Simultaneous antisense inhibition of two starch-synthase isoforms in potato tubers leads to accumulation of grossly modified amylopectin

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A chimaeric antisense construct was used to reduce the activities of the two major starch-synthase isoforms in potato tubers simultaneously. A range of reductions in total starch-synthase activities were found in the resulting transgenic plants, up to a maximum of 90 % inhibition. The reduction in starch-synthase activity had a profound effect on the starch granules, which became extremely distorted in appearance compared with the control lines. Analysis of the starch indicated that the amounts produced in the tubers, and the amylose content of the starch, were not affected by the reduction in activity. In order to understand why the starch granules were distorted, amylopectin was isolated and the constituent chain lengths analysed. This indicated that the amylopectin was very different to that of the control. It contained more chains of fewer than 15 glucose units in length, and fewer of between 15 and 80 glucose units. In addition, the amylopectin contained more very long chains. Amylopectin from plants repressed in just one of the activities of the two starch-synthase isoforms, which we have reported upon previously, were also analysed. Using a technique different to that used previously we show that both isoforms also affect the amylopectin, but in a way that is different to when both isoforms are repressed together.

Key words: amylopectin branching, amylopectin structure, phosphate, starch granule, starch synthesis.

INTRODUCTION

The starch granule is a complex structure consisting of two glucose polymers, branched amylopectin and the much less branched amylose. At present, however, we do not know exactly how the different enzymes involved in starch biosynthesis contribute to producing the highly structured amylopectin molecule. In storage organs, starch is made within amyloplasts by the action of several enzymes [1,2]. Analysis of both mutant and transgenic plants has shown that amylose, which is essentially linear, is the product of a specific type of starch synthase (SS) that is exclusively bound to the granule, hence its name granule-bound SS (GBSS) [1,2]. Genes coding for several other SS isoforms have been cloned and these isoforms appear to be present either both on the starch granule and in the soluble fraction surrounding it, or solely in the soluble fraction, and are referred to collectively as SSs [3–6]. As the GBSS protein appears to be almost exclusively responsible for amylose production it is assumed that the SS proteins play a role in making amylopectin and by altering their activities it may, therefore, be possible to affect amylopectin structure.

Recently, two mutant genes have been cloned from different plant species, one in pea (*rug5*) [7] and one in maize (*dull1*) [8], both of which were shown to be mutations in SS enzymes. In addition, a mutation in the green alga *Chlamydomonas rheinhardtii* (*sta3*) [9], has been isolated which causes the organism to have a reduced SS activity. The starches from all of these mutants have an increased amylose content, and the pea and *Chlamydomonas* mutants have been shown to contain an amylopectin with an altered branching pattern. These data suggest that, in some systems, alterations in the amounts of SS proteins can affect the amylopectin molecule.

In potato, three cDNAs encoding SS isoforms have been cloned, all producing proteins of different sizes that contribute different amounts towards the total activity in the tuber. Both immunoprecipitation and antisense experiments have shown that one isoform (SSIII) [10,11] contributes the major activity (80 % of the total) in potato tubers, with a second one (SSII) [5] being responsible for the majority (15 % of the total) of the remainder. When the activity of either of these isoforms is reduced using antisense techniques no differences are found in the constituent chains within starch [5,10,12]. In this paper we report on the reduction in activity of both of these isoforms simultaneously, which leads to a gross alteration in the amylopectin. Using a technique that was not used previously we show also that plants inhibited only in the SSII or SSIII isoforms contain amylopectin with an altered branching pattern, but which is different to that when both isoforms are reduced. This is the first time that the structure of amylopectin has been shown to be altered by the reduction in activity of a plant's native enzyme using genetic-engineering techniques.

EXPERIMENTAL

Materials

Solanum tuberosum L. var. Désirée was obtained from Vereinigte Saatzuchten eG (Ebstorf, Germany). All restriction enzymes used for manipulation of DNA came from Boehringer Mannheim (Mannheim, Germany). Isoamylase used for digestion of amylopectin was brought from Megazyme International Ireland Ltd. (Bray, Ireland). All other chemicals were obtained from the Sigma Chemical Company (St. Louis, MO, U.S.A.).

Abbreviations used: dp, degree of polymerization; SS, starch synthase; GBSS, granule-bound SS; HPAEC-PAD, high-performance anion-exchange chromatography using a pulsed amperometric detector; MALDI–TOF-MS, matrix-assisted laser desorbtion ionization–time-of-flight MS; FW, fresh weight.

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Preparation of chimaeric antisense plasmid and transformation of potato

A plasmid containing a 1546 bp fragment (*Eco*RV–*Hin*cII digestion) of the SSII cDNA and a 1356 bp fragment (*Bam*HI digestion) of the SSIII cDNA was prepared using standard molecular-biological techniques [13]. Both fragments were ligated into the same pBINAR transformation vector in antisense orientation between the cauliflower mosaic virus 35 S promoter and the *ocs* terminator. This plasmid was named p35SαGBSSII-αSSIII-Kan. The Désirée potato cultivar was transformed with this plasmid using *Agrobacterium tumefaciens*-mediated gene transfer [14].

