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Mutations in the Gene Encoding Starch Synthase II Profoundly Alter Amylopectin Structure in Pea Embryos

Josephine Craig,[1] James R. Lloyd,[2] Kim Tomlinson,[3] Lorraine Barber, Anne Edwards, Trevor L. Wang, Cathie Martin, Cliff L. Hedley, and Alison M. Smith[4]

John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom

Mutations at the *rug5* (*rug*osus*5*) locus have been used to elucidate the role of the major soluble isoform of starch synthase II (SSII) in amylopectin synthesis in the developing pea embryo. The *SSII* gene maps to the *rug5* locus, and the gene in one of three *rug5* mutant lines has been shown to carry a base pair substitution that introduces a stop codon into the open reading frame. All three mutant alleles cause a dramatic reduction or loss of the SSII protein. The mutations have pleiotropic effects on the activities of other isoforms of starch synthase but apparently not on those of other enzymes of starch synthesis. These mutations result in abnormal starch granule morphology and amylopectin structure. Amylopectin contains fewer chains of intermediate length (B_2 and B_3 chains) and more very short and very long chains than does amylopectin from wild-type embryos. The results suggest that SSII may play a specific role in the synthesis of B_2 and B_3 chains of amylopectin. The extent to which these findings can be extrapolated to other species is discussed.

INTRODUCTION

The starch synthases that catalyze the synthesis of the branched amylopectin component of the starch granule are poorly understood. It is well established that a specific class of granule-bound starch synthases (known as granule-bound starch synthase I or GBSSI) is responsible for the synthesis of the unbranched amylose component of the granule. Two or more distinct isoforms other than GBSSI are present in storage organs of the species examined to date, and these together with starch branching enzymes (SBEs) are responsible for the synthesis of amylopectin. However, it is not clear whether different isoforms play qualitatively distinct roles in amylopectin synthesis (Smith et al., 1997). Study of the roles of such isoforms is hampered by a lack of mutations that affect them specifically and exclusively. Analysis of transgenic potato plants in which activities of specific isoforms have been reduced (Edwards et al., 1995; Abel et al., 1996; Marshall et al., 1996) has thus far not revealed whether these isoforms have qualitatively distinct roles.

In this study, we elucidate the role of the major isoform of starch synthase present in the soluble fraction of the developing pea embryo. Starch synthase II (SSII) is a protein of 77 kD that accounts for 60 to 70% of the soluble starch synthase activity of the pea embryo. It is also present within the matrix of the starch granule (Smith, 1990; Denyer and Smith, 1992; Dry et al., 1992; Denyer et al., 1993; Edwards et al., 1996). Analysis of mutant lines of peas from which GBSSI is absent has shown conclusively that SSII is not involved in amylose synthesis (Denyer et al., 1995a). It is reasonable to assume, therefore, that SSII is important in the synthesis of amylopectin.

Amylopectin is a highly branched polymer consisting of linear chains of α(1,4)-linked glucose residues joined together by α(1,6)-linkages. Within the granule, the chains are thought to be arranged in clusters at intervals of 9 nm, within which chains associate to form double helices. These helices pack in ordered arrays to give concentric crystalline lamellae. The distribution of branch lengths in amylopectin is believed to be important in determining the extent and nature of the crystallinity of the granule, and this influences the physical properties of the starch (French, 1984; Hizukuri, 1986; Gidley, 1992; Jenkins et al., 1993).

Our approach to understanding the role of SSII in amylopectin synthesis was to look for mutant lines of pea likely to be deficient in this enzyme. The study of lines of mutant peas, identified on the basis of altered amount or composition of starch in the embryo, has already proven to be highly successful in elucidating the roles of enzymes in the pathway of starch synthesis. Mutations that specifically reduce or eliminate the activity of sucrose synthase (Craig, 1996; *rug4* [*rug*osus*4*] locus), plastidial phosphoglucomutase (Harrison et al., 1998; *rug3* locus), ADP–glucose pyrophosphorylase (Hylton and Smith, 1992; Martin and Smith, 1995; *rb* locus), GBSSI (Denyer et al., 1995a; *lam* [*l*ow *am*ylose] locus), and the A

[1] Current address: Zeneca Plant Sciences, Jealott's Hill Research Station, Bracknell, Berks RG42 6ET, UK.
[2] Current address: Max-Planck Institut für Molekulare Pflanzenphysiologie, D-14476, Golm, Germany.
[3] Current address: Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RA, UK.
[4] To whom correspondence should be addressed. E-mail smitha@bbsrc.ac.uk; fax 44-1603-456844.

isoform of the SBE (Bhattacharyya et al., 1990; *r* locus) have been characterized extensively. We reasoned that a deficiency in SSII is likely to reduce the starch content of the embryo and to affect the structure of amylopectin. By comparing the effects of other mutations that reduce starch content and affect amylopectin synthesis in the embryo, we expected a deficiency in SSII to result in a wrinkled seed shape and an altered starch granule morphology. All of the mutations that reduce starch content cause normally round seeds to become wrinkled at maturity (Wang and Hedley, 1991). Mutations at the *r* locus that specifically reduce the rate of synthesis of amylopectin and change its average branch length and molecular size (Colonna and Mercier, 1984; Bhattacharyya et al., 1990; Burton et al., 1995; Lloyd et al., 1996) cause the normally ovoid granule to become deeply divided into lobes.

Lines of peas carrying mutations at the *rug5* locus fit the above criteria. Three mutant alleles at the *rug5* locus—*rug5-a*, *rug5-b*, and *rug5-c*—have been identified by selection of wrinkled-seeded peas (Wang et al., 1990) and allelism testing (Wang and Hedley, 1993) after chemical mutagenesis of a round-seeded line. Preliminary analysis of *rug5* mutants showed that mature embryos have a lower starch content than do wild-type embryos. Mature *rug5* embryos also have a unique, convoluted starch granule morphology (Lloyd, 1995; Hedley et al., 1996).

In this study, we demonstrate that the gene encoding SSII lies at the *rug5* locus. We analyze the effects of mutations on amylopectin structure and on enzymes required for starch synthesis other than SSII. The role of SSII in amylopectin synthesis is discussed, and the extent to which we can extrapolate our conclusions with pea to cover starch synthesis in general is considered.

RESULTS

The SSII Protein Is Reduced or Missing in Mutant Embryos

The occurrence of the SSII protein in *rug5* peas was investigated by using SDS-PAGE of granule-bound proteins from mature and developing embryos and by immunoblotting with an antiserum raised against SSII. The SSII protein is relatively abundant in starch granules of wild-type embryos (Smith, 1990; Denyer et al., 1993; Edwards et al., 1996), and SDS-PAGE of proteins solubilized from granules of mutant embryos provided a convenient screen for altered levels of SSII. The concentration of SSII protein was severely reduced relative to wild-type levels in the starch of *rug5-b* and *rug5-c* embryos and was not detected in the starch of *rug5-a* embryos (Figure 1A). This was true for developing as well as mature embryos (data not shown). Immunoblotting of SDS–polyacrylamide gels of crude, soluble extracts and of amyloplasts prepared from developing embryos failed to detect any SSII protein in either the granule-bound or soluble fractions of *rug5-a* embryos under conditions of protein loading and antiserum concentration at which SSII was readily detectable in similar preparations from wild-type embryos (Figure 1B and data not shown). We have shown previously that mutations at two other loci that affect the structure of starch in developing pea embryos—the *lam* and *r* loci—do not affect the amount of SSII protein in the embryo (Denyer et al., 1995a; Edwards et al., 1996).

