Multiple, Distinct Isoforms of Sucrose Synthase in Pea[1]

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Genes encoding three isoforms of sucrose synthase (Sus1, Sus2, and Sus3) have been cloned from pea (*Pisum sativum*). The genes have distinct patterns of expression in different organs of the plant, and during organ development. Studies of the isoforms expressed as recombinant proteins in *Escherichia coli* show that they differ in kinetic properties. Although not of great magnitude, the differences in properties are consistent with some differentiation of physiological function between the isoforms. Evidence for differentiation of function in vivo comes from the phenotypes of *rug4* mutants of pea, which carry mutations in the gene encoding Sus1. One mutant line (*rug4-c*) lacks detectable Sus1 protein in both the soluble and membrane-associated fractions of the embryo, and Sus activity in the embryo is reduced by 95%. The starch content of the embryo is reduced by 30%, but the cellulose content is unaffected. The results imply that different isoforms of Sus may channel carbon from sucrose towards different metabolic fates within the cell.

The aim of this work was to discover and characterize isoforms of Suc synthase (Sus) in pea (*Pisum sativum*). We reported previously that mutations at the *rug4* locus of pea lie in a gene (S*us1*) encoding Sus (Craig et al., 1999). The mutations result in the loss of most of the Sus activity from developing embryos and *Rhizobium leguminosarum*-containing root nodules, but leaves and developing testas retain at least half of the normal Sus activity. This phenotype indicates that the pea plant has more than one isoform of Sus, and that the isoforms are differently expressed. This idea contradicts earlier speculation that legumes have only one *Sus* gene (Heim et al., 1993), but is supported by a report of a *Sus* cDNA from pea testa that is only 67% identical in sequence to *Sus1* (Buchner et al., 1998).

The roles of Sus isoforms in plants are the subject of uncertainty and debate. There is good reason to believe that Sus exists both free in the cytosol and in association with the cellulose synthase complex on the plasmalemma. The cytosolic form may supply products of Suc metabolism to the hexose phosphate pool for general metabolism, whereas the plasmalemma-associated form may supply UDP-Glc directly for cellulose synthesis (Amor et al., 1995; Carlson and Chourey, 1996; Delmer et al., 2000). However, the mechanism that partitions Sus between the cytosol and the plasmalemma is unknown. On the one hand, there are indications that cellular conditions may determine the extent to which Sus is soluble or associated with actin filaments and membranes (Winter et al., 1997, 1998; Winter and Huber, 2000). From in vitro experiments, it has been suggested that membrane association depends upon the phosphorylation status of the enzyme (Winter et al., 1997). On the other hand, studies of maize (*Zea mays*) lines carrying mutations in genes encoding the two isoforms of Sus in the endosperm (at the S*hrunken1* [S*h1*] and S*us1* loci) indicate that different isoforms may have distinct roles in cellular metabolism. Both mutations affect starch content in the endosperm but only *sh1* also confers a cell degeneration phenotype (Chourey et al., 1998). Thus, the Sh1 isoform may be more important than the Sus1 isoform in providing carbon for cellulose synthesis.

The idea that different isoforms fulfill distinct metabolic functions is supported by the existence in many species of plants of multiple isoforms of Sus that are strongly differentially expressed: spatially, developmentally, and in response to low temperature, anoxia, and elevated Suc (Springer et al., 1986; Chen and Chourey, 1989; Heinlein and Starlinger, 1989; Marana et al., 1990; Martin et al., 1993; Zeng et al., 1998). For example, the S*us3* and S*us4* genes of potato (*Solanum tuberosum*) encode Sus isoforms that are expressed most strongly in the stems and roots and in the tubers, respectively (Fu and Park, 1995). Specific reduction of the activity of the Sus4 isoform, through expression of antisense RNA, severely reduces starch content in the tuber (Zrenner et al., 1995). Use of the *sh1* and *sus1* maize lines and the *sus4* antisense potatoes for further elucidation of the roles of Sus isoforms is hampered by significant alterations in activities of several other enzymes of primary

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metabolism in these plants (Dancer and ap Rees, 1989; Zrenner et al., 1995; Singletary et al., 1997).