Growth of plants and tuber sampling

Plants were grown from tubers in top soil comprising 33% (w/w) sand. Until flowering the plants were fertilized twice weekly using Hakaphos Special fertilizer and then using Hakaphos Rot fertilizer (both from BASF, Hannover, Germany). The samples from tubers were taken using a cork borer as previously described [11], immediately frozen in liquid nitrogen and stored at −80 °C until used for making protein extracts or for analysing the amounts of starch. The remaining tuber was also frozen in liquid nitrogen and stored at −80 °C until used for RNA extraction.

Preparation of RNA and RNA blots

RNA was prepared by a previously published method [15] with the addition of 1 M β-mercaptoethanol in the extraction buffer. Total RNA (20 μg) was denatured in 40% (v/v) formamide and separated on a 1.5% (w/v) agarose gel containing formaldehyde [16]. The RNA was blotted on to a nylon membrane (Hybond N, Amersham, Amersham, Bucks, U.K.), which was hybridized in 0.25 M sodium phosphate, pH 7.2, 1 mM EDTA, 1% (w/v) BSA and 7% (w/v) SDS at 60 °C for 12 h. The filters were twice washed with 1 × saline sodium citrate and 0.5% (w/v) SDS for 15 min at 60 °C.

Extraction of protein

Proteins were extracted by grinding 400–600-mg samples under liquid nitrogen with 4 vols. of extraction buffer (50 mM Tris/HCl, pH 7.6/10% glycerol/2 mM EDTA/2 mM dithiothreitol/4 mM PMSF). The samples were further extracted in a glass homogenizer and then spun at 10000 ***g*** for 10 min at 4 °C. Following determination of the protein content [17] the supernatant was assayed for SS and starch-branching-enzyme activities, and for the production of native gels. The pellet was washed twice with extraction buffer and finally resuspended, again in extraction buffer. This resuspended pellet was assayed for starch-synthase activity associated with the granule.

Assays of enzyme activities and production of native gels

Both the activity of the soluble SS, and the production of non-denaturing gels used to visualize the SS activities of the different isoforms, were measured using methods published previously [10] except that 500 mM sodium citrate was added to the assay buffer for the soluble SS activity. GBSS activity was measured using the same method as for the soluble activity. Total branching-enzyme activity was assayed as follows. Extract (50 μl) was incubated in a total volume of 300 μl containing 100 mM sodium citrate, pH 6.0, 1 mM AMP, 50 mM [U-^{14}C]glucose 1-phosphate (1.5 kBq/μmol) and 0.02 units of glycogen phosphorylase *b* from rabbit muscle at 30 °C. Samples (50 μl) were taken after 30 and 60 min of incubation and heated at 95 °C for 2 min to stop the reaction. The glucans were precipitated at −20 °C for 1 h in 20 vols. of 75% methanol/1% potassium chloride and harvested by centrifuging at 10000 ***g*** for 10 min. The precipitate was washed with 1 ml of the methanol/potassium chloride mix and re-centrifuged as before. The precipitate was then dissolved in 400 μl of 100 mM sodium hydroxide, which was put in a scintillation vial with 5 ml of scintillant (Ready Safe, Beckman, Munich, Germany) and measured in a scintillation counter for 5 min.

Measurement of starch and its amylose and phosphate contents

The starch content of the potato tubers was measured using a previously published method [10]. The amylose content of the purified starch was measured by two different methods; first by quantitatively precipitating the amylopectin using the lectin Concanavalin A according to the instructions from a kit (Megazyme International), and secondly by an iodine-binding assay [18]. The glucose 6-phosphate content of the starch was measured using the enzyme glucose 6-phosphate dehydrogenase after acid hydrolysis of the starch [19].

Production of amylopectin and analysis by HPLC

Amylopectin and amylose were separated by selectively precipitating the amylose using thymol [20], and the amylopectin was analysed by HPLC. Two different HPLC systems were used to study the amylopectin. The first was a high-performance anion-exchange chromatography system using a pulsed amperometric detector (HPAEC-PAD, Dionex, Sunnyvale, CA, U.S.A.). Amylopectin (10 mg/ml) was dissolved in 40% DMSO by shaking, and then a 1/10 vol. of 100 mM sodium acetate, pH 3.5/0.4 units of isoamylase were added. The solution was digested at 37 °C for at least 4 h, passed through a 0.2 μm filter and 10 μl was injected on to a PA-100 column that was protected by a PA-100 guard column (Dionex). The elution conditions were as described previously [20].

The second HPLC system involved one guard column and three separation columns connected in series [21]. Amylopectin was dissolved in water to a concentration of 10 mg/ml by autoclaving and 1/10 vol. of 100 mM sodium acetate, pH 3.5, was added. Following addition of 0.4 units of isoamylase the solution was incubated at 37 °C for at least 4 h. The solutions were passed through a 0.2 μm filter before 100 μl was injected on to the columns. The eluent was 10 mM sodium acetate, pH 5.0, at a constant flow rate of 0.35 ml/min, and carbohydrate was detected by a refractive-index detector (Gynkotek, Berlin, Germany). To estimate the chain lengths of the eluting linear glucans, fractions were collected and the sizes of the carbohydrate chains within them determined using matrix-assisted laser desorption ionization–time-of-flight MS (MALDI–TOF-MS).