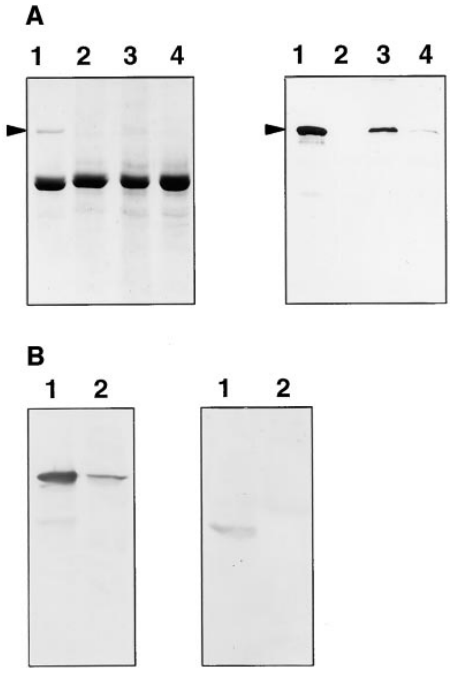

Figure 1. Occurrence of SSII Protein in Wild-Type and *rug5* Embryos.

(A) At left is an SDS–polyacrylamide gel (7.5%) of granule-bound proteins from mature embryos stained with Coomassie blue. Protein was extracted from starch by boiling in SDS-containing gel sample buffer. Each lane contains protein from ~0.5 mg of starch. At right is an immunoblot of a gel identical to that shown at left. The blot was developed with antiserum raised against the SSII protein of developing pea embryos at a dilution of 1:5000. No proteins were recognized by the preimmune serum (data not shown). In both panels, lane 1 contains protein from the wild type; lane 2, *rug5-a*; lane 3, *rug5-b*; and lane 4, *rug5-c*. Arrowheads indicate the position of the SSII protein.

(B) Immunoblots of SDS–polyacrylamide gels of soluble and insoluble fractions of amyloplast preparations from developing pea embryos. Amyloplasts were lysed by repeated passage through a fine needle and separated into soluble and insoluble fractions by centrifugation at 10,000*g* for 10 min. Soluble and insoluble material from the same proportion of the amyloplast preparation was loaded on the gel. Blots were developed with antiserum raised against SSII of developing pea embryos at a dilution of 1:5000. At left are results with wild-type embryos; at right, results with *rug5-a* embryos. In both panels, lane 1 contains insoluble material; lane 2, soluble material.

The *SSII* Gene Lies at the *rug5* Locus

Linkage analysis of the *rug5* locus and the *SSII* gene was undertaken to establish whether the *rug5* mutations are likely to lie in the *SSII* gene. To provide a suitable population of plants for linkage analysis, the *rug5-b* mutant line was crossed with line JI281 (John Innes Centre Germplasm Collection). A 2-kb EcoRI fragment of the *SSII* gene (Dry et al., 1992) was used to probe DNA gel blots of *rug5-b* and JI281 DNA digested with various restriction enzymes. A suitable restriction fragment length polymorphism (RFLP) was identified in DNA digested with HincII. Genomic DNA from 79 F_2 plants from the cross between the *rug5-b* and JI281 lines was then digested with HincII, blotted, and probed with the 2-kb EcoRI fragment. The 79 plants were scored according to whether they had the RFLP pattern of one or the other parent or a combination of the two, and whether the seeds from which they had grown were round or wrinkled. All of the plants with the RFLP pattern of the *rug5-b* parent came from wrinkled seeds, and all of those with the pattern of the JI281 parent or with a combined pattern came from round seeds. The F_2 plants were allowed to set seed. All of the plants with the RFLP pattern of the *rug5-b* parent produced wrinkled seeds, all of those with the pattern of the JI281 parent produced round seeds, and all of those with a combined pattern produced a mixture of round and wrinkled seeds in the next generation (data not shown). Linkage analysis with segregating alleles from 158 chromosomes thus provides strong evidence that the *SSII* gene lies at or very close to the *rug5* locus.

The *rug5-a* Mutation Lies in the *SSII* Gene

To investigate whether mutations at the *rug5* locus actually lie within the *SSII* gene, first-strand cDNA was synthesized from mRNA isolated from the *rug5-a* line, according to Frohman et al. (1988). *SSII* cDNAs were isolated by reverse transcription–polymerase chain reaction (RT-PCR), subcloned, and sequenced. The sequences from three independently derived clones were identical to the sequence of the *SSII* gene from wild-type peas reported by Dry et al. (1992; EMBL accession number X88790), except at position 2260, where the G/C pair in the wild-type sequence had been changed to an A/T. This change is typical of those caused by alkylating reagents (Pienkowska et al., 1993) and is consistent with results for mutations at the *r* locus generated in the same mutation program (MacLeod, 1994). The change converts tryptophan 651 into a stop codon and would thus result in premature termination of translation. The absence of SSII protein in the *rug5-a* line presumably means that the truncated protein is unstable. An equivalent truncation of the SSII isoform of starch synthase from potato is unstable relative to the full-length protein when expressed in *Escherichia coli* (C. Martin and A. Edwards, unpublished data).

The Mutations Have Complex Effects on Starch Synthase Activity during Embryo Development

To characterize the effects of the mutations on starch synthase activity, we measured soluble and granule-bound activity at three stages of embryo development of wild-type and *rug5-a* peas. Granule-bound activity was measured both per gram fresh weight, by subtracting soluble from total (homogenate) activity, and per milligram of starch from freshly prepared samples of starch (Table 1). Soluble starch synthase activity was ~35% lower in *rug5-a* than in wild-type embryos at the first two stages of development, but it was not significantly different at the third stage. Granule-bound starch synthase activity was statistically significantly higher in *rug5-a* than in wild-type embryos at all three developmental stages, as determined by both methods of measurement (Student's *t* test; $P < 0.05$).

The reduction in soluble starch synthase activity in *rug5* embryos is less than expected from the loss of SSII, which accounts for ~60% of soluble starch synthase activity of wild-type embryos (Denyer and Smith, 1992). This implies that the activity of one or more other isoforms is increased in mutant embryos. In an attempt to identify the isoform or isoforms responsible for the increased activity, starch synthase activity was partially purified from the soluble fraction of developing embryos of *rug5-a* mutants by a method based on those used for pea leaves (Tomlinson et al., 1998) and wheat endosperm (Denyer et al., 1995c). Activity was purified 300- to 800-fold (range of three purifications), to a final specific activity of ~0.5 μmol min^{-1} mg^{-1} protein. Immunoblotting experiments revealed that these partially purified preparations (Figure 2A) contained proteins antigenically related to the starch synthase III (SSIII) isoform of starch synthase from potato tuber (Marshall et al., 1996) and the SSIII isoform of pea leaves (Tomlinson et al., 1998). A protein of ~160 kD was clearly recognized by antisera raised against the potato tuber and pea leaf SSIII (Figures 2B and 2C). This protein was recognized only in fractions that contained starch synthase activity, and we suggest that it represents an isoform of the SSIII class.

The potato SSIII antiserum was used in immunoprecipitation experiments with crude, soluble extracts to measure the contribution of the SSIII-like isoform(s) to soluble starch synthase activity in wild-type and *rug5* embryos (Figures 3A and 3B). In two separate experiments using different extracts, the antiserum precipitated 20 to 30% of the activity from wild-type embryos and 35 to 40% of the activity from *rug5-a* embryos. Preimmune serum did not precipitate activity from either extract. From these data and the soluble starch synthase activities presented in Table 1, we estimated that at mid-development, the SSIII-like isoform(s) has an activity of 0.015 to 0.023 μmol min^{-1} g^{-1} fresh weight in wild-type embryos and 0.018 to 0.020 μmol min^{-1} g^{-1} fresh weight in *rug5-a* embryos of the same weight. The activity of the SSIII-like isoform(s) is thus not increased in the mutant embryos. The isoform(s) that is increased in activity in these embryos remains to be identified.

