Planta (1999) 208: 503–511

Planta

Cloning and functional analysis of a cDNA encoding a starch synthase from potato (*Solanum tuberosum* L.) that is predominantly expressed in leaf tissue

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Received: 19 August 1998 / Accepted: 17 December 1998

Abstract. Three isoforms of starch synthase (SS) were shown to be present in soluble potato tuber extracts by activity staining after native gel electrophoresis. A cDNA encoding SSI from rice was used as a probe to clone a corresponding cDNA from potato. The deduced amino acid sequence identified the protein as an SS from potato with an M_r of 70.6 kDa for the immature enzyme including its transit peptide. This novel isoform was designated SSI. An analysis of the expression pattern of the gene indicated that *SSI* is predominantly expressed in sink and source leaves, and, to a lower extent in tubers. In several independent transgenic potato lines, where the expression of *SSI* was repressed using the antisense approach, the activity of a specific SS isoform was reduced to non-detectable levels as determined through activity staining after native gel electrophoresis. The reduction in the amount of this isoform of SS did not lead to any detectable changes in starch structure, probably due to the fact that this isoform only represents a minor activity in potato tubers.

Key words: Antisense potato – Isoform (starch synthase) – *Solanum* (starch synthase) – Starch structure – Transgenic potato – Tuber

Introduction

Starch synthases (SSs; ADP-glucose: α-1,4-glucan 4-α-glucosyltransferase; EC 2.4.1.21) catalyse the glucosyl transfer from ADP-glucose to the non-reducing end of an α-1,4-glucan (Preiss 1991). Several different isozymes have been shown to be present in plant organs and can be distinguished into soluble and starch-granule-bound forms. Most isoforms are present both in the soluble fraction and bound to the granule and are referred to as being soluble SSs. One isoform, however, (granule-bound starch synthase I; GBSSI) is exclusively localised on the starch granules, and cannot be detected in soluble extracts.

In potato, three different soluble isoforms are present. These are detectable as distinct bands through activity staining after non-denaturing PAGE (Edwards et al. 1995; Abel et al. 1996; Marshall et al. 1996), and have been designated SSI, SSII and SSIII according to their migration behaviour on these gels, where SSI is the fastest migrating form. Two cDNAs have been isolated so far, which encode the SSII and SSIII isoforms from potato (Dry et al. 1992; Edwards et al. 1995; Abel et al. 1996; Marshall et al. 1996). Production of antisense plants reduced in activities of either of these isoforms, as well as immunoinhibition experiments using antibodies that recognise SSII or SSIII specifically, have shown that SSII contributes approximately 10–15% of the total SS activity (Edwards et al. 1995), while SSIII contributes approximately 80% (Abel et al. 1996; Marshall et al. 1996). Another cDNA, which seems to encode an isozyme distinct from SSII and SSIII in higher plants was isolated from rice and corn (Baba et al. 1993; Knight et al. 1998). It can be assumed, therefore, that any remaining SS activity in potato extracts might be assigned to a homologue of the cDNA from these species.

Here we report the cloning of a novel cDNA from potato encoding a homologue to the SS which was isolated from rice. Furthermore, the analysis of transgenic potato plants with reduced expression levels of the respective SS, as well as plants with reduced levels of SSII, is described. The effect of the reduction of these specific SS activities on starch metabolism is analysed.

Materials and methods

Plant material. Solanum tuberosum L. cv. Désirée was obtained from Saatzucht Fritz Lange KG (Bad Schwartau, Germany).

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Abbreviations: GBSS = granule-bound starch synthase; SS = starch synthase

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Plants were kept in tissue culture on MS medium (Murashige and Skoog 1962) containing 2% sucrose under a 16 h light/8 h dark regime. Plants were grown in the greenhouse under a 16 h light (ca. 250 µmol quanta m^{-2} s^{-1}; 22 °C) and 8 h dark (15 °C) regime.

Chemicals. The ADP[U-^{14}C]glucose at a specific activity of 7.4 GBq $mmol^{-1}$ and the immunodetection kit were from Amersham. The starch-determination kit and all the enzymes used for the modification and restriction of DNA were purchased from Boehringer Mannheim. Isoamylase from *Pseudomonas amyloderamosa* and Lugols solution were obtained from Sigma. All other chemicals were purchased from Sigma or Merck.

Cloning of a cDNA encoding SSI. For isolation of a full-length SSI cDNA, a potato leaf-specific expression library (Kossmann et al. 1992) was screened with a ^{32}P-labelled 1000-bp polymer chain reaction-(PCR)-amplified fragment encoding SSI from rice. About 500 000 recombinant plaque-forming units were screened. The phage DNA was transferred to Hybond N filters (Amersham). After denaturation and neutralisation the DNA was immobilised for 2 h at 80 °C, and filters were hybridised overnight with the ^{32}P-labelled fragment at 42 °C in polyethyleneglycol (PEG)-buffer (Amasino 1986). The filters were washed twice with saline sodium citrate (SSC = 0.15 M NaCl, 0.015 M Na_3-citrate, pH7) and 0.5% (w/v) SDS. Positive plaques were purified in two additional rounds and plasmids were recovered by in vivo excision.