Pea is potentially an excellent system in which to elucidate the roles of Sus isoforms. Sus is claimed to be of central importance in the metabolism of three organs with very different requirements for the regulation of Suc metabolism: the developing testa (Rochat and Boutin, 1992; Déjardin et al., 1997a, 1997b), the developing embryo (Déjardin et al., 1997b), and the *R. leguminosarum*-infected root nodule (Gordon, 1995; Streeter, 1995). The *rug4* mutations affect these organs differently, and have minimal pleiotropic effects on the activity of other enzymes of primary metabolism (Craig et al., 1999). In this paper, we report that the pea plant has multiple isoforms of Sus, with distinct spatial and temporal patterns of expression and kinetic properties. We present evidence that the Sus1 isoform is not required for normal cellulose synthesis.

RESULTS

Isolation of cDNAs for Three Sus Isoforms

To discover whether pea plants expressed more than one Sus gene, a 1-kb fragment of the *Sus* gene from *Lotus japonicus* (Skøt et al., 1996) was used to probe a leaf cDNA library at low stringency. Leaves were chosen because the *rug4* mutations reduce Sus activity by only 40% in this organ; hence, isoforms other than Sus1 may account for much of the activity. Seventeen clones were isolated, which, from sequence analysis, grouped into three classes designated *Sus1*, *Sus2*, and *Sus3. Sus1* was a full-length cDNA containing sequence identical to that of partial cDNAs described previously from pea testa and pea embryo (Déjardin et al., 1997a; Craig et al., 1999: EMBL database accession no. AJ012080) and 99.5% identical at the level of predicted amino acid sequence to a cDNA from pea root nodules (accession no. AF079851). This gene lies at the *rug4* locus (Craig et al., 1999). *Sus2* was a full-length cDNA-containing sequence identical to a cDNA previously isolated from pea testa (Buchner et al., 1998; accession no. AJ001071). *Sus3* was derived from a previously unreported gene (accession no. AJ311496). The longest *Sus3* cDNA clone, which was full length, was 2,652 bp. It encoded a peptide of 812 amino acids with a predicted molecular mass of 92 kD. Over the coding sequences, *Sus1* and *Sus2* are 69% identical, *Sus1* and *Sus3* are 82% identical, and *Sus2* and *Sus3* are 68% identical at the nucleotide level. The predicted amino acid sequences of Sus1 and Sus 3 are very similar (87% identical), and both are less similar to Sus2 (Sus1 is 71% identical and Sus3 70% identical to Sus2). A phylogenetic tree of predicted full-length amino acid sequences of Sus proteins reveals that Sus 1 and Sus3 fall on a different branch from Sus2 (Fig. 1). Sus1 is more similar to the other reported legume Sus proteins than it is to Sus3.

To assess whether there may be further isoforms of Sus in pea, blots of genomic DNA digested with various restriction enzymes were probed at low stringency with a fragment of the *Sus2* gene containing a region highly conserved between Sus genes. The blots revealed that pea contains at least five genes encoding Sus-like proteins (not shown). The extent and pattern of expression of genes other than *Sus1*, *2*, and *3* remain to be investigated.

Pattern of Expression of *Sus1*, *Sus2*, and *Sus3* Genes in the Pea Plant

Hybridization experiments performed on blots of total RNA from various organs of the plant revealed spatial and temporal differences in the patterns of expression of the three *Sus* genes (Fig. 2). *Sus1* transcripts were present in all of the organs examined. Levels were highest in *R. leguminosarum*-infected root nodules, roots, and young embryos and testas (seeds of 200–300 mg fresh weight). Levels of expression were lower in older embryos and testas (seeds of 300–400 mg fresh weight) and in flowers, stems, pods, and young leaves (not yet fully expanded). Expression was not detected in mature leaves. The highest expression of *Sus2* was in older testas, and expression was also detectable in both young and mature leaves. *Sus3* transcript was difficult to detect in total RNA samples, analysis of which detected expression only in flowers and young testas. Analysis of *Sus3* expression using poly(A^+) RNA rather than total RNA revealed that transcript was present in testas, embryos, mature leaves, and nodules (Fig. 2 and data not shown).