RESULTS

Production of antisense plants with reduced activity of SSII and SSIII

In an attempt to understand the role of SS enzymes in *S. tuberosum* tubers, potato cv. Désirée was transformed with the plasmid p35SaGBSSII-aSSIII-Kan, which leads to the repression of both the SSII and SSIII isoforms simultaneously. Plants were screened using native gels to visualize the reduction in activity of the two enzymes. We chose nine lines that had an apparent reduction in the activities of both enzymes for further analysis.

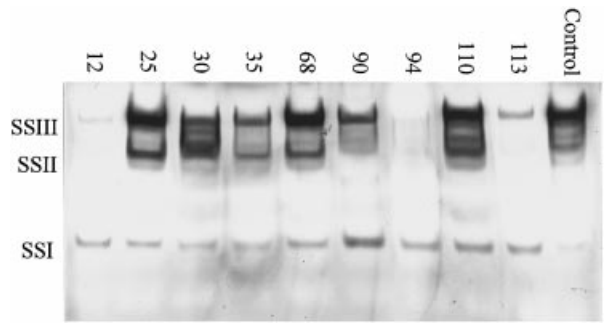

Figure 1 Reduction in activities of SSII and SSIII isoforms in the transgenic lines as shown by native gels

Total protein (40 μg) was loaded on to an 8% agarose gel not containing SDS. The gel was run at a constant 100 V for 2 h before being incubated overnight in 50 mM Tricine/sodium hydroxide, pH 8.5/500 mM sodium citrate/25 mM potassium acetate/2 mM dithiothreitol/2 mM EDTA/1 mM ADP glucose/2 mg/ml potato amylopectin at room temperature. Production of glucans by the different isoforms was visualized by staining with Lugols solution.

Table 1 Activities of SS and starch-branching enzyme in control and transgenic plants

Protein extracts were made from potato tuber samples and assayed for the activities of various enzymes involved in building the starch polymer. Values are means $\pm$ S.E.M. (with n in parentheses). *, $P < 0.05$, compared with control. FW, fresh weight.

Line	SS activity (nmol of ADP glucose/min per g of FW)	Granule-bound-starch activity (nmol of ADP glucose/min per g of FW)	Starch-branching-enzyme activity (μmol glucose 1-phosphate/min per g of FW)
Control	57.4 $\pm$ 9.1 (7)	22.7 $\pm$ 3.4 (7)	8.9 $\pm$ 0.3 (7)
12	4.8 $\pm$ 2.5 (4)*	18.9 $\pm$ 3.7 (4)	8.1 $\pm$ 0.7 (4)
25	25.9 $\pm$ 5.8 (6)*	20.4 $\pm$ 2.4 (7)	7.8 $\pm$ 0.1 (7)
30	8.3 $\pm$ 1.1 (6)*	26.9 $\pm$ 5.9 (6)*	8.5 $\pm$ 0.7 (5)
35	13.7 $\pm$ 2.3 (6)*	19.7 $\pm$ 1.3 (6)	9.8 $\pm$ 0.4 (6)
68	33.7 $\pm$ 5.0 (6)*	25.8 $\pm$ 2.3 (5)	10.9 $\pm$ 0.5 (6)*
90	36.5 $\pm$ 7.5 (6)	25.4 $\pm$ 2.4 (6)	9.9 $\pm$ 0.7 (6)
94	14.9 $\pm$ 5.4 (6)*	21.8 $\pm$ 1.8 (6)	8.5 $\pm$ 0.8 (6)
110	36.6 $\pm$ 7.2 (6)	24.3 $\pm$ 2.7 (6)	7.3 $\pm$ 0.1 (4)*
113	6.6 $\pm$ 0.8 (6)*	36.0 $\pm$ 4.9 (7)*	6.4 $\pm$ 0.3 (7)*

Native gels staining for SS activity showed three bands, as is normal for potato tubers [10] (Figure 1). The band migrating fastest was the SSI isoform, that migrating slowest was the SSIII isoform, whereas the SSII isoform migrated at intermediate speed. Different levels of repression for the SSII and SSIII enzymes were found in the different antisense lines (Figure 1). In one line (no. 12), the activity of the SSII isoform appeared to be almost completely eliminated, whereas that of SSIII was greatly reduced. RNA-blot analysis showed that these lines also exhibited a reduction in accumulation of the cDNAs coding for both isoforms (results not shown).

Changes in enzyme activities

To confirm that the plants did indeed have a reduction in SS activity, protein extracts were assayed for their total SS activity (Table 1). Those isolated from wild-type tubers contained a total activity of 57.4 nmol of ADP glucose/min per g of fresh weight (FW). Two lines (nos. 110 and 90) did not have significant alterations in SS activity compared with the control; however, in the other seven lines there was a range of reductions from an approximate 40% decrease in line 68 to a 90% decrease in line 12 (Table 1).