Expression of SSI in the Escherichia coli strain G6MD2. Plasmid pSSI-VK, containing the full-length sequence coding for the SSI protein, was transformed into the *E. coli* strain G6MD2 which lacks activity of all glycogen-synthetic enzymes due to deletion of the *glg* operon. The bacteria also contained a plasmid containing the *E. coli glgC16* gene which codes for an unregulated form of the enzyme ADP glucose pyrophosphorylase (Creuzat-Sigal et al. 1972). The gene was isolated from the *E. coli* strain LCB 618 by PCR amplification. This gene allows the bacteria to produce a lot of ADP glucose, the substrate for SS enzymes. The *glgC16* gene was present in the plasmid pACYC-184 (New England Biolabs). The bacteria were grown overnight at 37 °C on solid YT medium (Miller 1972) with the addition of 2% (w/v) glucose. Colonies were stained for accumulation of linear glucans by placing solid iodine on the lid of the agar plate with the bacterial colonies suspended above and waiting for gaseous iodine to sublime from the solid and stain the colonies.

Construction of a chimeric antisense genes for SSI or SSII and potato transformation. The *Eco*RI/*Hin*dIII fragment of pA7 (Von Schaewen 1989) containing the cauliflower mosaic virus (CaMV) 35S promoter, a multiple cloning site and the *ocs*-terminator was introduced into the *Eco*RI/*Hin*dIII site of pBIB-Hyg (Becker 1990) resulting in the plant expression vector pBinAR-Hyg. A 1.7-kb *Xba*I/*Asp*718 fragment, encoding the C-terminal region of SSI, and a 1.9-kb *Asp*718/*Sma*I fragment encoding the C-terminus of SSII, respectively, were introduced in antisense orientation into the vectors pBinAR-Hyg and pBinAR (Höfgen and Willmitzer 1990) between the CaMV 35S promoter and the *ocs*-terminator.

This chimeric antisense genes pBinAR-HygαSSI and pBinAR αSSII were introduced into *Solanum tuberosum* L. cv. Désirée by *Agrobacterium*-mediated transformation as described by Rocha-Sosa et al. (1989).

Extraction of RNA and Northern blot analysis. Total RNA was extracted from frozen plant material as described previously (Logemann et al. 1987). The RNA was denatured in 40% (v/v) formamide, separated on a 1.5% (w/v) agarose gel containing formaldehyde (Lehrach et al. 1977) and blotted onto a nylon membrane (Hybond N; Amersham). Hybridisation of membranes was performed in 0.25 mM sodium phosphate (pH 7.2), 1 mM EDTA, 1% (w/v) BSA and 7% (w/v) SDS. The filters were washed twice with SSC and 0.5% (w/v) SDS for 15 min at 68 °C.

Radioactively labelled probes were made by the random primed method using a commercially available kit (Boehringer Mannheim). The fragments used as probes were the entire coding sequence of the cDNAs from the different SS cDNAs. These were excised as follows: GBSSI was cut with *Bam*H1 and *Xho*1; SSI was cut with *Eco*R1 and *Xho*1; SSII was cut with *Eco*R1 and *Xho*1; SSIII was cut with *Eco*R1. The digests were separated on 1% (w/v) agarose gels and the DNA fragments isolated using a commercially available kit (Geneclean, Bio101 inc, La Jolla, Calif., USA)

Sucrose induction experiments. Potato leaf discs were incubated under illumination for 24 h in water containing 0, 1, 4 or 8% (w/v) sucrose at room temperature. A sample with 0% and 8% sucrose was kept in parallel in darkness. Prior to incubation, plants were kept for 24 h in darkness. The RNA isolated from leaves of greenhouse plants exposed for 6 h to daylight served as a control. The RNA was extracted and analysed as described above.

Native gel electrophoresis. Enzyme extracts were separated on a continuous gel [7.5% (w/v) polyacrylamide in 25 mM Tris-glycine, pH 8.4] without SDS. Starch synthase activity was detected after incubation of the gel in 50 mM Tricine-NaOH (pH 8.5), 0.5 M sodium citrate, 25 mM potassium acetate, 2 mM EDTA, 2 mM DTT, 0.1% (w/v) potato amylopectin and 1 mM ADP-glucose at room temperature for 12 h. Following incubation the gels were stained with Lugols solution.

Carbohydrate analysis. Tuber slices (ca. 50 mg) were extracted three times with 500 µl of 80% (v/v) ethanol at 80 °C for 30 min. Sucrose was determined according to Stitt et al. (1989). The ethanol-insoluble material was homogenised in 400 µl of 0.2 M KOH and incubated at 95 °C for 1 h. After the addition of 70 µl of 1 M acetic acid to neutralise the extract the starch content was measured using a commercially available kit (Boehringer Mannheim) based on the enzymatic hydrolysis of starch with the subsequent enzymatic determination of glucose.

The amylose content of the starch was measured iodometrically according to Hovenkamp-Hermelink et al. (1988). The phosphate bound to the starch at the C-6 position was determined as glucose-6-phosphate released after acid hydrolysis according to Nielsen et al. (1994). Amylopectin was purified according to the method of Tomlinson et al. (1997) while analysis of the sidechain distribution of debranched amylopectins by high-performance anion-exchange chromatography using a pulsed amperometric detector (HPAE-PAD) and gel permeation chromatography was performed as described by Lloyd et al. (1999).