Two sorts of experiments indicated that GBSSI accounted for all of the granule-bound starch synthase activity of *rug5-a* mutant embryos. First, SDS-PAGE did not reveal any granule-bound proteins that were present in *rug5* embryos and not in wild-type embryos or that were present in greater amounts per milligram of starch in *rug5* than in wild-type embryos (Figure 1 and data not shown). Second, GBSSI accounted for all of the starch synthase activity recovered after solubilization of granule-bound proteins from *rug5* starch with α-amylase followed by fractionation by Mono Q chromatography (data not shown; method described by Smith, 1990; Edwards et al., 1996).

These results indicated that the high granule-bound starch synthase activity of *rug5* embryos might be due to a high specific activity of GBSSI. The specific activity of GBSSI was estimated in experiments in which amounts of GBSSI protein in starch from *rug5* and wild-type embryos were measured using SDS-PAGE followed by scanning densitometry, and granule-bound starch synthase activity was measured using the same samples of starch. At two stages of embryo development, the amounts of GBSSI protein in *rug5* starch were approximately the same as or lower than those in wild-type starch, but the starch synthase activity was higher (Table 1). The apparent specific activity of GBSSI was thus 60 to 75% lower in wild-type than in *rug5* embryos.

The Mutations Do Not Affect Other Enzymes of Starch Synthesis

Information about pleiotropic effects of the *rug5* mutations on enzymes of starch synthesis other than starch synthases is important in interpreting their effects on amylopectin synthesis. Accordingly, we compared the maximum catalytic activities of enzymes involved in the conversion of sucrose to starch (Hill and Smith, 1991; Smith and Denyer, 1992; Craig, 1996; Harrison et al., 1998) in wild-type and *rug5-a* embryos at approximately the midpoint of embryo development, when the rate of starch synthesis is maximal (Denyer et al., 1995b). Apart from starch synthase, there were no statistically significant differences in enzyme activities between the two sorts of embryos (Table 2).

We also investigated whether there were differences between the two sorts of embryos in the isoform complements of the SBE. Developing pea embryos have two isoforms of SBE, referred to as A and B (Smith, 1988; Bhattacharyya et al., 1990; Burton et al., 1995). We used a partial purification followed by immunoblotting with antisera specific for SBE A (Smith, 1990) and SBE B (Denyer et al., 1993) to investigate whether the mutations at the *rug5* locus affect these isoforms. The results showed that there are no major differences between wild-type and *rug5* embryos. The relationship

Table 1. Starch Synthase Activity in Developing Embryos of Wild-Type and *rug5-a* Peas

	110 to 180 mg FW[a]		230 to 270 mg FW		330 to 370 mg FW	
Activity	Wild Type	*rug5-a*	Wild Type	*rug5-a*	Wild Type	*rug5-a*
Soluble starch synthase[b] (μmol min^{-1} g^{-1} FW)	0.111 ± 0.012 (7)	0.070 ± 0.003	0.077 ± 0.008	0.051 ± 0.010	0.037 ± 0.008 (4)	0.037 ± 0.010 (6)
Granule-bound starch synthase[b,c] (μmol min^{-1} g^{-1} FW)	0.115 ± 0.031 (7)	0.158 ± 0.020	0.104 ± 0.023	0.196 ± 0.017	0.133 ± 0.026 (4)	0.274 ± 0.041 (6)
Granule-bound starch synthase[b,d] (nmol min^{-1} mg^{-1} starch)	0.44 ± 0.09	1.14 ± 0.025	0.27 ± 0.06	0.69 ± 0.06 (6)	0.28 ± 0.05 (4)	0.62 ± 0.10 (6)
Amount of GBSSI protein[e] (μg mg^{-1} starch)	ND[f]	ND	0.54	0.45	0.82	0.50
Specific activity of GBSSI[g] (μmol min^{-1} mg^{-1} protein)	ND	ND	0.50	1.53	0.34	1.24

[a] FW, fresh weight.

[b] Activities are means ±SE of five independent measurements or of the number of independent measurements given within parentheses. Each measurement was made on a separate extract or starch sample.

[c] Granule-bound activity per gram fresh weight is the difference between total activity, measured on a homogenate, and soluble activity, measured after centrifugation of the homogenate.

[d] Granule-bound activity per milligram of starch was measured directly on freshly prepared starch samples.

[e] Measurements of amounts of protein were made by pooling the samples of starch used for measurement of activity. Each pooled sample contained starch from 20 to 30 embryos. Protein was solubilized from the pooled samples and subjected to SDS-PAGE and staining with Coomassie blue. Amounts of protein were estimated by densitometric comparison of the major 59- to 60-kD protein band from starch, with samples of known amounts of BSA run on the same gel.

[f] ND, not determined.

[g] Specific activity was calculated from the measurements of activity and amount of protein.

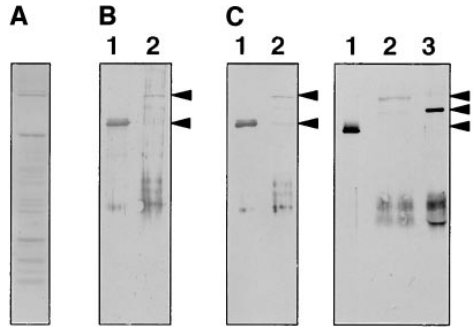

Figure 2. Partial Purification of an SSIII-like Isoform of Starch Synthase from Developing Embryos of *rug5-a* Peas.

Starch synthase activity was purified from the soluble fraction of developing *rug5-a* mutant embryos by ammonium sulfate and polyethylene glycol fractionation and chromatography on Red Agarose, 1,4-diaminobutane agarose, and Mono Q.

(A) SDS–polyacrylamide gel (7.5%) of proteins in a fraction containing the highest starch synthase activity eluted from the final Mono Q column in a typical purification.

(B) Immunoblot of an SDS–polyacrylamide gel (7.5%) developed with antiserum raised against the SSIII isoform of starch synthase from pea leaves at a dilution of 1:1000. Lane 1 contains purified SSIII from pea leaves; lane 2, a fraction containing the highest starch synthase activity eluted from the final Mono Q column in a purification of starch synthase from developing embryos of the *rug5-a* mutant of pea (as in **[A]**). Other proteins recognized by the antiserum in lane 1 were also present in all other fractions eluted from the Mono Q column, including those with no starch synthase activity. The upper arrowhead indicates the protein of ~160 kD recognized by the antiserum; the lower arrowhead indicates the SSIII protein of pea leaves.

(C) Immunoblots of SDS–polyacrylamide gels developed with antiserum raised against the SSIII isoform of starch synthase from developing potato tubers at a dilution of 1:500. The two gels represent two different preparations of starch synthase from developing embryos. Lanes 1 contain purified SSIII from pea leaves; lanes 2, fraction containing the highest starch synthase activity eluted from the final Mono Q column in a purification of starch synthase from developing embryos of the *rug5-a* mutant of pea (as in **[A]**); and lane 3, granule-bound proteins from the starch of developing tubers of potato cultivar Desiree. Other proteins recognized by the antiserum in lanes 2 were also present in all other fractions eluted from the Mono Q column, including those with no starch synthase activity. The upper arrowheads indicate the protein of ~160 kD recognized by the antiserum, the middle arrowhead indicates the SSIII protein of potato starch, and the lower arrowheads indicate the SSIII protein of pea leaves.

between the elution profiles of activity and of SBE A and B proteins from a Polyanion anion exchange column is very similar for wild-type and *rug5* embryos (Figures 4A and 4B).

The Mutations Profoundly Affect Amylopectin Structure

Embryos of all three *rug5* mutant lines displayed a highly convoluted starch granule morphology from an early stage of development through to maturity. Starch contents of mature *rug5* seeds were 30 to 40% lower than those of wild-type seeds (Figure 5 and data not shown).