The activity of SS enzymes associated with the starch granule were also assayed (Table 1). The control plants had an activity of 22.7 nmol of ADP glucose/min per g of FW, and there was some variation in the transgenic lines. One line (no. 113) had a significantly increased activity, in comparison with the control lines, of 36.0 nmol of ADP glucose/min per g of FW; however, none of the other lines showed a significant alteration in comparison with the controls.

Starch-branching enzyme is directly involved in the synthesis of the starch polymer and changes in its activity have been shown to alter the amylopectin of some mutants. In order to test whether there was a pleiotropic effect of the reduction in SS activity, starch-branching enzyme activity was measured using the method of phosphorylase stimulation (Table 1). Extracts from the control tubers had an activity of 8.9 μmol of glucose 1-phosphate/min per g of FW and the activities of three of the transgenic lines (nos. 110, 68 and 113) were slightly altered in comparison with this (Table 1). These alterations were probably due to experimental error, rather than being true changes, for two reasons. One of the altered lines (no. 110) contained no significant reduction in total SS activity (Table 1) whereas, of the other two lines, one showed an increase in activity (no. 68) while the other showed a decrease (no. 113). This shows that there is no correlation between changes in starch-branching enzyme activity and reductions in SS activity.

Measurement of the starch, and of the amylose and phosphate contents of the starch

The amylose contents of the different starches were determined by two different methods, one based on iodine binding and the other based on quantitive precipitation of the amylopectin fraction using the lectin Concanavalin A. No consistent differences were found between the two methods (results not shown). The phosphate bound to glucose residues in the starch at the C_6 position was determined using an enzymic assay. This showed a direct relationship between the reduction in soluble SS activity and reduction in phosphate contents to the extent that in line 12 the phosphate content of the starch was reduced to 30% that of the untransformed control (results not shown).

Alterations to the starch

In order to study the structure of the starch several approaches were taken. First, starch granules were isolated from the tubers and examined by light microscopy, which showed a range of phenotypes in the different lines (Figure 2). The control lines contained starch granules which were regular and oval in appearance, typical of potato starches, and in lines where the total SS activity was reduced by only 60% compared with controls the granules were very similar to those of the control. In lines where the repression was greater, cracks appeared in the granules, as had previously been observed in starch granules of lines where the SSIII isoform was reduced in activity [10,11]. Starch granules from the lines which had over 80% of their activity reduced were extremely distorted in shape, much more so than had been observed previously in potato lines with reduced SS activity.

Secondly, amylopectin was isolated from the starch by selectively precipitating the amylose using thymol. This allowed

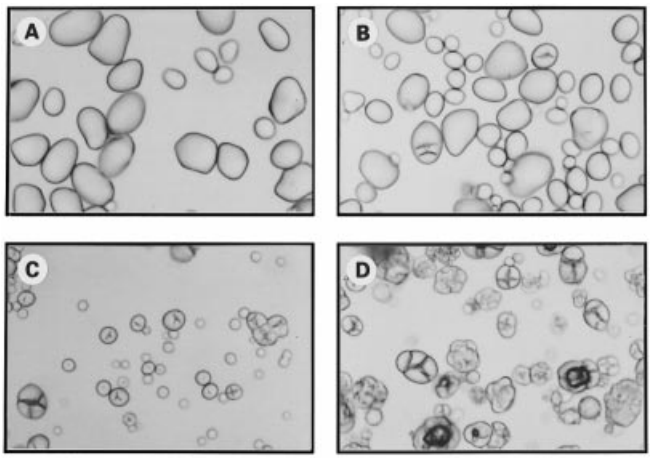

Figure 2 Observation of starch granules under the light microscope

(**A**) Control; (**B**) line 68; (**C**) line 30; (**D**) line 12 (all lines are from the present experiment). All pictures were taken under the same magnification ($\times 50$).

the study of the structure of the amylopectin using two different HPLC techniques, but without interference from amylose. Initially, amylopectin was examined from lines produced for an earlier experiment [10,12], which had the activity of either the SSII or SSIII isoform reduced, using HPAEC-PAD (Figures 3A–3C). The debranched amylopectin from these lines eluted as two main peaks, as is typical for amylopectin. There was a first main peak, comprising chains with degrees of polymerization (dp) between 6 and 35, followed by a second peak of longer chains of dp between 36 and 50, which were not so well defined as those in the first peak. Differences between amylopectins of these transgenic lines and the controls could be seen in very short chains of dp 6–10 when compared with the untransformed control. In the line where the SSIII isoform was reduced this was especially true for the peak representing glucose chains of dp 6, which was greatly increased in comparison with the untransformed control.