Computer analysis. Sequence analysis was performed with the Wisconsin GCG-package (Devereux et al. 1984). Amino acid sequences encoding glycogen and SSs were aligned by PILEUP and used to generate the dendrogram with the help of GROWTREE and DISTANCES. Accession numbers were for GBSSI: from potato, X58453; from cassava, X74160; from barley, X07931; from maize, X03935; from rice, X65183; from wheat, X57233; for SSI: from potato, Y10416; from maize, AF036891; from rice, D16202; for SSII; from potato, X87988; from pea, X88790; from maize, AF019296; for SSIII: from potato, X94400. Glycogen synthase from *Agrobacterium tumefaciens*, AF033856; from *Escherichia coli*, Y02616.

Results

Cloning of a novel SS from potato. In order to clone novel sequences encoding SSs from potato, a cDNA library from potato tubers was screened using a fragment encoding a soluble SS from rice as a heterologous probe. In order to avoid the re-cloning of cDNAs coding for other SSs, replicate filters were hybridised

with probes encoding GBSSI and SSII from potato, and clones were isolated which solely hybridised with the cDNA from rice. Several clones were isolated and sequenced. The clones had inserts of different lengths (the longest having a size of 1700 bp), all of them encoding a novel type of SS from potato tubers; however, none of them contained the entire coding region for the protein. A potato leaf-specific expression library was screened with a 5′-fragment of one of these clones (pSSI1.7) in order to obtain a full-length clone. In turn, three larger independent cDNA clones differing in length were isolated. The longest cDNA-clone pSSI-VK with an insert size of 2360 bp was sequenced on both strands, showing an identity of 99.79% to the clone pSSI1.7, which was isolated from the tuber-specific cDNA expression library. The presence of a termination codon, which is in frame with the initiation codon, upstream of the coding region suggests that this cDNA contains the full coding region for SSI.

The clone has an open reading frame of 1992 bp, encoding a protein of 70.6 kDa. A comparison of the predicted amino acid sequence of the cDNA, in comparison with those of the SSI proteins from maize and rice is shown in Fig. 1. This demonstrates the similarity in both sizes and sequences of the three proteins. The deduced amino acid sequence was aligned with other starch and glycogen synthases. The alignment was used to compute a dendrogram (Fig. 2), showing that SSI represents a novel SS from potato, which is most closely related to the sequences reported for soluble SSs from rice and maize. The three homologous boxes present in other glycogen and SSs (Van der Leij et al. 1991; Baba et al. 1993) are also contained within the sequence of SSI. From the sequence data it was not possible to identify the cleavage site of the transit peptide, which is necessary for the import of the protein into the plastids.

If it is assumed that there is a transit peptide of between 50–80 amino acids then it is likely that SSI may not contain a significant N-terminal extension (“flexible arm”; Martin and Smith 1995), which is present in SSII and SSIII, in comparison to GBSSI. Furthermore, no consecutive proline-residue stretches, which have been interpreted as the anchor of the flexible arm, are present in the sequence upstream of the first conserved region. This indicates that the presence of an N-terminal extension is not necessarily an intrinsic characteristic of a soluble SS. Knight et al. (1998) have, however, shown that with the maize SSI protein the transit peptide is only 40 amino acids long and, thus, if this were also the case with this potato enzyme it would be possible that it would contain an N-terminal extension.

Using the plasmid pSSI-VK it is possible to complement a mutant of *E. coli* (G6MD2; Schwartz 1966) which carries a chromosomal deletion for all of the *glg* genes. It was transformed with a plasmid carrying a mutated form of glgC with altered allosteric properties (glgC16; Creuzat-Sigal et al. 1972) in order to constitutively supply the SS with substrate. Cells transformed with the plasmid containing the SS cDNA give a heavy blue staining with iodine after growth on glucose-containing media, whereas cells transformed with an empty plasmid do not stain at all (data not shown).

Expression analysis of the gene for SSI. Northern blot analysis was performed to investigate the tissue-specific expression pattern of the gene for SSI. In contrast to the genes for GBSSI, SSII and SSIII, the expression of the gene for SSI was high in leaves and comparatively very low in tubers of different developmental stages (Fig. 3; Edwards et al. 1995; Abel et al. 1996; Marshall et al. 1996), a distribution which also contrasted with that of other starch biosynthetic enzymes (Müller-Röber et al. 1990; Kossmann et al. 1991).

Sucrose is a precursor of starch biosynthesis in detached leaves (Müller-Röber et al. 1990). Leaf discs were used to examine the sugar-responsiveness of the gene encoding the SSI. Leaf discs, which were derived from plants kept in darkness for a prolonged period, were incubated in sucrose solutions of varying concentration, either under illumination, or in darkness. The RNA was extracted and analysed on Northern blots (Fig. 4). In this experimental system, the expression level of *SSI* was constant or rather reduced in comparison to samples taken during the light period from plants under normal greenhouse conditions. This is again in contrast to other genes encoding starch biosynthetic enzymes (ADP glucose pyrophosphorylase subunit S; Müller-Röber et al. 1990; branching enzyme, Kossmann et al. 1991; GBSSI and SSIII, Abel et al. 1996).