Kinetic Properties of Sus Isoforms

We attempted to purify individual isoforms of Sus from various organs of the pea plant for kinetic analyses. Although purification of total Sus to near-homogeneity was achieved, it proved extremely difficult to achieve effective separation of isoforms (not shown). This problem was circumvented by expression of Sus1, 2, and 3 in *Escherichia coli* (see "Materials and Methods"). Each of the three isoforms was expressed in an active, soluble form. Preparations of at least 90% purity were obtained by ammonium sulfate fractionation followed by FPLC chromatography on HiLoad Superdex 200 and Mono Q. All three purified isoforms behaved as proteins of 92 kD on SDS-PAGE, and migrated in the same position on native polyacrylamide gels as the enzyme purified from the plant (a tetramer; data not shown).

For kinetic analyses, activity was measured by continuous assays coupled to NAD reduction (cleavage reaction) or NADH oxidation (synthesis reaction). For both the synthesis and the cleavage reaction of the enzyme, activity was measured at eight concentrations of one substrate at each of eight concentra-

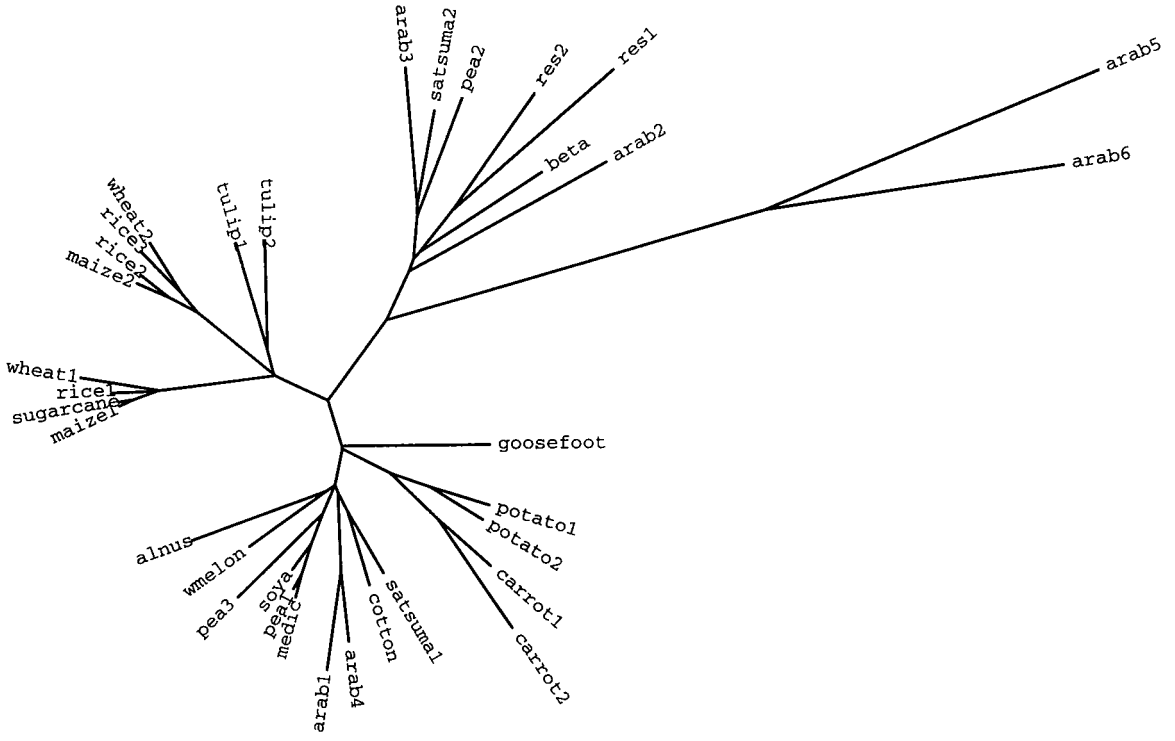

Figure 1. Unrooted phylogenetic tree of predicted amino acid sequences of Sus proteins, assembled using Phylogeny Inference Package, version 3.5. Maximum likelihood tree was calculated using PUZZLE and the tree drawn using Neighbor. EMBL database accession nos. of the displayed Sus are as follows: *Alnus glutinosa*, X92378; Arabidopsis (arab)1, X70990; arab2, Q00917; arab3, AL161494; arab4, AL353871; arab5, BAB11375; arab6, AAG30975; *Beta vulgaris* (beta), A41498; carrot (*Daucus carota*)1, P49035; carrot2, Y16091; goosefoot (*Chenopodium rubrum*), X82504; maize1, P04712; maize2, P49036; pea1, AJ012080; pea2, AJ001071; pea3, AJ311496; potato1, P10691; potato2, P49039; *Medicago sativum* (medic), AF049487; cotton (*Gossypium hirsutum*), Q9XGB7; rice (*Oryza sativa*) 1, P30298; rice2, P31924; rice3, Q43009; tulip (*Tulipa gesneriana*) 1, Q41608, tulip2, Q41607; *Craterostigma