When we examined amylopectin from lines where both the SSII and SSIII isoforms were reduced we found a range of differences (Figures 3D–3F). The degree of alteration appeared to be directly correlated with the severity of the antisense inhibition of the total SS activity, as plants with the lowest degree of inhibition had only slightly altered structure, whereas those with a greater than 90% decrease in the activity had the greatest alteration. The change appeared to be due to a progressive decrease in chains between dp 11 and 50 in length, the reduction of which was correlated with the reduction in SS activity (Figures 3D–3F). There was no resolution of chains longer than dp 50 using the pulsed amperometric detector.

Debranched amylopectin was also separated on a series of size-exclusion HPLC columns [21]. This allowed the quantification of the amount of carbohydrate that was separated; however, it did not allow separation of individual chains. The columns were calibrated by detecting carbohydrates in collected

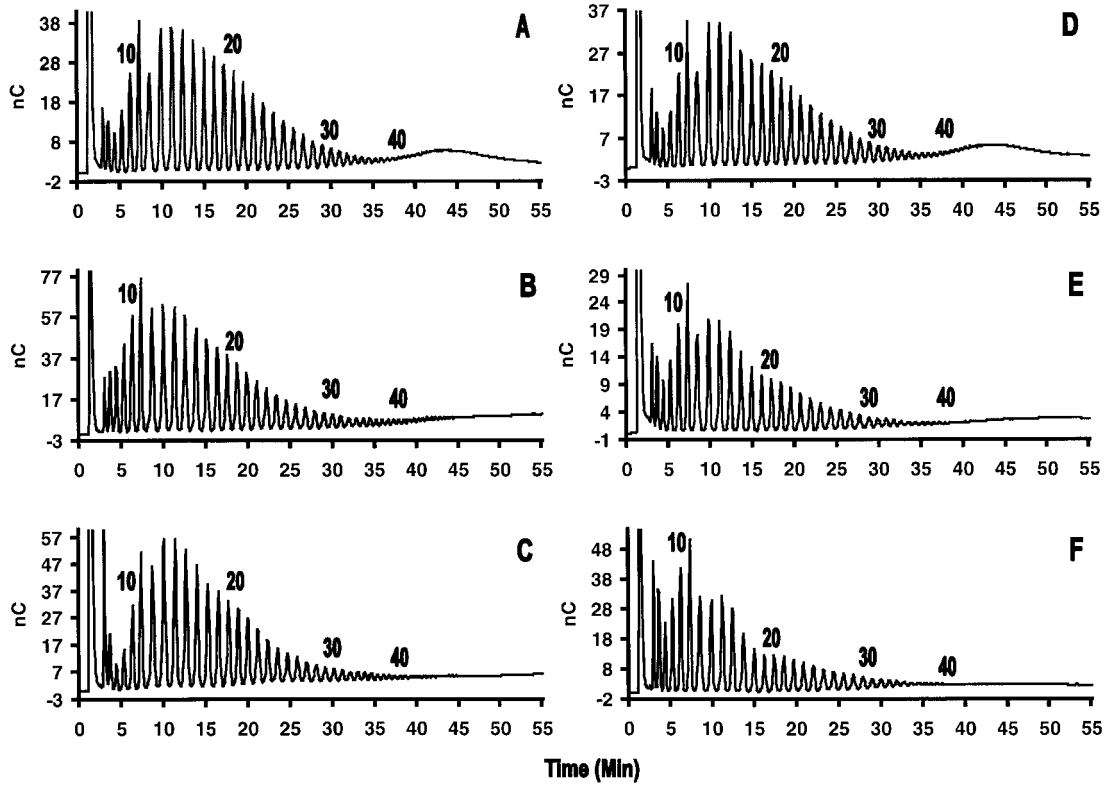

Figure 3 Analysis of constituent chain lengths of amylopectin using HPAEC-PAD

Amylopectin was purified by precipitating amylose using thymol. The amylopectin was dissolved in 40% DMSO and debranched using isoamylase. The debranched sample was loaded on to a HPAEC-PAD HPLC system and separated on a PA-100 column. (**A**) Control; (**B**) SSII antisense line; (**C**) SSIII antisense line; (**D**) line 68; (**E**) line 30; (**F**) line 12 (lines A, D, E and F are from the present experiment; lines B and C are from [10,12]). Numbers represent the dp of carbohydrate eluting from the respective peaks as calculated by comparison with oligosaccharide standards.