Generation of plants with reduced levels of SSI and SSII. In order to assign specific functions to SSI and SSII in starch metabolism, 80 transgenic potato plants, which were transformed with the construct pBinAR-HygαSSI or pBinAR-HygαSSII, were transferred to the greenhouse and leaves were analysed for decreased amounts of mRNA for SSI and SSII, respectively. Three lines were selected with a drastic reduction in mRNA for each of the two isozymes as compared to wild-type plants (data not shown). The reduction in mRNA in these lines (1, 4 and 67 for SSI, and 14, 35 and 44 for SSII) was also easily demonstrated in tubers (Fig. 5A,B).

To demonstrate that the reduction in mRNA for the respective SS proteins correlates with the decrease of a specific soluble SS isoform in the tubers, protein extracts were separated on a native gel and analysed for SS activity (Fig. 6A,B). The transgenic lines 1, 4 and 67 showed drastically lower activities of a specific SS, which had the highest mobility in the native gel, whereas the lines 14, 35 and 44 showed lower activities of another specific SS, which had an intermediate mobility. The other activity that was visualised after iodine staining could be assigned to the other isoform of starch synthase (SSIII), as has been demonstrated by the generation of transgenic potato plants in which the expression of the respective gene was reduced using the same approach (Abel et al. 1996; Marshall et al. 1996). The contribution of SSI and SSII to the total SS activity present in soluble extracts from potato tubers was determined by measuring the enzyme in the

```
           1                                                        50
SSI Maize   ...MATPSAV GA.ACLLLAR AAWPAAVGDR ARPRRLQRVL RRRCVAELSR
SSI Rice  . ..MAT.... .A.AGMGIGA ACLVAPQVRP GRRLRLQRV. RRRCVAELSR
SSI Potato MGSLQTPTNL SNKSCLCVSG RVVKGLRVER QVGLGFSWLL KGRRNRKVQS

           51                                                      100
SSI Maize  E.GPAPRPLP PA.LLAPPLV PGFLAPPAEP TG.....EPA STPPPVPDAG
SSI Rice   DGGSAHGPLA PAPLVKQPVL PTFLVPTSTP PAPTQSPAPA PTPPPLPDSG
SSI Potato LCVTSSVSDG SSIAENKNVS EGLLLGAERD GS........ ........GS

           101                                                     150
SSI Maize  LGDLGLEPEG IAEGS..IDN TVVVASEQD. .SEIVVGKEQ ARAKVTQSIV
SSI Rice   VGEIEPDLEG LTEDS..IDK TIFVASEQE. .SEIMDVKEQ AQAKVTRSVV
SSI Potato VVGFQLIPHS VAGDATMVES HDIVANDRDD LSEDTEEMEE TPIKLTFNII

           151                                                     200
SSI Maize  FVTGEASPYA KSGGLGDVCG SLPVALAARG HRVMVVMPRY LNGT.SDKNY
SSI Rice   FVTGEASPYA KSGGLGDVCG SLPIALALRG HRVMVVMPRY MNGA.LNKNF
SSI Potato FVTAEAAPYS KTGGLGDVCG SLPMALAARG HRVMVVSPRY LNGGPSDEKY
                              I
           201                                                     250
SSI Maize  ANAFYTEKHI RIPCFGGEHE VTFFHEYRDS VDWVFVDHPS YHRPGNLYGD
SSI Rice   ANAFYTEKHI KIPCFGGEHE VTFFHEYRDS VDWVFVDHPS YHRPGNLYGD
SSI Potato ANAVDLDVRA TVHCFGDAQE VAFYHEYRAG VDWVFVDHSS YCRPGTPYGD

           251                                                     300
SSI Maize  KFGAFGDNQF RYTLLCYAAC EAPLILELGG YIYGQNCMFV VNDWHASLVP
SSI Rice   NFGAFGDNQF RYTLLCYAAC EAPLILELGG YIYGQKCMFV VNDWHASLVP
SSI Potato IYGAFGDNQF RFTLLSHAAC EAPLVLPLGG FTYGEKCLFL ANDWHAALVP

           301                                                     350
SSI Maize  VLLAAKYRPY GVYKDSRSIL VIHNLAHQGV EPASTYPDLG LPPEWYGALE
SSI Rice   VLLAAKYRPY GVYRDARSVL VIHNLAHQGV EPASTYPDLG LPPEWYGALE
SSI Potato LLLAAKYRPY GVYKDARSIV AIHNIAHQGV EPAVTYNNLG LPPQWYGAVE

           351                                                     400
SSI Maize  WVFPEWARRH ALDKGEAVNF LKGAVVTADR IVTVSKGYSW EVTTAEGGQG
SSI Rice   WVFPEWARRH ALDKGEAVNF LKGAVVTADR IVTVSQGYSW EVTTAEGGQG
SSI Potato WIFPTWARAH ALDTGETVNV LKGAIAVADR ILTVSQGYSW EITTPEGGYG

           401                                                     450
SSI Maize  LNELLSSRKS VLNGIVNGID INDWNPATDK CIPCHYSVDD LSGKAKCKGA
SSI Rice   LNELLSSRKS VLNGIVNGID INDWNPSTDK FLPYHYSVDD LSGKAKCKAE