plantagineum* (resurrection plant: res)1, Q9ZPC6; res2, Q9ZPC5; *Citrus reticulata* (*Citrus unshiu*: satsuma)1, Q9SLY1; satsuma2, Q9SLY2; wheat (*Triticum aestivum*) 1, AJ001117; wheat2, AJ000153; watermelon (*Citrullus vulgaris*), Q9SBL8; *Glycine max* (soya), AF030231; and sugarcane (*Saccharum officinarum*), AF263384. For clarity, the following full-length sequences have been omitted: mung bean (*Phaseolus aureus*), Q01390; *Medicago truncatula*, Q9TOM6; barley (*Hordeum vulgare*), P31922 and P31923; and tomato (*Lycopersicon esculentum*), P49037 and AJ011535. If included, mung bean and *M. truncatula* sequences would fall very close to pea1, soya, and medic; barley sequences would fall very close to wheat; and tomato sequences would fall very close to potato.

tions of the other substrate, in a microtitre plate. Measurements were made on three independent preparations of each isoform, each from a separately grown batch of *E. coli*. Data from one of the preparations are displayed in Table I and Figures 3 and 4: The same relative differences between isoforms were seen in all three data sets. Figure 3 displays a data set typical of those from which the kinetic constants were derived: All three isoforms showed normal, hyperbolic kinetics with respect to all substrates.

The three isoforms displayed small differences with respect to several kinetic constants, but two differences were particularly striking. First, there was a 4-fold overall difference in $^{\text{UDP-Glc}}K_{\text{m}}$ between the isoforms. Second, Sus1 was significantly inhibited in the synthetic direction by high concentrations of the substrate Fru (Fig. 4). At inhibitory Fru concentrations, the degree of inhibition increased with increasing UDP-Glc concentration (Fig. 4B). These data are indicative of inhibition resulting from the formation of a dead-end Enzyme.Fru.UDP complex. This inhibition is described by the kinetic constant $^{\text{Fru}}K_{\text{si}}$ (Table I). The other two isoforms were not markedly inhibited in the range of Fru concentrations we used.