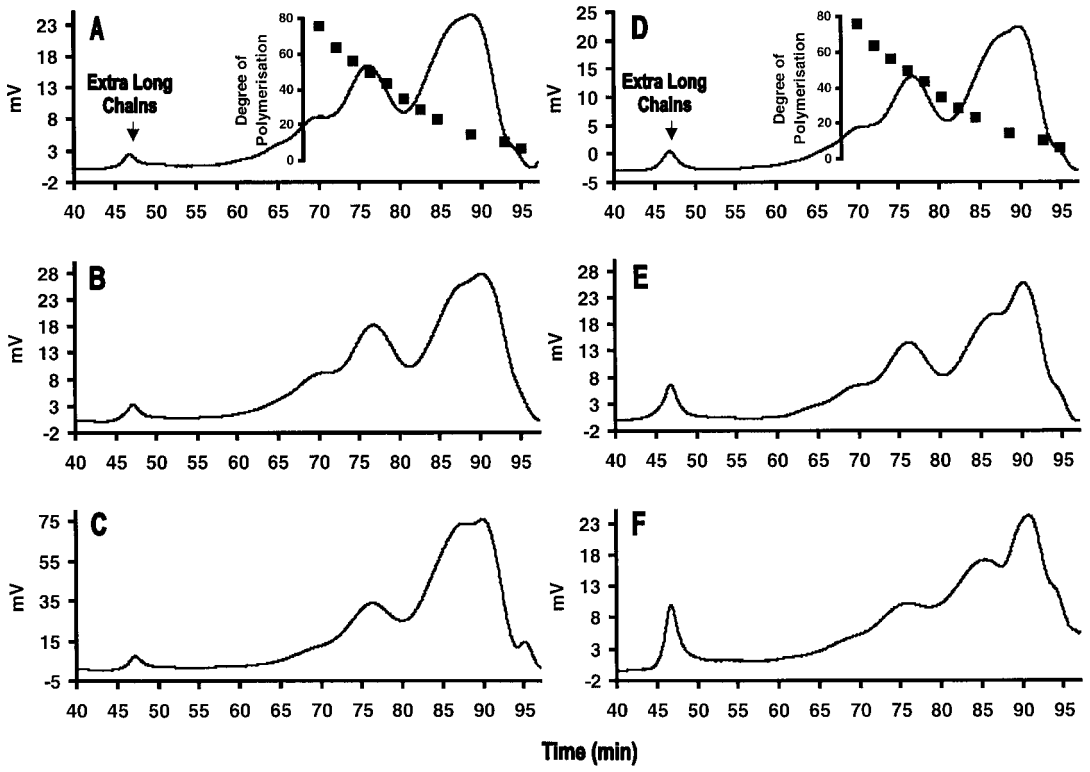

Figure 4 Analysis of constituent chain lengths of amylopectin using gel-permeation chromatography

Amylopectin was purified by precipitating amylose using thymol. The amylopectin was dissolved in water and debranched using isoamylase. The debranched sample was loaded on to an HPLC and separated through two TSK-Gel 2000SW columns and one TSK-Gel 3000SW column connected in series. (**A**) Control; (**B**) SSII antisense line; (**C**) SSIII antisense line; (**D**) line 68; (**E**) line 30; (**F**) line 12 (lines A, D, E and F are from the present experiment; lines B and C are from [10,12]). Chain lengths were calculated by collecting fractions and analysing the size of the chains within using MALDI–TOF-MS.

Table 2 Proportions of chains in amylopectin isolated from transgenic plants with reduced SS activities

Amylopectin was debranched and separated on a series of gel-permeation HPLC columns. The relative proportions of the chains within the polymodal distributions were determined. Fraction 1 represents A and B1 chains as defined previously [21], whereas those chains in fraction 2 are chains B2–B4 [21]. Extra-long chains are those eluting earlier than the B4 fraction, as shown in Figure 4. SSII and SSIII are lines previously produced that are reduced in the activities of the SSII or SSIII isoforms respectively [10,12].

Line	Fraction 1 (%)	Fraction 2 (%)	Extra-long chains (%)	Ratio of fractions 1:2
Control	58.7	40.3	1.0	1.5:1
SSII	62.6	36.5	0.9	1.7:1
SSIII	72.4	26.3	1.3	2.8:1
12	66.9	27.5	5.6	2.4:1
25	68.5	30.5	1.0	2.2:1
30	61.5	35.1	3.4	1.8:1
35	63.8	33.9	2.3	1.9:1
68	58.1	40.2	1.7	1.4:1
90	61.0	38.0	1.0	1.6:1
94	61.3	35.2	3.5	1.7:1
110	58.7	39.5	1.8	1.5:1
113	62.5	34.5	3.0	1.8:1

fractions using MALDI–TOF-MS; however, this was only able to resolve chains of less than dp 80 in length. Typical results for amylopectin from lines where only SSII or SSIII isoforms are repressed are shown in Figures 4(A)–4(C). As with the previous HPLC system a polymodal distribution can be seen. A large peak (containing chains with lengths of dp 6–30) was preceded by a smaller peak and a tail (representing chains greater than dp 30). These peaks have previously been classified into five different fractions (A and B1–B4) [21], representing where the chains are thought to be present within the amylopectin molecule. We call the main peak which elutes second (containing the short A and B1 chains) fraction 1 and the second smaller peak and tail (which represents chains B2–B4) fraction 2 (Figures 4A and 4D). In these experiments a fraction of chains much longer than those in the typical polymodal distribution were found, which we call extra-long chains.