SSI Potato LHELLSSRQS VLNGITNGID VNDWNPSTDE HIASHYSIND LSGKVQCKTD

           451                                                     500
SSI Maize  LQKELGLPIR PDVPLIGFIG RLDYQKGIDL IQLIIPDLMR EDVQFVMLGS
SSI Rice   LQKELGLPIR PDVPLIGFIG RLDYQKGIDL IKLAIPDLMR DNIQFVMLGS
SSI Potato LQKELGLPIR PDCPLIGFIG RLDYQKGVDI ILSAIPELMQ NDVQVVMLGS

           501                                                     550
SSI Maize  GDPELEDWMR STESIFKDKF RGWVGFSVPV SHRITAGCDI LLMPSRFEPC
SSI Rice   GDPGFEGWMR STESGYRDKF RGWVGFSVPV SHRITAGCDI LLMPSRFEPC
SSI Potato GEKQYEDWMR HTENLFKDKF RAWVGFNVPV SHRITAGCDI LLMPSRFEPC
                                                              II
           551                                                     600
SSI Maize  GLNQLYAMQY GTVPVVHATG GLRDTVENFN PFGENG.EQG TGWAFAPLTT
SSI Rice   GLNQLYAMQY GTVPVVHGTG GLRDTVENFN PFAEKG.EQG TGWAFSPLTI
SSI Potato GLNQLYAMRY GTIPIVHSTG GLRDTVKDFN PYAQEGIGEG TGWTFSPLTS
                                  III
601                                                   650
SSI Maize  ENMF....VD IANCNIYIQG TQVLL..... ...GRANEAR H....VKRLH
SSI Rice   EKNA....VG IADGNFDIQG TQVLL..... ...GGSNEAR H....VKRLY
SSI Potato EKLLDTLKLA IGTYTEHKSS WEGLMRRGMG RDYSWENAAI QYEQVFTWAF

           651
SSI Maize  VGPCR...
SSI Rice   MGPCRLTV
SSI Potato IDPPYVR.
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Fig. 1. Comparison of the deduced amino acid sequence of potato SSI with those of rice and maize. The three consensus sequences for glycogen and starch synthases are in *bold*. Accession numbers of the nucleotide sequences at the EMBL database: potato, Y10416; rice, X65183; maize, AF036891

respective transgenic and control plants. No significant reduction was measured in the plants with lowered SSI activity, whereas a 10–20% reduction was detected in the plants with decreased SSII activity (data not shown), which is in agreement with previously reported data (Edwards et al. 1995). This is also in accordance with the view that SSIII contributes the major proportion of SS activity in potato tubers (Abel et al. 1996; Marshall et al. 1996).

Influence of reduced levels of SSI and SSII on starch metabolism. In order to investigate the role of SSI and SSII in starch biosynthesis, independent transgenic lines with reduced levels of either SSI or SSII activity were analysed with respect to the amount of starch accumulating in the tubers and to the structure of the starch synthesised. No differences with respect to the wild type were observed when the total tuber yield and number per plant, and the starch content were determined (data not

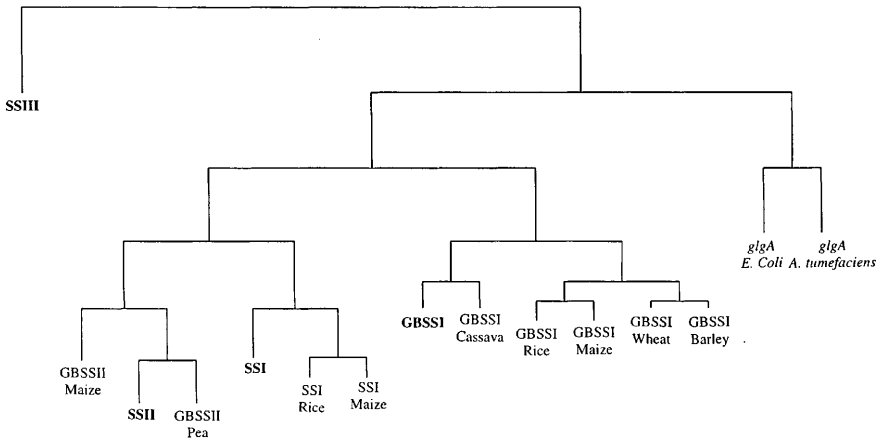

Fig. 2. Dendrogram showing the evolutionary distance of the different starch and glycogen synthases. Amino-acid sequences were aligned by the program PILEUP. This alignment was used for the generation of the dendrogram by the DISTANCES (uncorrected distances) and GROWTREE (UPGMA-method) program

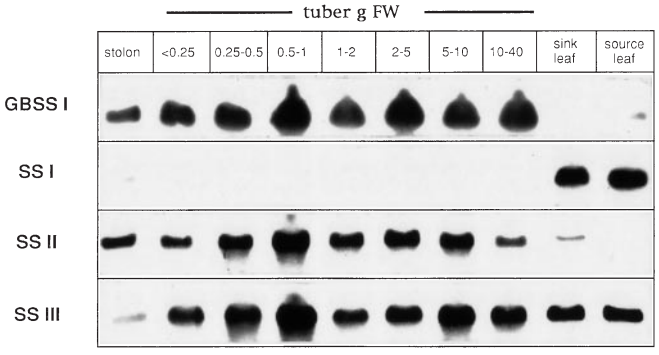

Fig. 3. Northern blot analysis of the tissue-specific gene expression of SSI in comparison to GBSSI, SSII, and SSIII. Total RNA was extracted from stolons, tubers of different fresh weights, and sink and source leaves of a three-month-old potato plant from the greenhouse. An aliquot of 50 µg RNA was separated and probed with ^{32}P-labeled fragments of the respective cDNAs

shown), indicating that the reduction of either SSI or SSII has no effect on the net flux of carbon into starch under the growth conditions in these studies. The sucrose contents of transgenic tubers were also unchanged in comparison to wild-type tubers (data not shown), which substantiates this observation. Furthermore, no reproducible differences were detected when the amylose content of the starch was investigated (data not shown) and, for the SSI antisense lines, when the chain-length distributions of enzymatically debranched amylopectin samples were analysed (Fig. 7). However, significant changes were observed when the amount of phosphate that is covalently bound to the C-6 position of the glucose monomers was determined. Here, an approximately 50% decrease could be measured in plants with reduced SSII activity, whereas no change was found in plants with lowered levels of SSI (Table 1). Although the levels of starch phosphorylation varied greatly between experiments for both control and transgenic plants, the percentage decrease in the