The Effect of the *rug4* Mutations on Sus Transcript and Protein

To discover the role of Sus1 in the pea plant, we characterized the *rug4* mutant alleles and examined their effects on Sus transcript and protein levels. s*us1* cDNAs were isolated from each of the *rug4* mutant lines (*rug4-a*, *rug4-b*, and *rug4-c*) by reverse transcriptase-PCR, subcloned, and sequenced. As reported previously (Craig et al., 1999), the *rug4-b sus1* cDNA contained a single point mutation resulting in the conversion of Arg 578 to Lys. In *rug4-a*, a single point mutation resulted in the conversion of Leu 164 to Phe. For *rug4-c*, sequencing of several clones

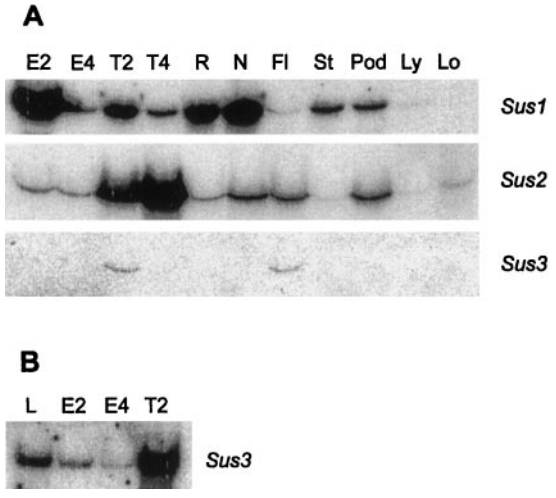

Figure 2. RNA gel-blot analysis of RNA from different organs of pea. A, Blots of total RNA, probed with *Sus1*, *Sus2*, and *Sus3* as indicated. Each lane contains 25 μg total RNA. B, Blot of poly(A^+) RNA, probed with *Sus3*. Each lane contains 3 μg poly(A^+) RNA. E2 and E4, Embryos from seeds of approximately 200 and 400 mg fresh weight, respectively. T2 and T4, Testas from seeds of approximately 200 and 400 mg fresh weight, respectively. R, Root. N, *R. leguminosarum*-infected root nodule. Fl, Flower. St, Top two nodes of stem from mature plants. Ly, Young leaf (not yet fully expanded). Lo, Older leaf (fully expanded).

showed that bases encoding either four or 39 amino acids were deleted. This suggested a problem with splicing of an intron. Genomic DNA therefore was isolated from wild-type and *rug4-c* plants and sequenced around the deleted area. A single base mutation was detected at the intron donor site, predicted to prevent correct intron recognition. The deletions observed in the cDNA indicate that two alternative splice sites are used. Both give rise to frame shifts and predicted truncated protein products.

Hybridization experiments performed on blots of total RNA revealed that *Sus1* transcripts were present in *rug4-a*, *rug4-b*, and *rug4-c* plants, at higher levels than in wild-type plants (not shown). This demonstrated that none of the lines carries an RNA null allele. To examine levels of Sus protein, blots of SDS-polyacrylamide gels of embryo extracts were probed with an antiserum that recognizes all three isoforms of Sus (see "Materials and Methods"). Because Sus is reported to be present in both soluble and membrane-associated forms, crude extracts were subjected to centrifugation at 100,000*g* and both the supernatant and the pellet were used for immunoblotting. Levels of Sus protein were comparable in wild-type, *rug4-a*, and *rug4-b* embryos. In all three lines, the amount of Sus protein in the pellet fraction was very approximately one-fourth of that in the supernatant fraction on a fresh weight basis (Fig. 5; supernatant lanes contain four times as much fresh weight of tissue as pellet lanes). In extracts of *rug4-c* embryos, levels of Sus protein in both supernatant and pellet fractions were drastically reduced (Fig. 5). Taken as a whole, these data suggest that Sus1 protein is expressed in normal amounts in *rug4-a* and *rug4-b* mutants, but is not present in *rug4-c* mutants. Remaining Sus protein and activity in *rug4-c* embryos are likely to be either Sus2 or Sus3, and not Sus1.