When the relative proportions of chains present in each fraction were measured it was found that lines reduced in either the SSIII or SSII isoforms were altered in comparison with the control. The relative proportions of the chains eluting in fraction 1 were increased in relation to those eluting later (Figures 4B and 4C; Table 2) and was more severe for the line reduced in the SSIII isoform than for that reduced in the SSII isoform. In addition, the distribution of chains within fraction 1 changed. A shoulder appeared on the peak, indicating an increase in chains of between dp 20 and dp 40, leading to an increase of the relative proportions of chains in fraction 1 to those in fraction 2 from 1.5:1 in the control to 1.7:1 in the lines reduced in the SSII isoform and 2.8:1 in the lines reduced only in the SSIII isoform. This indicates a progressive switch from production of chains eluting in fraction 2 to those in fraction 1 in these plants, which can be correlated

with the reductions in total SS activity previously measured in these lines [10,12].

Amylopectin from lines with both SSII and SSIII isoforms reduced also contained altered amylopectin. Although there was no absolute relationship between total SS activity and the alteration in amylopectin structure, some trends could be seen. The three lines with the least inhibition of SS activity (nos. 68, 90, and 110) contained amylopectin which was very similar to that of the control plants. In the other six lines, however, changes were noted which differed for each line. In these lines there was a general decrease in the relative proportions that fraction 2 chains contributed to the total sample. For example, these were reduced from approximately 40 % of the total in the control lines to 28 % in line 12 (the line with the least total SS activity). The profile of fraction 1 also changed as the total SS activity decreased. In the control, fraction 1 eluted as a single peak (Figure 3D); however, in lines containing lower SS activity a shoulder in the peak appeared (Figures 4D–4F) which, in the line with the least activity, was resolved into a second peak (Figure 4F). This shoulder indicates a decrease in the number of chains of dp between 20 and 40 that were produced, in contrast with the lines reduced only in the SSIII isoform where an increase in these chains was seen (see Figure 4F in contrast with Figure 4C). In addition, an increase in the relative proportions of extra-long chains in the amylopectin from some of the transgenic lines was seen. For example, in line 12 they comprised 5 % of the total, compared with only 1 % in the control (Table 2).

DISCUSSION

In this paper we have described how the constituent chain lengths within amylopectin are altered when the activity of either the SSII or SSIII isoforms is removed on its own. This allows the dissecting out of the roles of these individual isoforms in the production of amylopectin in potato tubers. Previously the activities of these two isoforms have been reduced in transgenic plants [5,10–12], but no differences in the constituent chains of starch between the transgenic plants and the controls were noted, and there are two possible reasons for this. In the present study amylopectin was isolated from the starch, rather than unfractionated starch being used, which may have led to interference from the amylose fraction that is slightly branched. Secondly, the carbohydrate was previously only studied using an HPAEC-PAD system, which has difficulty in resolving chains longer than dp 50, and which uses a detector that cannot give a response proportional to the amount of carbohydrate that passes through it.

In this study, it was possible to show a difference between lines where the activity of only the SSII or SSIII isoform was reduced using HPAEC-PAD (Figures 3A–3C), but only as an increase in very short chains, of dp between 6 and 10. This is somewhat similar to results found with two mutants affected in SS activity in pea [7] and *Chlamydomonas* [9] and, therefore, this may be a general feature of a reduction in SS activity. Unlike the two mutants, however, reductions in the relative proportion of chains of dp between 20 and 40 within the amylopectin of the transgenic lines were not so apparent. When a gel-permeation HPLC system was used (Figures 4A–4C) it was possible to show that a reduction in the activity of either the SSII or SSIII isoform led to a reduction in these longer chains that eluted from fraction 2, which are too long for the HPAEC-PAD system to resolve and, in the SSIII antisense line, which also led to an increase in specific chains within fraction 1 of dp between approximately 20 and 40. The shift from the production of relatively long chains to relatively short chains was greater in the line lacking the SSIII isoform than in that which lacked the SSII isoform, consistent with the fact that the SSIII isoform contributes a much greater proportion of the total SS activity than the SSII isoform [5,10–12]. There are several possible explanations to explain these data. For example, SS isoforms may be involved generally in the elongation of all chains and a reduction in their activity might first lead to a reduction in the number of relatively longer chains being produced, but not a decrease in the total number of chains, thus enriching fraction 1. A second possibility is that the isoforms elongate chains represented in fractions 2 in preference to those in fraction 1, which would be elongated specifically by other isoforms. This would also lead to an increase in chains eluting in fraction 1 at the expense of longer chains.

As well as analysing lines where the SSII and SSIII isoforms were repressed individually, a chimaeric construct was also used to reduce the activities of both isoforms simultaneously. In these lines a range of reductions in total SS activities were found (Table 1), from lines containing no significant reduction (nos. 90 and 110) to those with an approximately 90 % reduction in activity compared with the control (no. 12). Analysis of the activities using native gels staining for SS activity (Figure 1) showed a variation in the relative amounts by which the SSII and SSIII isoforms were reduced in the different lines. In these lines, as the SS activity was reduced, the number of chains eluting in fraction 2 was also reduced but, also, specific chains of dp between approximately 20 and 40 in length within fraction 1 were decreased in number (Figures 3D–3F and 4D–4F). As such, these lines produce amylopectin that is more similar to that found in the *rug5* and *sta3* mutants [7,9] than when only the SSIII or SSII isoform is reduced.