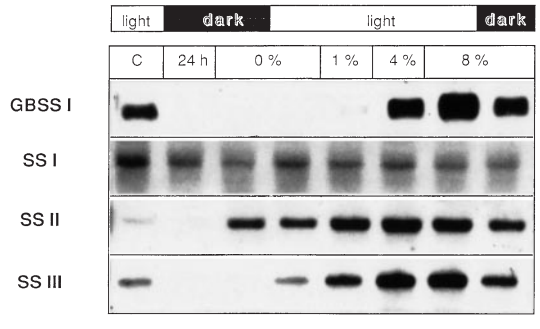

Fig. 4. Induction of SSI, GBSSI, SSII, and SSIII gene expression in leaves by sucrose. Potato leaf discs were incubated in water with varying concentrations of sucrose with or without light for 24 h. Total RNA was subsequently extracted and probed with a ^{32}P-labeled fragment of the respective cDNA. In each case, 40 µg of total RNA was subjected to Northern blot analysis. *C*, Expression in leaves of an intact plant after 6 h illumination; *24 h*, expression in the leaf of an intact plant after 24 h without light. Sucrose concentrations (%, w/v) are indicated

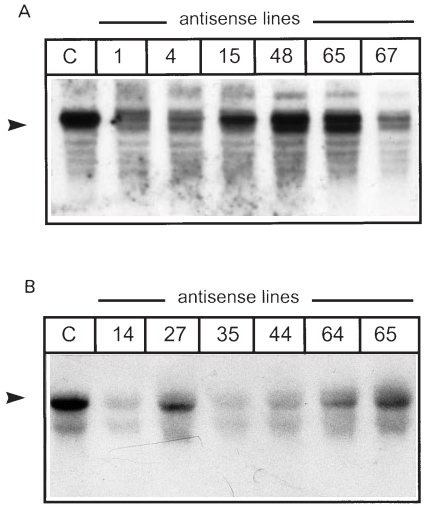

Fig. 5A,B. Northern blot analysis of transgenic potato plants with reduced levels of SSI (**A**) and SSII (**B**). Total RNA was extracted from tubers of a control plant (*C*) and several transgenic lines. Fifty micrograms of total RNA was loaded per lane and probed with a ^{32}P-labeled fragment of the SSI and SSII cDNAs

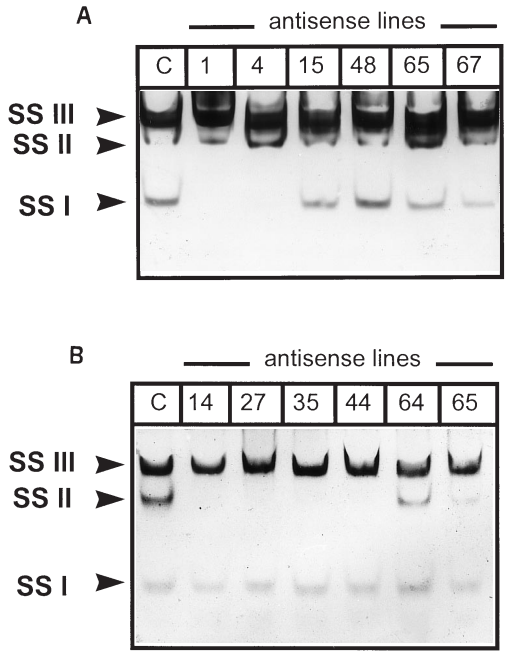

Fig. 6A,B. Assay for SS activity on a native gel. Soluble tuber extracts (100 μg) from a control (*C*) and selected transgenic lines (**A** for SSI, and **B** for SSII) were separated on a native gel [7.5% (w/v) polyacrylamide; 8 cm × 7.3 cm] for 2 h at a current of 15 mA. Starch synthase activity was assayed overnight in the presence of 0.5 M citrate and 0.1% amylopectin at room temperature. Synthesised glucans were stained with iodine

phosphate contents in starch from the SSII antisense lines was reproducible between experiments (data not shown).

Discussion

Isolation of a cDNA encoding a-70 kDa SS. A heterologous probe, encoding a soluble SS from rice was used to isolate several partial cDNAs which encode a novel isoform of SS from potato, as identified by sequence comparison. Subsequently, a full-length clone of 2360 bp was isolated from a leaf-specific cDNA library. The cDNA encodes a protein of predicted M_r of 70.6 kDa, which is in agreement with data obtained from transgenic plants that express reduced levels of the protein. This protein has not previously been described from potato and was designated SSI. A protein of higher molecular weight (approximately 90 kDa) with SS activity has, however, previously been identified from potato tubers (Hawker et al. 1972). This is most likely to have been SSII, or a degradation product of SSIII, since the small amount of activity that SSI contributes to the total would not make it the easiest isoform to purify. In contrast to SSII and SSIII, SSI may well contain no N-terminal extension compared with GBSSI. Expression studies using several cDNA clones for SSI were performed in mutants of *E. coli*, which are defective for glycogen synthase. Glycogen synthesis was only restored with the full-size cDNA containing any putative transit peptide (data not shown).

Starch synthase I is expressed differently from other starch biosynthetic enzymes. The expression pattern of *SSI* shows major differences from those of *GBSSI*, *SSII*, and *SSIII*. Its expression level in tubers of different developmental stages is drastically lower than that of other starch biosynthetic genes. Furthermore, the expression level of SSI is appreciably higher in sink and source leaves than in tubers. A similar expression pattern has not been reported for any other starch biosynthetic enzyme in potato, which suggests only a minor role for SSI in starch synthesis in storage organs. Its contribution to the deposition of transient starch in chloroplasts of leaves might be higher. However, under

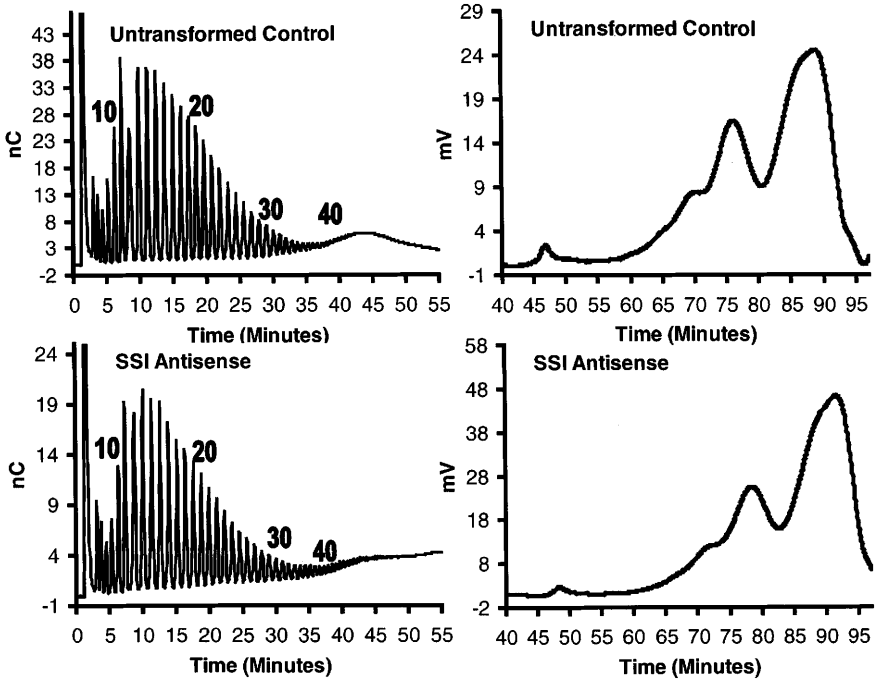