The Effect of the *rug4* Mutations on Cellulose Content in the Embryo

To assess whether loss of Sus1 affects cellulose synthesis in the embryo, mature wild-type and *rug4-c* embryos were assayed for cellulose. Tissue was digested with an acid treatment that hydrolyses polysaccharides other than cellulose. The residue was washed, and cellulose content determined by a phenol-sulfuric acid method against a standard curve of pure cellulose.

The cellulose contents of mature wild-type and mutant embryos were the same (1.47% ± 0.04% and

Table I. *Kinetic constants of recombinant Sus isoforms determined from initial-rate kinetic analysis*

Values are best fit estimates ± SE from 128 assays, except for those for Sus2 and Sus3 in the direction of sucrose cleavage that are each derived from 256 assays. Using Student's unpaired *t* test, the following differences between isoforms are statistically significant at the 95% level ($^{UDP}K_i$) or the 99% level (other values). $^{UDP}K_i$: Sus2 versus Sus 3. $^{Sus}K_m$: Sus1 versus Sus2, and Sus 2 versus Sus3, and Sus3 versus Sus1. $^{UDP\text{-}Glc}K_m$: Sus1 versus Sus2. $^{Fru}K_m$: Sus1 versus Sus2 and Sus2 versus Sus3. For $^{Fru}K_{si}$, the value for Sus1 is significantly lower than 100 mM; therefore, differences between Sus1 and Sus2, and Sus1 and Sus3, are statistically significant.

Kinetic Parameter	Kinetic Constant of Isoform		
	Sus1	Sus2	Sus3
		mM	
$^{UDP}K_m$	0.082 ± 0.009	0.145 ± 0.012	0.114 ± 0.005
$^{UDP}K_i$	0.107 ± 0.024	0.175 ± 0.026	0.072 ± 0.007
$^{Suc}K_m$	32.1 ± 2.1	42.7 ± 2.2	67.5 ± 1.6
$^{UDP\text{-}Glc}K_m$	0.209 ± 0.027	0.058 ± 0.006	0.090 ± 0.008
$^{UDP\text{-}Glc}K_i$	0.041 ± 0.018	0.059 ± 0.014	0.126 ± 0.031
$^{Fru}K_m$	10.4 ± 1.2	20.9 ± 1.6	9.1 ± 0.9
$^{Fru}K_{si}$	42.1 ± 11.1	>100	>100

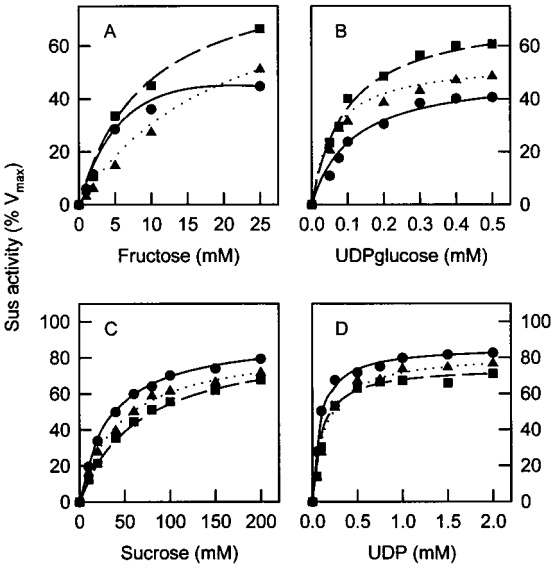

Figure 3. Effect of substrate concentration on Sus activity. Recombinant forms of the three isoforms of Sus purified from *E. coli* were assayed in the direction of Suc synthesis (A and B), or Suc cleavage (C and D). The concentration of the substrate was varied as indicated in the presence of near-saturating concentrations of the second substrate. A, 1 mM UDP-Glc; B, 25 mM Fru; C, 1 mM UDP; D, 200 mM Suc. Sus1, circles; Sus2, triangles; Sus3, squares. All values are expressed relative to V_{max} determined from initial rate kinetic analysis following systematic variation of the concentration of both substrates. The lines are theoretical curves for enzyme activity described by the kinetic constants presented in Table I for: Sus1, solid line; Sus2, dotted line; and Sus3, dashed line.

