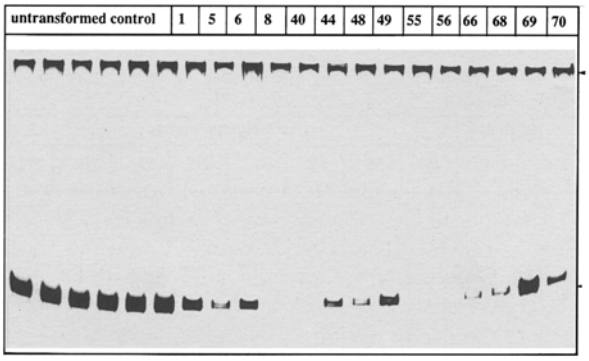


Fig. 6. Antisense inhibition of starch phosphorylase L-type activity in leaves of transgenic potato plants. Total leaf protein extracts from untransformed control plants and transgenic plants showing strongly reduced STP-1 transcript levels (40 μg each lane) were separated in a polyacrylamide gel containing glycogen. After electrophoresis and incubation, starch phosphorylase activity was visualized by iodine staining (see Materials and methods). The upper band represents the retarded high affinity H-type isoform, whereas the lower band represents the unretarded low-affinity L-type isoforms.

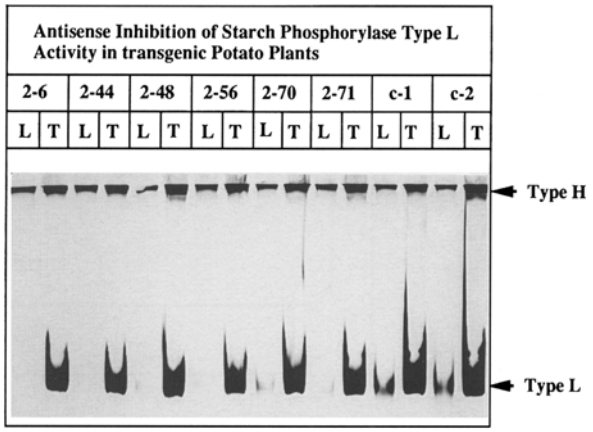


Fig. 7. Starch phosphorylase activity in leaves and tubers of transgenic potato plants. Total leaf and tuber protein was extracted from untransformed control plants (c-1 and c-2) and transgenic plants (2–6, 44, 48, 56, 70 and 71). The protein (40 μg each lane leaf protein and 5 μg each lane tuber protein) was separated in a polyacrylamide gel containing glycogen. Starch phosphorylase activity was visualized as described in Materials and methods. The upper band represents the retarded high-affinity H-type isoform, whereas the lower band represents the unretarded low-affinity L-type isoforms.

Reduction of starch phosphorylase type L activity in leaves has no influence on leaf starch accumulation

To investigate the possible function of starch phosphorylase type L activity during the metabolism of leaf starch, the light-dependent starch accumulation of transgenic and wild-type plants was analysed. Five plants of the individual transformant 2–56 and wild-type control plants were clonally propagated in tissue culture and transferred to the greenhouse. Under normal growth conditions (16 h light/8 h dark) no significant reduction of the starch content was observed in leaves of wild-type and transgenic potato plants (data not shown). To measure differences in the starch content the accumulation of starch was measured in source leaves (> 15 cm length) of 4-week old plants after a prolonged dark period of 24 h (Table 1). If starch phosphorylase type L would be involved in the turnover of starch in leaves, starch accumulation would have been expected. As evident from the data presented in Table 1, no change in starch content was detected.

Table 1. Antisense inhibition of STP-1 does not lead to significant changes in leaf starch accumulation. Greenhouse-grown plants were kept in complete darkness for 24 h. After transferring the plants into the light, leaf samples were taken every four hours and the starch content was determined.

Hours after illumination	STP-56	Untransformed control
0	3.68 ± 1.42	3.43 ± 1.91
4	3.75 ± 1.01	3.01 ± 1.32
8	5.56 ± 1.60	5.47 ± 2.17
12	8.89 ± 3.50	7.53 ± 2.01

Values are the mean of five independent plants each ± standard deviation ($n = 10$). Starch content in mmol hexose per m^{-2}.

Moreover, no significant alterations in the light-dependent starch accumulation were found. Due to variations in the starch content of individual plants minor changes cannot be excluded. This finding suggests that starch breakdown in leaves is probably catalysed by amylases rather than phosphorylases. The function of starch phosphorylase type L still remains unknown.

Conclusion

At least two different starch phosphorylase L-type isoforms are present in potato plants. Both forms differ in their tissue specificity, indicating different roles during starch breakdown. The polypeptides are homologous to each other, but the insertion sequence, specific for plant L-type starch phosphorylases, is highly variable. These differences might reflect changes in substrate specificities and specific activities of the enzymes. Antisense inhibition of one starch phosphorylase L-type isoform proves that the two forms differ in their tissue specificity. Reduction of the starch phosphorylase L-type activity had no significant influence on the accumulation of starch in leaves of transgenic potato plants. Future experiments will include the mutagenesis of the insertion sequence and the inhibition of the second L-type starch phosphorylase in transgenic potato plants.