Fig. 7. High performance anion exchange chromatography using a pulsed amperometric detector (HPAE-PAD) and gel permeation chromatography of debranched starch samples from wild-type tubers and the transgenic line 4 with reduced expression of SSI. *Numbers* on the HPAE-PAD traces represent degree of polymerisation

Table 1. Analysis of phosphate bound at the C-6 position in starch isolated from potato tubers of lines lacking either the SSI or SSII isoforms reduced by antisense techniques

	Control	SSI Antisense Lines	
		Line 1	Line 4
Phosphate content at the 6 position [nmol (mg starch)$^{-1}$]	9.5 ± 2.6	8.9 ± 0.4	9.3 ± 0.4

	Control	SSII Antisense Lines		
		Line 14	Line 35	Line 44
Phosphate content at the 6 position [nmol (mg starch)$^{-1}$]	7.0 ± 0.2	4.5 ± 0.2*	4.1 ± 0.1*	3.8 ± 0.1*

*Data different from the control at the 5% probability level (Students *t*-test)

conditions where starch synthesis and the expression of other starch biosynthetic genes are substantially induced in leaves (incubation on sucrose), the gene for SSI responded in the opposite manner, being repressed. In these experiments it was found that GBSSI was not expressed in sink leaves and this may be for several reasons. Granule-bound SSI is thought to be responsible for production of amylose, and leaf starch has previously been shown in pea and *Arabidopsis* to contain small amounts of amylose (Tomlinson et al. 1997; Zeeman et al. 1998). Lack of expression of the gene coding for the protein that manufactures amylose may simply reflect this. In addition, in pea, two GBSSI cDNAs have been identified (Dry et al. 1992; Denyer et al. 1997), one of which is preferentially expressed in pea leaves. If there were a second GBSSI cDNA in potato, which replaced the expression of the gene for which we probed in sink leaves, that could also explain this result.

Plants with reduced levels of SSI do not show any drastic changes in starch metabolism. Several antisense plants for SSI show a nearly complete suppression of SSI expression. Starch synthase I can be identified as the isoform with the highest mobility on a native gel, since it is specifically absent in extracts derived from tubers of the antisense plants.

The almost complete reduction of a major SS activity in transgenic plants does not affect the amount of starch or structure of amylopectin, which accumulates in the storage organs. This is probably due to the fact that SSI contributes only minor proportions to the total SS activity present in potato tubers, since no appreciable differences in total SS activity were measured in the transgenic as compared to control tubers. Potato tubers lacking activity of either the SSII or SSIII isoforms individually, or simultaneously, do show major alterations in amylopectin structure when analysed using the methods in this present paper (Lloyd et al. 1999). This suggests that it is indeed the small amount of activity that SSI contributes that leads to the lack of alterations in carbohydrate metabolism in SSI antisense tubers, as SSII and SSIII contribute a much greater proportion of the activity to the tuber. It is of course possible that the lack of changes noted in this study are due to the other SS isoforms being up-regulated to compensate for the lack of SSI. A second possibility is that the enzyme is active extra-plastidially. This may be a possibility as Tacke et al. (1991) showed that there was glucan synthase activity both within and outside of chloroplasts in spinach leaves. This possibility is further supported by the fact that it was not possible to identify a transit peptide in the cDNA sequence that we isolated. The presence of a transit peptide has, however, been identified in the SSI protein from maize (Knight et al. 1998). The functional significance of any activity outside of chloroplasts is not known.