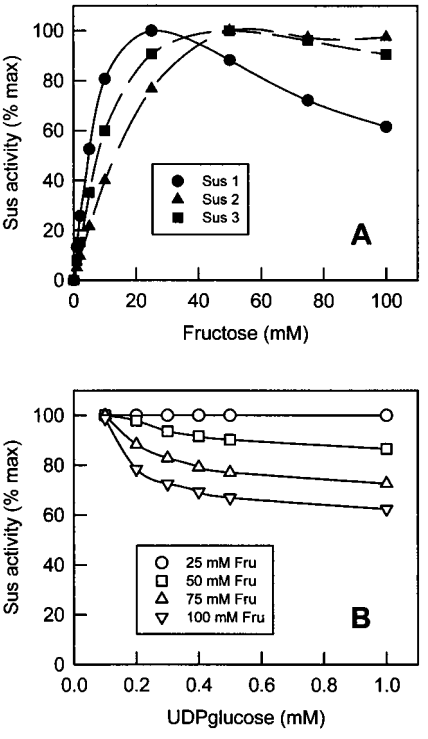

Figure 4. Effect of Fru on Sus activity. A, The activities of Sus1 (circles), Sus2 (triangles), and Sus3 (squares) were measured in the direction of Suc synthesis in the presence of 1 mM UDP-Glc. Fru was varied as shown. B, Sus1 activity was measured in the presence of 25 (circles), 50 (squares), 75 (triangles), or 100 (inverted triangles) mM Fru. UDP-Glc was varied as shown. All values are expressed relative to the maximum activity measured at the concentration of UDP-Glc used in the assay.

1.40% ± 0.03% of the dry weight, respectively; means ± SE of measurements on six randomly selected seeds from a large harvest).

DISCUSSION

The three isoforms of Sus from pea for which genes have been cloned lie on two different branches of the phylogenetic tree. All of the dicotyledonous Sus for which full-length sequences are available lie on one of these two branches, whereas all of the monocotyledonous Sus lie on a third branch. Two of the six Sus-like proteins encoded in the Arabidopsis genome constitute a separate group on the branch on which pea Sus2 is located (Arabidopsis Sus5 and Sus6; Fig. 1). Our results suggest that the pea genome contains at least five genes encoding Sus-like proteins, which opens the possibility that isoforms related to this group may also be present in pea. Nothing is known at present about the location and properties of either the Arabidopsis Sus5 and Sus6 isoforms or the products of the two or more uncharacterised *Sus* genes in pea.

The mutations at the *rug4* locus provide new information about residues of the enzyme essential for normal activity. A change from Arg to Lys at position 578 of Sus1 (the *rug4-b* mutation), and from Leu to Phe at position 164 (the *rug4-a* mutation) both reduce activity very significantly and, in the case of the latter change, alter kinetic properties. Activity in the embryo and nodule of the *rug4-a* mutant is very low in the cleavage direction but is 40% to 70% of that in wild-type peas in the synthetic direction (Craig, 1996), and recombinant Sus1 in which Leu 164 is changed to Phe shows a similar change (D.H.P. Barratt, unpublished data). The equivalent residue to that at position 578 is conserved among reported Suc synthases, including the enzyme from the cyanobacterium *Anabaena* sp. strain PCC7119, which shows