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References

1. Amasino RM: Acceleration of nucleic acid hybridisation rate by polyethylene glycol. Anal Biochem 152: 304–307 (1986).
2. Bevan M:binary Agrobacterium vectors for plant transformation. Nucl Acids Res 12: 8711–8721 (1984).
3. Bradford MM: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 243–254 (1976).
4. Brisson N, Giroux H, Zollinger M, Camirand A, Simard C: Maturation and subcellular compartmentation of potato starch phosphorylase. Plant Cell 1: 559–566 (1989).
5. Bullock WO, Fernandez JM, Short JM: XL-1 Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques 5: 376 (1987).
6. Conrads J, van Berkel J, Schächtele C, Steup M: Non-chloroplast α-1,4-glucan phosphorylase from pea leaves: characterization and *in-situ* localization by indirect immunofluorescence. Biochim Biophys Acta 882: 452–463 (1986).
7. Debleare R, Bytebier B, DeGreve H, Debroek F, Schell J, Van Montagu M, Leemans J: Efficient octopine Ti plasmid-derived vectors of *Agrobacterium* mediated gene transfer to plants. Nucl Acids Res 13: 4777–4788 (1985).
8. Dickinson DB, Preiss J: Presence of ADP-glucose pyrophoshorylase in shrunken-2 and brittle-2 mutants of maize endosperm. Plant Physiol 44: 1058–1062 (1969).
9. Franck A, Guilley H, Jonrad G, Richards K, Hirth L: Nucleotide sequence of cauliflower mosaic virus DNA. Cell 21: 285–294 (1980).
10. Gielen J, De Beuckeleer M, Seurinck J, Debroeck H, DeGreve H, Lemmers M, Van Montagu M, Schell J: The complete nucleotide sequence of the TL-DNA of the *Agrobacterium tumefaciens* plasmid pTiAch5. EMBO J 3: 835–846 (1984).
11. Greve B: Immunochemische Analyse und submolekulare Charakterisierung pflanzlicher Heteropolymere mit Hilfe

mono- und polyklonaler Antikörper. Dissertation, Universität Münster (1992).
12. Höfgen R, Willmitzer L: Storage of competent cells for Agrobacterium transformation. Nucl Acids Res 16: 9877 (1988).
13. Kossmann J, Müller-Röber B, Dyer TA, Raines CA, Sonnewald U, Willmitzer L: Cloning and expression analysis of plastidic fructose-1,6-bisphosphatase coding sequence from potato: circumstantial evidence for the import of hexoses into chloroplasts. Planta 188: 7–12 (1992).
14. Lin T-P, Caspar T, Sommerville C, Preiss J: Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADPglucose phosphorylase activity. Plant Physiol 86: 1131–1135 (1988).
15. Lin T-P, Caspar T, Sommerville C, Preiss J: A starch deficient mutant of *Arabidopsis thaliana* with low ADP-glucose pyrophosphorylase activity lacks one of the two subunits of the enzyme. Plant Physiol 88: 1175–1181 (1988).
16. Lin C-T, Yeh K-W, Lee PD, Su JC: Primary structure of sweet potato starch phosphorylase deduced from its cDNA sequence. Plant Physiol 95: 1250–1253 (1991).
17. Logemann J, Schell J, Willmitzer L: Improved method for the isolation of RNA from plant tissues. Anal Biochem 163: 21–26 (1987).
18. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
19. Mori H, Tanizawa K, Fukui T: Potato tuber type H phosphorylase isozyme. J Biochem 266: 18446–18453 (1991).
20. Müller-Röber B, Sonnewald U, Willmitzer L: Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J 11: 1229–1238 (1992).
21. Murashige T, Skoog F: A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497 (1962).
22. Nakano K, Mori H, Fukui T: Molecular cloning of cDNA encoding potato amyloplast α-glucan phosphorylase and the structure of its transit peptide. J Biochem (Tokyo) 106: 691–695 (1989).
23. Preiss J: Regulation of the biosynthesis and degradation of starch. Annu Rev Plant Physiol 33: 431–454 (1982).
24. Proudfoot NJ, Brownlee GG: 3′ Non-coding region sequences in eukaryotic messenger RNA. Nature 263: 211–214 (1976).
25. Proudfoot NJ: The end of message and beyond. Nature 307: 412–413 (1984).
26. Shimomura S, Nagai M, Fukui T: Comparative glucan specificities of two types of spinach leaf phosphorylase. J Biochem 91: 703–717 (1982).
27. Rocha-Sosa M, Sonnewald U, Frommer W, Stratmann M, Schell J, Willmitzer L: Both developmental and metabolic signals activate the promoter of a class I patatin gene. EMBO J 8: 23–29 (1989).
28. Steup M, Latzko E: Intracellular localization of phosphorylases in spinach and pea leaves. Planta 145: 69–75 (1979).
29. Steup M: Starch degrading enzymes. Meth Plant Biochem 3: 103–128 (1990).
30. Stitt M, Wirtz W, Heldt HW: Pathway of starch breakdown in photosynthetic tissue of *Pisum sativum*. Biochim Biophys Acta 544: 200–214 (1978).
31. Tsai CY, Nelson OE: Starch deficient maize mutant lacking adenosine diphosphate glucose pyrophosphorylase activity. Science 151: 341–343 (1966).
32. Vervliet G, Holsters M, Teuchy H, Van Montagu M, Schell J: Characterization of different plaque-forming and defective temperate phages in *Agrobacterium* strains. J Gen Virol 26: 33–48 (1975).
33. Yang Y, Steup M: Polysaccharide fraction from higher plants which strongly interacts with the cytosolic phosphorylase isozyme. Plant Physiol 94: 960–969 (1990).