Effects on the Amylose and Amylopectin Fractions

The iodine-binding capacity of starch from mature *rug5* embryos was significantly greater than that of starch from wild-type embryos. The absorbance at 600 nm of the starch–iodine complex (measured as the absorbance of the flour–iodine complex divided by the concentration of starch as milligrams per cubic centimeter in the sample) for *rug5-c* was 13.7 $\pm$ 0.2 compared with 10.2 $\pm$ 0.1 for wild-type embryos (means $\pm$SE of measurements on three seeds; Student's *t* test, $P < 0.01$). This could indicate that *rug5* starch has a high content of amylose, which is the essentially linear polymer of high iodine-binding capacity that comprises ~35% of wild-type pea starch. However, iodine-binding capacity is also affected by the lengths of chains within the amylopectin fraction of the starch.

To investigate the fraction of the starch responsible for the high iodine-binding capacity of *rug5* starch, we subjected solubilized starch to gel permeation chromatography on a column of Sepharose CL-2B. This technique separates the

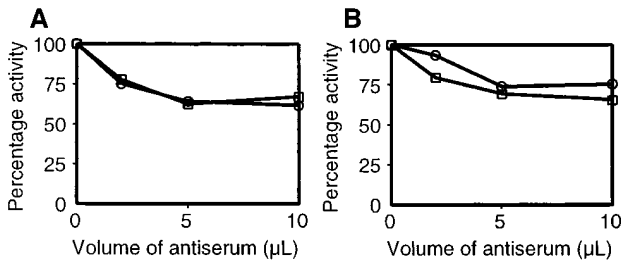

Figure 3. Immunoprecipitation of Soluble Starch Synthase Activity from Developing Pea Embryos by an Antiserum Raised against the SSIII Isoform of Starch Synthase from Potato Tubers.

Samples of soluble extracts of developing embryos (each 240 to 300 mg fresh weight) prepared as they were for enzyme assays were incubated at room temperature with antiserum raised against SSIII of potato tubers or preimmune serum from the same rat or mixtures of antiserum and preimmune serum. The total volume of rat serum in each incubation was 10 µL. Each incubation also contained antiserum raised in rabbit against rat IgG and protein A–Sepharose. After incubation, mixtures were centrifuged, and the supernatants were assayed for starch synthase activity. Starch synthase activity in incubations with differing amounts of antiserum is expressed as a percentage of that in incubations with preimmune serum alone. The experiment was performed twice with two separately prepared extracts: results of one are displayed as open squares, and the results of the other are shown as open circles. Values are means of measurements on duplicate incubations at a given concentration of antiserum.

(A) Extracts from developing embryos of *rug5-a* peas.

(B) Extracts from developing embryos of wild-type peas.

Table 2. Maximum Catalytic Activities of Enzymes Involved in the Synthesis of Starch in Developing Embryos of Wild-Type and *rug5-a* Peas

Enzyme	Enzyme Activity[a] (μmol min^{-1} g^{-1} fresh weight)	
	Wild Type	*rug5-a*
Sucrose synthase	0.99 ± 0.07 (4)	1.13 ± 0.10 (4)
Alkaline invertase	0.114 ± 0.014 (5)	0.091 ± 0.012 (4)
UDP–glucose pyrophosphorylase	40 ± 8 (3)	32, 32
Phosphoglucomutase	13.9 ± 2.1 (4)	15.6 ± 1.9 (4)
ADP–glucose pyrophosphorylase	1.20 ± 0.26 (3)	1.25 ± 0.16 (3)
Starch synthase: total[b,c]	0.193 ± 0.012 (5)	0.323 ± 0.048 (4)
Starch synthase: soluble	0.061 ± 0.017 (5)	0.043 ± 0.004 (4)
SBE[d]	6.73 ± 0.77 (6)	5.26 ± 0.64 (6)
Alkaline pyrophosphatase	1.50 ± 0.13 (3)	1.55 ± 0.19 (4)

[a]Activities were assayed on extracts of single embryos of 250 to 350 mg fresh weight. Values are means ±SE of measurements made, with the number of embryos (each from a separate pod) shown within parentheses. For UDP–glucose pyrophosphorylase activity in *rug5-a* embryos, two individual measurements were made.
[b]Total starch synthase activity was assayed on homogenates; other activities were assayed using soluble extracts.
[c]Activities for wild-type and *rug5-a* mutant embryos are statistically significantly different (Student's *t* test; $P < 0.05$). Differences for all other enzymes are not statistically significant ($P > 0.05$).
[d]Activity is measured as stimulation by the extract of incorporation of glucose units from glucose 1-phosphate into methanol-KCl–insoluble glucan via phosphorylase a.

amylopectin and amylose components from the starch of mature wild-type peas, the former eluting as material of high molecular mass with a maximum wavelength of absorbance of the starch–iodine complex (λ_{max}) of 580 nm and the latter as material of lower molecular mass with a λ_{max} of 620 nm (Figure 6A; Denyer et al., 1995a). Starch from mature and developing embryos of *rug5* mutants also separated into material of high and lower molecular mass; however, the λ_{max} value of the material of high molecular mass was much higher than that of wild-type amylopectin and comparable to that of amylose (Figures 6A and 6B). The high λ_{max} of this first peak could reflect the presence of unusually long chains in amylopectin molecules or the presence of linear glucans of unusually high molecular mass in the amylopectin fraction.

Two experiments were performed to distinguish between these possibilities. First, solubilized starch from mature embryos was treated with thymol, a reagent that selectively precipitates long, linear glucan chains and is used to separate amylose from amylopectin (Bourne et al., 1948). Chromatography of material soluble in thymol, using a Sepharose CL-2B column, revealed a single peak, corresponding to amylopectin, from wild-type starch but two peaks from *rug5* starch (Figure 6C). The first of these peaks coeluted with amylopectin, and the second contained material of considerably lower molecular mass that eluted later than did amylose (Figure 6C). Material in the first peak had a λ_{max} of ~590 nm. This value is lower than that of the amylopectin peak from native *rug5* starch (610 nm; Figure 6B). This result suggests that thymol precipitated either linear glucans of high molecular mass present within the amylopectin fraction of untreated *rug5* starch or a fraction of amylopectin with more or longer long chains than those of the amylopectin that remained soluble in thymol. However, the thymol-soluble amylopectin from *rug5* starch still had a considerably higher λ_{max} value than did the amylopectin from wild-type starch, implying that it had more or longer long chains than did the wild-type amylopectin.

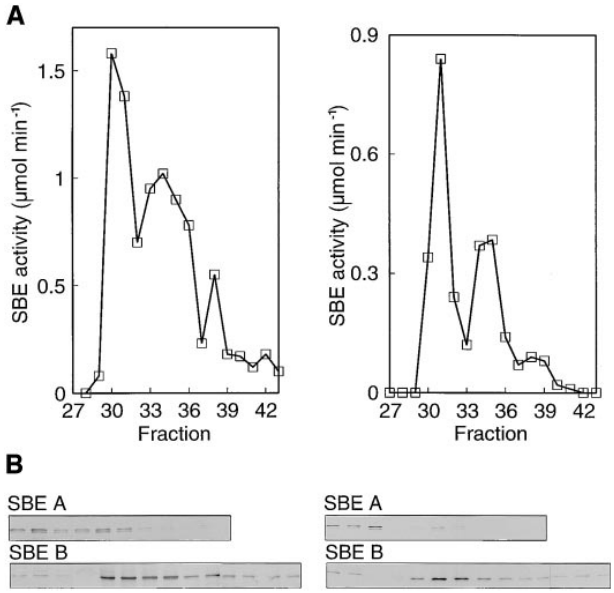

Figure 4. Elution of Partially Purified SBE A and SBE B from a Polyanion Anion Exchange Column.