At the moment the possibility still remains that SSI plays a major role in potato leaf starch metabolism, as it is predominantly expressed in leaves. However, no remarkable changes were observed when the starch content was measured in transgenic and wild-type leaves after different periods of illumination (data not shown). The further thorough investigation of the structure of the starches synthesised in the different leaves might elucidate the role of SSI.

The fact that SSI seems to play only a minor role in potato starch metabolism is intriguing, since at least in rice and in corn it is the major SS purified from endosperm tissue (Baba et al. 1993; Knight et al. 1998). It is possible that a mutation in the respective gene would result in greater effects in rice or corn, similar to the *dull1* or *rug5* mutation in corn or pea, respectively, both mutations in a gene coding for a soluble SS (Craig et al. 1998; Gao et al. 1998).

Plants with reduced levels of SSII show only slight changes in starch metabolism. The SSII isoform can be identified as that which migrates between SSI and SSIII in a native gel, since the corresponding band is specifically absent in extracts derived from tubers of several independent antisense plants. Plants lacking activity of SSII have previously been manufactured by another group (Edwards et al. 1995) who, as in this study, did not find alterations in the amylose or starch contents of the transgenic plants. In this present study, major changes were, however, measured when the phosphate that is bound to the C-6 position of the

glucose monomers was determined. Here a 50% reduction was observed in the starches derived from the transgenic plants with reduced levels of SSII as compared to control preparations. Phosphate is either located at the C-3 or C-6 position of the glucose monomers and is exclusively found in the amylopectin fraction (Hizukuri et al. 1970; Muhrbeck and Tellier 1991). The biochemical pathway leading to the phosphorylation of starch is not known (Nielsen et al. 1994). One possibility for the incorporation of phosphate into starch is that the substrate of the SSs, ADP-glucose, also occurs in a phosphorylated form, e.g. ADP-glucose-6-phosphate, which might be derived from glucose-1,6-bisphosphate, and could be used as a precursor for chain elongation. Assuming that different α-1,4-glucan synthases have varying affinities towards this substrate, the differences in phosphorylation could be explained through the changes in the relative contribution of the different isoforms to total starch synthesis in the transgenic plants. Plants with lowered levels of SSIII showed a 70% increase of starch phosphate (Abel et al. 1996). This would argue for SSII being the main SS responsible for the incorporation of phosphorylated precursors into growing α–1,4-glucans. However, lowered phosphate levels were measured in starches derived from transgenic potato plants in which the glycogen synthase from *E. coli* was overexpressed (Shewmaker et al. 1994). In this case, other changes in starch structure were also observed, e.g. the amylose content was reduced and the amylopectin was more highly branched. If starch phosphorylation occurs after the polymerisation process, it is possible that the enzyme responsible acts in a context-dependent manner and utilises only very defined chains as acceptors for the phosphate groups. In all the cases discussed it is possible that structural changes have occurred, leading to altered contents of 'phosphate-acceptors'. A second possibility is that the SSII, but not the SSIII isoform, interacts directly during starch synthesis with a recently identified protein called R1 (Lorberth et al. 1998), which may play a role in phosphorylating potato starch. Upon antisense inhibition of this protein the phosphate content in potato tubers was found to be decreased by 90%. The plants described above may, in the future, help to identify the biochemical mechanism of starch phosphorylation.

The authors are grateful to Jessyca Dietze, Claudia Hofmann for technical assistance, Regina Breitfeld, Birgit Burose and Tile Nieland for greenhouse work, and Antje Voigt for photographic work.

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