This decrease in the relative number of longer chains appeared to be somewhat correlated both with the reduction in SS activity and with the severity of the abnormality of the starch granule's shape. These data lead to the attractive hypothesis that the SS enzymes are involved in elongating chains longer than dp 11 and, therefore, when their activities are reduced, so are the amounts of these chains. This proposition cannot, however, be sustained when the results of the plants where only the SSIII or SSII isoform was repressed (see above) are taken into account. Although from our analysis it cannot be quantified how the SSII and SSIII isoforms were reduced individually in the lines repressed with the chimaeric construct, some lines were identified which had a similar total SS activity as when the SSIII isoform was reduced on its own [10,11] (approximately 80 %). If the observed phenotypes were simply due to the reduction in total SS activities then it would be expected that the changes seen in the amylopectin of these SSIII antisense plants would be similar to those of plants from the lines where both the SSII and SSIII isoforms were repressed, and which have 80 % of their SS activity inhibited. This is not the case, since although fraction 2 was reduced in a similar manner, the chains in fraction 1 were not (contrast Figure 4C, representing a line repressed in the SSIII isoform only, with Figure 4E, where line 30 is repressed in both SSII and SSIII isoforms, but which has a total reduction of SS activities of approximately 80 %). In the SSIII antisense plants these were increased in comparison with the controls, whereas in the double-antisense plants they were decreased. This clearly indicates that the SSII and SSIII isoforms interact with each other in the production of amylopectin and, thus, when they are simultaneously reduced there is a synergistic effect on the amylopectin. One explanation for this would be if each isoform was responsible for manufacturing different structures within amylopectin. When the activity of one isoform is removed on its own, then what is seen is the product of synthesis of the other

isoform. When the activities of both isoforms are reduced simultaneously, however, the specific structures that each isoform produces are also lost simultaneously, and that interferes somehow with the normal synthesis of amylopectin, leading to an alteration in the amylopectin which is not merely an additive effect of the reduction in activities.

SS enzymes generally consist of a catalytic domain at the C-terminal end of the protein and an extension at the N-terminus, and it has been suggested that this extension may lead to the enzyme interacting with other enzymes involved in starch biosynthesis, such as isoamylases [8]. It is possible that, despite there being no pleiotropic differences in the activities of other starch biosynthetic enzymes (Table 1), the reduction in both SS isoforms may have led to alterations in the activities of other enzymes, which we did not examine, in a manner that is different to when the genes were repressed individually. It is also possible that the ratio of branching-enzyme activity to SS activity is responsible for the phenotype that we see, as this will be increased in all of the lines. We think that this is unlikely, however, as when the activity of starch-branching enzyme in potato tubers was reduced by 99% in transgenic lines, which would also alter the ratio, no effect was seen on the amylopectin [22].

In the present study, as in the pea *rug5* mutant and the *C. rheinhardtii sta3* mutant [7,23], the alteration in SS activity led to a change in the shape of the starch granule (Figure 2). As was suggested for the pea mutant we think that this is likely to be due to the alteration in branching structure of the amylopectin, which may lead to a change in the crystallinity of the granule. The *Chlamydomonas* mutant also accumulates an amylopectin containing a preponderance of relatively short chains and manufactures a starch granule where the crystallinity is decreased from approximately 30% to less than 5% [23].

Although the structures of amylopectins from both the pea and *Chlamydomonas* mutants are similar to those in our most repressed lines, the enzymological changes are not similar, in that the reductions in SS activity in the mutants are smaller compared with some of the transgenic lines in this study, which were inhibited up to approximately 90% of the total SS activity (Table 1). In pea the mutation has been shown to be in a gene coding for a SS isoform that is similar to SSII from potato [7]. Studies have shown that this enzyme contributes up to 60% of the SS activity in the pea embryo [24,25]. In the mutant, however, the total SS activity in the embryo is at most 40% reduced compared with the wild type, presumably because other isoforms compensate for the mutation. In the algal mutant, one SS isoform can be seen not to be present on a native gel, and this leads to a reduction in total SS activity of 50%. Both mutants do, however, accumulate amylopectin which has an increased wavelength of maximum absorbance when complexed with iodine (λmax) compared with that of the wild type. This increase in the pea mutant is probably caused by an accumulation of extra-long chains in the amylopectin, which was also observed in our lines where both the SSII and SSIII isoforms were reduced (Figures 4E and 4F). These chains are probably manufactured by the GBSS enzyme, which has been demonstrated previously to introduce very long chains into amylopectin [26], although we cannot rule out that they are produced by the SSI isoform which is also still present.

In our experiments we found that the phosphate contents of the starches were reduced in plants where both the SSII and SSIII isoforms were reduced, and that this was dependent on the total reduction in soluble SS activity. One of the important aims of this work was to produce amylopectins with altered branching structures, and then to try and relate this to altered physical properties of the starches. This may be difficult as the physical properties of the starches are also greatly affected by the phosphate content [27] and, therefore, as both the branching of the amylopectin and the phosphate contents are altered it would be difficult to separate out the effect of altered branching from alterations in phosphate contents.

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