Shown at left are results from a purification from wild-type embryos; at right are the results using *rug5-a* embryos.
(A) Elution profile of SBE activity from a 1-mL Polyanion SI column. SBE activity was measured as stimulation by the fraction of incorporation of glucose units from glucose 1-phosphate into methanol-KCl–insoluble glucan via phosphorylase a. Fractions were 0.5 mL. There was a linear gradient from 0.65 to 0.85 M NaCl across the fractions shown.
(B) Elution profile of SBE A and SBE B proteins from a 1-mL Polyanion SI column. SDS–polyacrylamide gels (7.5%) of protein from fractions shown in **(A)** were blotted onto nitrocellulose and probed with antisera raised against SBE A and SBE B. The SBE A antiserum was used at 1:1000 dilution, and the SBE B antiserum was used at 1:1500 dilution. Preimmune sera showed no cross-reaction with the proteins recognized by the antisera. Each lane contains protein from one-tenth of the fraction indicated. At top are fractions 29 to 38 developed for SBE A. There were no immunoreactive proteins in fractions eluting after fraction 38. At bottom are fractions 29 to 42 developed for SBE B.

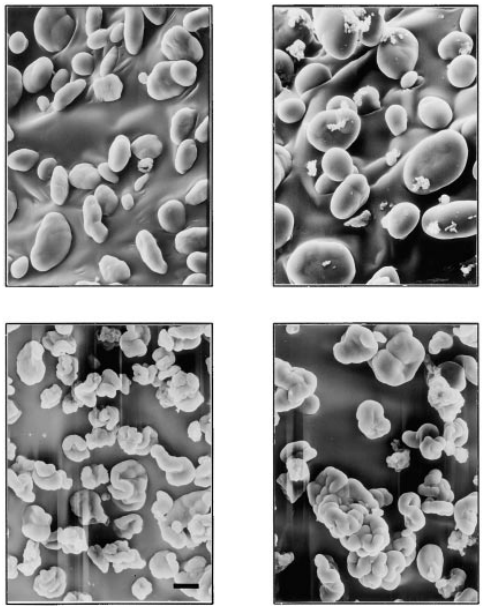

Figure 5. Starch Granules from Developing Wild-Type and *rug5* Peas.

(Top) Starch from wild-type embryos.
(Bottom) Starch from *rug5* embryos.
At left, embryos are ~250 mg fresh weight. At right, embryos are ~350 mg fresh weight. Bar = 10 μm.

The λ_{max} of the second peak of thymol-soluble material from *rug5* starch was ~590 nm. This value is indicative of material that is more highly branched than amylose (Figure 6C). Branched glucans of low molecular mass are also found in the starch of *r* mutant peas and have been termed intermediate material (Colonna and Mercier, 1984; Tomlinson et al., 1997). The presence of this material in starch from mature *rug5* embryos may explain why the λ_{max} of the second peak from native starch declines from 620 nm, which is typical of amylose, to <590 nm with decreasing molecular mass (Figure 6B).

Second, the amylopectin fraction of wild-type and *rug5* starch (prepared by Sepharose CL-2B chromatography) was debranched with isoamylase, and the chains were subjected to chromatography on a Sepharose CL-2B column (Figure 6D). Debranching treatment does not affect the molecular mass of linear glucans; hence, this treatment would not affect the elution position of linear glucans of high molecular mass that coeluted with amylopectin from untreated starch. In fact, almost all of the isoamylase-treated material from both *rug5* and wild-type starch eluted from the column as material of low molecular mass, indicating that it was derived from branched glucans. This result suggests strongly that the high λ_{max} of the amylopectin fraction of *rug5* starch is due to long chains within the amylopectin molecules and not to the presence of linear glucans of high molecular mass.

Effects on the Chain Length Profile of Amylopectin

To examine the chain length profile of amylopectin in more detail, we debranched the amylopectin fraction of wild-type and *rug5* starch (prepared by Sepharose CL-2B chromatography)

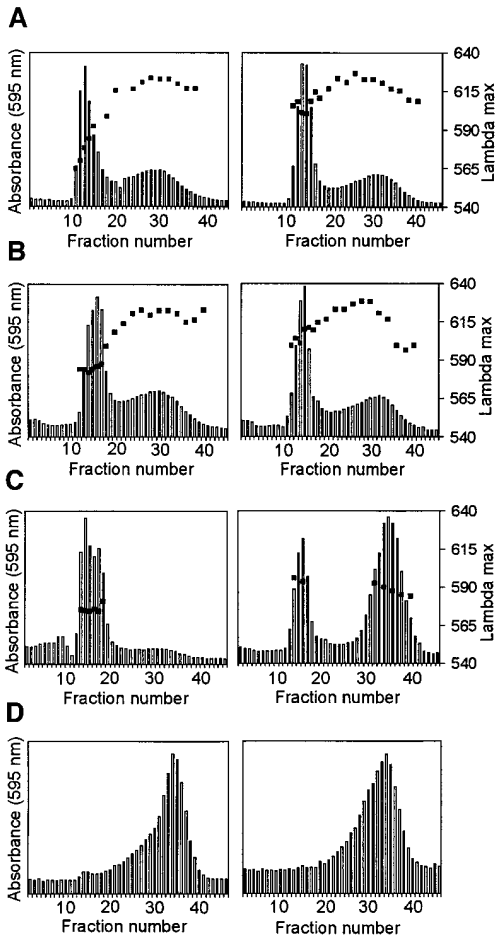

Figure 6. Gel Permeation Chromatography of Starch from Embryos of Wild-Type and *rug5-a* Peas.

Solubilized starch and glucans derived from starch were subjected to chromatography on Sepharose CL-2B columns. Fractions eluted from the columns were mixed with an iodine solution, and absorbance was measured at 595 nm (bars). The wavelength of maximum absorbance of the starch–iodine complex (λ_{max}) was also determined (squares). At left, starch or glucans are from wild-type embryos. At right, starch or glucans are from *rug5-a* embryos.
(A) Starch from developing embryos of ~350 mg fresh weight.
(B) Starch from mature embryos.
(C) Thymol-soluble fraction of starch from mature embryos.
(D) Amylopectin fraction of starch from mature embryos after debranching with isoamylase. Amylopectin was prepared by pooling fractions from gel permeation chromatography of solubilized starch, as given in **(B)**.

with isoamylase and subjected it to size exclusion HPLC. The chain length profile for wild-type amylopectin was typical of the profile of storage starches generally. Such profiles are divided into A and B_1–B_3 fractions (Hizukuri, 1986), as indicated in Figure 7A. The profile for *rug5* amylopectin differed from that of wild-type amylopectin in three main ways. First, *rug5* amylopectin contained a far higher proportion of very long chains (with a degree of polymerization [dp] of ~1000) than did wild-type amylopectin. These chains accounted for ~10% of the *rug5* amylopectin. Second, the ratio of short (A and B_1) to long (B_2 and B_3) chains was much higher in *rug5* amylopectin. The ratio was ~4:1 for wild-type and 8:1 for *rug5* amylopectin. Third, the average degree of polymerization of A chains was considerably lower in *rug5* than in wild-type amylopectin. Whereas there was a clear maximum at dp 15 in the distribution of chain lengths from wild-type amylopectin, *rug5* starch had large numbers of chains of smaller degree of polymerization than this.

Differences between *rug5* and wild-type amylopectin in the distribution of chain lengths within the A chain fraction were examined further by high-performance anion exchange chromatography (HPAEC) of debranched amylopectins (Figures 7B and 7C). The profile for wild-type amylopectin from both mature and developing embryos showed a maximum at dp 12 to 15, with no pronounced shoulder. The profile for *rug5* amylopectin showed a maximum at a considerably lower dp, in the range of 7 to 13. The abundance of shorter relative to longer chains in this range decreased during embryo development so that the maximum was at dp 7 to 10 in amylopectin from embryos of 250 mg and at dp 8 to 13 in mature embryos. The profile for *rug5* amylopectin also showed clear shoulders at dp ~15 and 20.

DISCUSSION

Our results show unequivocally that mutations at the *rug5* locus of pea directly affect the gene encoding the SSII isoform of starch synthase. Linkage analysis revealed that the *SSII* gene lies at or very close to the *rug5* locus, and we have shown that peas carrying the *rug5-a* allele have a point mutation within the DNA sequence encoding the SSII protein. Lines of peas carrying each of the three mutant alleles at the *rug5* locus lack SSII protein or have much reduced levels during development and at maturity.

Mutations at the *rug5* locus have three major effects on the structure of amylopectin in the pea embryo. First, the relative abundance of short chains is increased and the average length of short chains is reduced. The *rug5* amylopectin appears to be strongly deficient in B_2 and B_3 chains such that the ratio of A and B_1 chains to these longer B chains is twice as great in *rug5* amylopectin as in wild-type amylopectin. Very short chains of 7 to 9 glucose units are abundant in *rug5* amylopectin but form only a very minor fraction in wild-type amylopectin.

Second, the abundance of very long chains within the amylopectin fraction is considerably increased. These very long chains probably account for the fact that *rug5* amylopectin has a considerably higher λ_{max} than wild-type amylopectin. We investigated the alternate possibility that the high λ_{max} is due to the presence of very long linear glucan molecules—extra-long amylose molecules—with the same mass as amylopectin molecules from *rug5* starch; however, overall, our results indicate that this is not the case. Although some of the material causing high λ_{max} is apparently precipitated by thymol, gel permeation chromatography of debranched amylopectin provides no evidence for the presence of linear molecules with the same mass as amylopectin.

Third, solubilized *rug5* starch contains amylopectin-like material of lower molecular mass than does normal amylopectin. Although this material may be present in the starch granule in vivo, it is also possible that it is generated from amylopectin during the extraction and solubilization of starch. The abundance of this material precludes an accurate assessment by gel permeation chromatography of the amylose content of *rug5* starch. The presence of amylose in *rug5* starch can, however, be deduced from the fact that λ_{max} of soluble material of low molecular mass is reduced by thymol treatment.

The distinctive shape of starch granules in embryos of peas carrying mutations at the *rug5* locus is likely to be a consequence of the profound effects of the mutations upon the structure of amylopectin. Both the abundance of very short chains in *rug5* amylopectin and the deficiency in B_2 and B_3 chains may be contributory factors. For pure maltooligosaccharides in solution, double-helix formation leading to crystallization requires a chain length of at least 10, although chains as short as 6 glucose units may participate in crystal formation in the presence of longer chains (Gidley and Bulpin, 1987; Pfannemüller, 1987). Within an amylopectin molecule, at least two residues adjacent to a branch point cannot participate in double helix formation due to steric constraints (Umeki and Kainuma, 1981). Therefore, it seems likely that the proportion of A chains able to participate in double-helix formation is smaller in *rug5* amylopectin than in wild-type amylopectin. The B_2 and B_3 chains are believed to span, respectively, two and three of the clusters formed by A and B_1 chains (Hizukuri, 1986). A deficiency in these chains would most likely alter the stability of the cluster structure within the granule. The effects of the *rug5* mutations upon granule structure are the subject of current investigations.

The extent to which the effects of the *rug5* mutations upon amylopectin can be used to deduce the role of SSII in amylopectin synthesis is conditioned by pleiotropic effects on other enzymes of starch synthesis. The mutations appear to have no effects on the maximum catalytic activities of enzymes involved in the conversion of sucrose to ADP-glucose and SBEs or on the individual isoforms of SBEs. However, the mutations affect the activities of starch synthases other than SSII. These effects may influence the synthesis of amylopectin as follows.

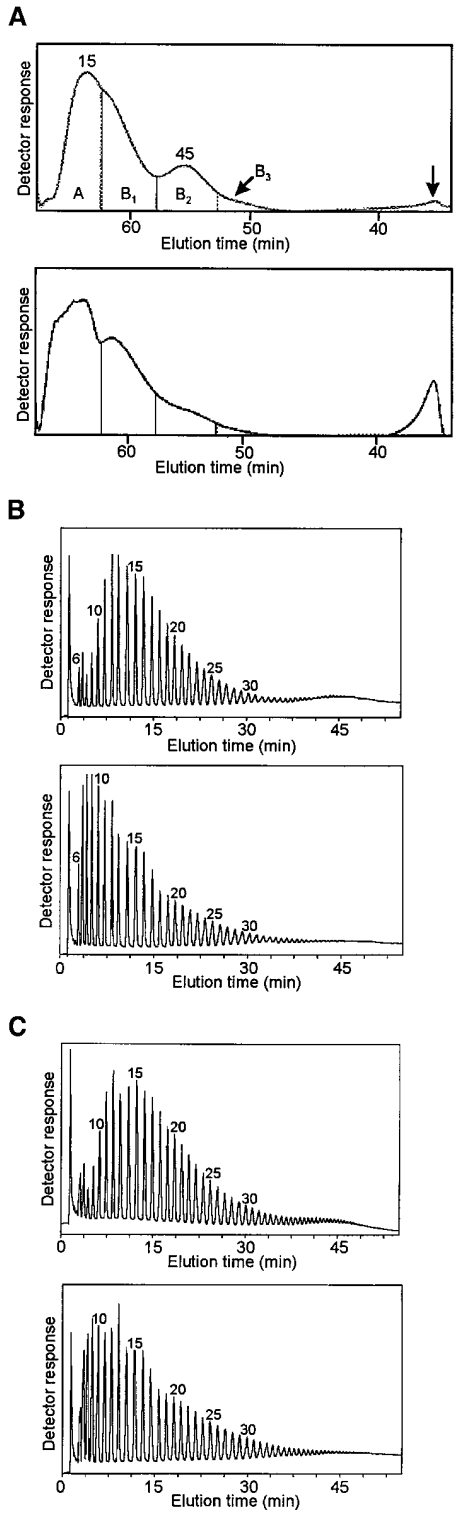

Figure 7. HPLC of Debranched Amylopectin from Embryos of Wild-Type and *rug5-a* Peas.

Amylopectin was prepared by pooling fractions from gel permeation chromatography of solubilized starch, as given in Figures 6A and 6B, followed by debranching with isoamylase.

(A) HPLC of debranched amylopectin. The eluate was monitored with a refractive index detector. Numbers above the peaks indicate the degree of polymerization (dp) of chains eluting at that point. The arrow at ~35-min elution time indicates very long chains, with a dp of ~1000. At top is amylopectin from mature wild-type embryos; at bottom, amylopectin from mature *rug5-a* embryos.

(B) HPAEC of debranched amylopectin on a Dionex HPLC column with pulsed amperometric detection. Numbers above the peaks indicate the dp of chains eluting in that peak. At top is amylopectin from developing wild-type embryos of ~350 mg fresh weight. At bottom is amylopectin from developing *rug5-a* embryos of ~350 mg fresh weight.

(C) As given for **(B)**, except that at top is amylopectin from mature wild-type embryos and at bottom is amylopectin from mature *rug5-a* embryos.

The activity of soluble starch synthase is lower in developing embryos of *rug5-a* mutants than in wild-type embryos, but the reduction is less than expected from the loss of SSII in the mutant embryo. This implies that another isoform or isoforms are increased in activity in *rug5* relative to wild-type embryos. In an attempt to discover the isoform that is increased in activity in *rug5* embryos, we have shown that ~40% of the activity in *rug5* embryos and 20 to 30% of that in wild-type embryos are accounted for by proteins immunologically related to the SSIII isoform of starch synthase from potato tubers (Marshall et al., 1996) and pea leaves (Tomlinson et al., 1998). Immunoprecipitation experiments indicated that the activity of an SSIII-like isoform or isoforms in embryos is not affected by the *rug5* mutations; thus, the isoform that is increased in activity in *rug5* relative to wild-type embryos remains to be identified. Together with our previous discoveries of the GBSSI and SSII isoforms (Denyer and Smith, 1992; Dry et al., 1992; Edwards et al., 1996), the demonstration of an SSIII-like isoform and at least one additional unidentified isoform of starch synthase in the pea embryo means that at least four isoforms must be active in the wild-type organ.

Granule-bound starch synthase activity is higher in *rug5* than in wild-type embryos. This higher activity is not due to greater amounts of GBSSI protein in these embryos. It appears instead to result from a higher specific activity of GBSSI. We suggest that a higher specific activity of granule-bound starch synthase may be a secondary consequence of the effects of the mutation upon the structure of amylopectin and hence upon the environment of the enzyme. This could result, for example, in greater accessibility of the enzyme to its substrates or favorable conformational changes in the enzyme or its glucan substrate.

In light of these alterations in isoforms of starch synthase, we suggest that the simplest interpretation of the effects of the

rug5 mutations upon amylopectin is as follows. The deficiency in B_2 and B_3 chains in *rug5* amylopectin is consistent with a role for SSII in the production of these chains. The abundance of short chains in *rug5* amylopectin may reflect the activity of the remaining isoforms of starch synthase, which may preferentially elongate short chains rather than synthesizing longer B_2 and B_3 chains. The fact that soluble starch synthase activity is reduced by only a small amount in *rug5* embryos makes it likely that the production of normal numbers of B_2 and B_3 chains specifically requires SSII. Other isoforms remaining in the *rug5* embryo appear to be unable to substitute for the function of SSII in this respect.

The *rug5-a* mutation has relatively little effect on the ratio of soluble starch synthase to SBE activity, and we consider it very unlikely that the reduction in average chain length in the mutants is solely due to an altered ratio of activities of starch synthase and the SBE. However, the mutations affect the soluble starch synthase activity to a greater extent in early rather than late development, and their effects on short chains are more pronounced at the midpoint of development rather than at maturity. It is possible, therefore, that part of the effect of the mutations upon chain length is due to an altered ratio of activities of starch synthase and the SBE early in development rather than to the specific loss of SSII.

We suggest that the increased abundance of very long chains in the amylopectin of *rug5* mutants may be due to the action of either the isoform of starch synthase that is increased in activity in *rug5* embryos or GBSSI. Our failure to identify the former isoform precludes further speculation about its role. There are, however, good reasons to suppose that GBSSI can contribute to the synthesis of long chains in amylopectin. In washed, isolated starch granules, GBSSI is responsible for the elongation of a specific population of long chains within the amylopectin fraction of the starch (Baba et al., 1987; Denyer et al., 1996). The amylopectin of *waxy* mutants of cereals, which lack GBSSI, lacks a fraction of very long chains present in the amylopectin of wild-type cereals (Yeh et al., 1981; Hizukuri et al., 1989; Reddy et al., 1993). The fact that the activity of GBSSI is considerably higher in *rug5* than in wild-type embryos is consistent with the idea that this isoform may be responsible for the increase in very long chains brought about by the mutations.

The *rug5* mutations provide clear evidence that individual isoforms of starch synthase play distinct roles in the synthesis of amylopectin in higher plants. The similarities between the effects of the *rug5* mutations and mutations at the *sta3* locus of the unicellular green alga Chlamydomonas encourage us to think that different isoforms of starch synthase may play distinct roles in amylopectin synthesis in all starch-synthesizing systems. One type of isoform of soluble starch synthase (named SSII, but the extent of its similarity to higher plant starch synthases is not known) present in wild-type Chlamydomonas is lacking in *sta3* mutants, and the amylopectin of the mutants is similar to that of the *rug5* mutants in that it has more very short chains and fewer chains of intermediate length than does that of the wild type (Fontaine et al., 1993).

The extent to which our conclusions about the role of SSII in amylopectin synthesis in pea can be extrapolated in detail to other higher plants is unclear. A primary difficulty is that the contributions of distinct classes of starch synthase isoforms to total activity vary considerably from one plant organ to another. Although SSII-like isoforms have been reported in a number of plant organs (pea leaf, Tomlinson et al., 1998; wheat endosperm, Denyer et al., 1995c), their contribution to total activity is in some cases low. For example, ~85% of the soluble starch synthase activity in the potato tuber is contributed by SSIII, and potato SSII (an isoform similar in primary sequence to pea SSII) contributes only 10 to 15% of the activity of the tuber. The starch of transgenic tubers with reduced SSII activity was not obviously different from that of normal tubers (Edwards et al., 1995; Marshall et al., 1996). However, it is premature to conclude that the role of potato SSII in the synthesis of potato starch is qualitatively different from that of pea SSII in the pea embryo. The transgenic tubers retained some SSII, and this may have been sufficient to perform the normal function of SSII in amylopectin synthesis. Transgenic tubers with reduced SSIII activity had altered granule morphology, but this was not accompanied by changes in amylopectin structure equivalent to those caused by the *rug5* mutations of pea (Abel et al., 1996; Marshall et al., 1996). The possibility that SSII plays the same role in elongating B_2 and B_3 chains in amylopectin in pea and potato cannot be ruled out.

Although several mutations affecting starch synthase activity and/or amylopectin structure in higher plants other than peas have been described, these do not currently provide information about the roles of individual isoforms. A mutation at the *shx* locus of barley reduces the soluble starch synthase activity in the endosperm through effects on one isoform (Schulman and Ahokas, 1990; Tyynelä and Schulman, 1993), and mutations at the *dull* locus of maize are reported to reduce (Boyer and Preiss, 1981) or increase (Singletary et al., 1997) the soluble starch synthase activity in the endosperm. Both mutations also affect the activities of other starch-synthesizing enzymes. The effects of the *dull* and the *sugary2* mutations of maize on amylopectin structure in the endosperm are somewhat analogous to those of the *rug5* mutations. Both increase the proportion of short (A and B_1) chains relative to B chains of intermediate length and the average chain length of long chains in the amylopectin of the endosperm. The amylopectin of *sugary2* mutants has a higher proportion of long chains and a higher λ_{max} than does normal maize (Takeda and Preiss, 1993; Wang et al., 1993). Detailed analysis of starch from the endosperm of *shx* barley revealed no striking effects of the mutation upon amylose or amylopectin structure (Schulman et al., 1995). Full elucidation of the biochemical effects and molecular basis of the *shx*, *dull*, and *sugary2* mutations should provide further information about the roles of isoforms of starch synthase in amylopectin synthesis.

METHODS

Plant Material

Allelic lines of pea (*Pisum sativum*) carrying the wild-type and three mutant alleles at the *rug5* (*rug*osus*5*) locus were derived from individual mutants isolated by chemical mutagenesis of a round-seeded (wild-type) line of peas, BC1/RR (Wang et al., 1990), and were given the symbols *rug5-a*, *rug5-b*, and *rug5-c*, according to the Pisum Genetics Association (University of Tasmania, Hobart, Australia), after complementation analysis (Wang and Hedley, 1993). For measurements of starch content and absorbance of starch–iodine complexes, mutant seeds were selected from the sixth backcrosses of the mutant lines to the wild-type line. Other experiments were done with heterozygote-derived pairs of near-isolines that had been purified by at least eight generations of selfing and reselection. No differences were detected between the three wild-type lines derived in this way; where comparisons are made between the wild type and the three mutant lines, data from only one of the wild-type lines are displayed.

Plants were grown in a soil-based compost in a greenhouse at minimum temperatures of 15°C (day) and 10°C (night) and provided with supplementary light for 16 hr day^{-1} from October to March.

Measurement of Enzyme Activities

Freshly harvested embryos were homogenized with a pestle and mortar followed by an all-glass homogenizer at 0°C in ~5 volumes of 100 mM 3-(*N*-morpholino)propanesulphonic acid (Mops), pH 7.2, 5 mM DTT, 5 mM $MgCl_2$, and 10 g L^{-1} BSA. Homogenates were centrifuged at 10,000*g* at 4°C for 10 min to produce a soluble extract.

Enzymes were assayed at 25°C in conditions previously determined to give the maximum activity in embryo extracts from lines of peas near-isogenic to those used in this study. Sucrose synthase was assayed as the fructose-dependent incorporation of carbon-14 from ADP—U-^{14}C–glucose into sucrose, according to Craig (1996). Alkaline invertase was assayed at pH 7.5 by determining the glucose and fructose present after incubation of extract with sucrose (Craig, 1996). UDP–glucose pyrophosphorylase, ADP–glucose pyrophosphorylase, and phosphoglucomutase were assayed spectrophotometrically, according to Smith et al. (1989) and Foster and Smith (1993). Starch synthase was assayed as the incorporation of carbon-14 from ADP—U-^{14}C–glucose into glucan. For soluble starch synthase, assays were processed by the resin method described by Jenner et al. (1994). For granule-bound starch synthase, assays were processed by the methanol–KCl method described by Smith (1990). Alkaline pyrophosphatase was assayed according to Gross and ap Rees (1986), except that the assay contained 200 mM Bicine, pH 8.9, 20 mM $MgCl_2$. The starch branching enzyme (SBE) was assayed by the phosphorylase stimulation method described by Smith (1988).

Preparation of Amyloplasts

Amyloplasts were prepared from developing pea embryos, according to Hill and Smith (1991).

Preparation of Starch

Starch was prepared from developing embryos, according to Smith (1990). Starch from mature seeds was prepared by two methods. For SDS-PAGE of granule-bound proteins, dry cotyledons were homogenized to a fine powder. The powder was suspended in 20 g L^{-1} aqueous SDS and filtered through two layers of Miracloth (Calbiochem, La Jolla, CA). Insoluble material in the filtrate was washed three times by suspension and centrifugation in the SDS solution, followed by one wash in water and two in acetone at −20°C. Purified starch was air dried and stored at −20°C.

For analysis of starch polymers, mature peas were soaked in excess water overnight at 3°C and then homogenized. The homogenate was washed with water through a 53-μm filter, and the filtrate was allowed to sediment for 2 hr. The starch sediment was resuspended and allowed to sediment first in water and then in 0.1 M NaCl. The sediment was then filtered, washed twice with ethanol, and dried.

Analysis of Starch

Starch contents and the absorbance of starch–iodine complexes were measured according to Denyer et al. (1995a). Gel permeation chromatography on a Sepharose CL-2B column, thymol precipitation, debranching of amylopectin, and Dionex high-performance anion exchange chromatography (HPAEC) were all performed according to Tomlinson et al. (1997). For analysis by HPLC, 100-μL samples of debranched amylopectin at 5 mg mL^{-1} were applied to three HPLC columns in series (TSK-gel, 300 × 7.5 mm: G3000SW_{x1}, G2000SW_{x1}, and G2000SW_{x1}; TOSOH Ltd., Linton, Cambridge, UK), according to Hizukuri (1986). Elution was with 10 mM sodium acetate, pH 5.0, at 1.5 mL min^{-1}. The eluent was monitored with a refractive index detector. The columns were calibrated with linear dextrins of known degrees of polymerization (dp) and pullulan standards of narrow molecular weight distribution.

Electron Microscopy

Dry starch samples were coated in gold and viewed in a CamScan series 4 scanning electron microscope (Cambridge Instruments, Cambridge, UK).

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting procedures were performed according to Marshall et al. (1996). Amounts of granule-bound starch synthase I (GBSSI) protein on gels stained with Coomassie Brilliant Blue R 250 were determined with a scanning densitometer by reference to known amounts of BSA (0.15 to 1.5 μg) run on the same gel.

Preparation of Antiserum

The starch synthase SSIII isoform from pea leaves was purified according to Tomlinson et al. (1998). Strips containing the SSIII protein were excised from SDS–polyacrylamide gels, and the protein was electroeluted, dialyzed extensively against water, and freeze dried. Samples were dissolved in half-strength PBS, mixed with Freund's complete adjuvant, and injected intramuscularly into a rat. The

immunization was repeated at 2-week intervals, using Freund's incomplete adjuvant. Blood samples were taken at 2-week intervals after the third immunization. Crude serum was used in all experiments.

Immunoprecipitation

Immunoprecipitation procedures were performed according to Marshall et al. (1996) and Tomlinson et al. (1998).

Partial Purification of Soluble Starch Synthase

Purification of soluble starch synthase was from 100 to 200 g of embryos, each of 250 to 350 mg fresh weight, and was developed from the methods described by Tomlinson et al. (1998) for pea leaves and Denyer et al. (1995c) for wheat endosperm. The supernatant resulting from homogenization in 5 volumes of extraction medium (50 mM Tris, pH 7.2, 10 mM EDTA, 2.5 mM DTT, and 50 mL L^{-1} glycerol), filtration, and centrifugation was subjected to ammonium sulfate precipitation. The fraction precipitating at 20 to 40% saturation was redissolved and brought to a polyethylene glycol (PEG 600) concentration of 10% (w/v). Material remaining soluble was subjected to chromatography on successive columns of Reactive Red Agarose type 3000-CL (Sigma), Mono Q (Pharmacia Ltd., Milton Keynes, UK) 1,4-diaminobutane–agarose (Sigma), and Mono Q. For SDS-PAGE of partially purified starch synthase, fractions from the final Mono Q column were dialyzed overnight against water, freeze dried, and redissolved in 100 μL of gel sample buffer.

Partial Purification of the SBE

Purification was from 10 g of embryos, each of 300 to 400 mg fresh weight, and was a scaled-down modification of the method described by Smith (1988). The supernatant resulting from homogenization in 2 volumes of extraction medium (50 mM Tris, pH 7.8, 0.5 mM EDTA, and 1 mM DTT) and centrifugation was subjected to chromatography on DEAE Sepharose Fast Flow by a batch method and then, successively, to ammonium sulfate precipitation and Mono Q and Polyanion SI (Pharmacia) chromatography, according to Smith (1988). For SDS-PAGE of partially purified SBEs, fractions from the Polyanion column were dialyzed overnight against water, freeze dried, and redissolved in 100 μL of gel sample buffer.

Linkage Analysis

Genomic DNA was extracted from pea leaves, purified, and concentrated according to Ellis et al. (1984). DNA digested with restriction enzymes was separated on 7 g L^{-1} agarose gels and blotted onto nitrocellulose. The radioactive probe was prepared from the 2-kb EcoRI fragment of the *SSII* gene of pea (Dry et al., 1992), according to Feinberg and Vogelstein (1984). Blots were washed with 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 5 g L^{-1} of SDS at 65°C.

Isolation and Sequencing of cDNA

Total RNA was isolated from 1.0 g of pea seeds (each of 100 to 200 mg fresh weight), according to Edwards et al. (1995). Polyadenylated mRNA was purified from total RNA by using an oligo(dT)–cellulose spin column (Pharmacia). First-strand cDNA was synthesized using a cDNA synthesis kit (Amersham International).

Three pairs of oligonucleotides were synthesized to generate three overlapping fragments by polymerase chain reaction (PCR) (1, 0.95, and 0.84 kb): 5′-GATTCATACAAACACCACTTTCTTCAATTGGTC-3′ and 5′-CCATAATGAGGTGCAACAATCATAACTCTATG-3′; 5′-CTTGGTCGAAAACAGGCGGGCT-3′ and 5′-TCACGGACAGGCAAACCGAGCTCCCTTTGCAAAG-3′; 5′-GGAATTGTGAACGGTGTCGACACAAAAGATTGG-3′ and 5′-GTTCGATCTAGTAAGAGAAAATTTCTCGTTC-3′.

PCR was performed using Pfu DNA polymerase (Stratagene, La Jolla, CA), according to the manufacturer's instructions. Each fragment was cloned into vector pCR2.1 (Invitrogen, Leek, The Netherlands). The sequence of three clones for each fragment was determined using a dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Norwalk, CT) and an ABI 373 sequencer (Applied Biosystems, Warrington, Cheshire, UK). For all three fragments, the sequences of the three clones were identical.

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