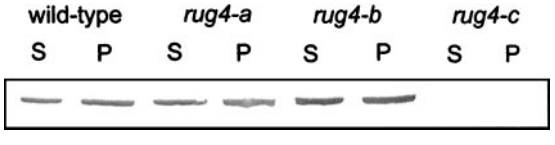

Figure 5. Immunoblots of pellet and supernatant fraction of extracts of embryos from wild-type, *rug4-a*, *rug4-b*, and *rug4*-c peas, probed with an antiserum which recognizes all three Sus isoforms. Pellet and supernatant fractions from embryos of seeds of 300 to 400 mg fresh weight were loaded onto SDS-polyacrylamide gels such that each lane contained the same amount of protein. For supernatant fractions, this amount was equivalent to approximately 1.25 mg fresh weight. For pellet fractions, this material was equivalent to approximately 5 mg fresh weight. The antiserum was used at a dilution of 1:25,000.

only 30% to 40% identity with higher plant Sus proteins (Curatti et al., 2000). The equivalent residue to that at position 164 is conserved in all reported Suc synthases except Arabidopsis Sus5 and Sus6. Neither Arg 578 nor Leu 164 is within putative hydrophobic domains or actin-binding domains, or at or immediately adjacent to phosphorylation sites, all of which have been proposed to influence the intracellular location of the enzyme (Nakai et al., 1999; Zhang et al., 1999; Winter and Huber, 2000).

The analysis of expression patterns of the three isoforms and the effects of the *rug4* mutations on Sus1 activity show that the contribution of each isoform to Sus activity differs between organs and through development. In the embryo, for example, Sus1 accounts for 95% of the activity (based on comparison of activity in wild-type and *rug4-c* mutant embryos; Craig et al., 1999), but both *Sus2* and *Sus3* genes are also expressed. In the testa, Sus1 accounts for 50% of the activity. The relative contribution of Sus2 and Sus3 to the remaining activity probably changes through testa development because *Sus3* transcript levels fall and *Sus2* transcript levels rise as the testa matures. The contribution of Sus1 to activity in leaves is low (Craig et al., 1999), and probably falls as leaves mature. Transcript is detectable in younger but not fully mature leaves. In contrast, both *Sus2* and *Sus3* genes are expressed in mature leaves.

The Sus isoforms differ in kinetic properties. The nature and magnitude of the differences we report do not mirror the degree of relatedness of the amino acid sequences of the isoforms in an obvious way, in that there were clear differences between Sus1 and Sus3 (87% identical in sequence) as well as between Sus2 and Sus1 and 3 (71% and 70% identical, respectively). Although not of great magnitude, the differences in kinetic properties are consistent with differentiation of physiological function between the isoforms. For example, the inhibition of Sus1 by Fru suggests that Sus1 activity is likely to be disproportionately restricted in tissues containing high concentrations of Fru. In addition, differences in $^{\text{UDP-Glc}}K_{\text{m}}$ between isoforms suggest that cells with different complements of Sus isoforms can maintain different steady-state concentrations of UDP-Glc under conditions when fluxes and concentrations of other metabolites are essentially the same. The kinetic data do not, however, allow detailed speculation about the roles of the isoforms in metabolism in vivo.

An important conclusion about the roles of Sus isoforms arises from study of the phenotype of the *rug4-c* mutant. The effects of this mutation indicate strongly that Sus1 in embryos is necessary for the provision of carbon to starch synthesis but not for the provision of carbon to cellulose synthesis. The mutation eliminates Sus1 protein from the embryo, and results in a 95% loss of Sus activity (Craig et al., 1999). Although the mutation reduces the starch content of the embryo by 30% (Wang and Hedley, 1993), it has no effect on cellulose content or on anatomical appearance of the developing embryo (not shown). These data are consistent with the idea derived from the maize *sh1* and *sus1* mutants (Chourey et al., 1998) that different isoforms of Sus may channel carbon from Suc towards different metabolic fates within the cell.

MATERIALS AND METHODS

Plant Material

Four near-isogenic lines of peas (*Pisum sativum*), carrying the wild-type and three mutant alleles at the r*ug4* locus (*rug4-a*, *rug4-b*, and *rug4-c*; Wang et al., 1990; Wang and Hedley, 1993; Craig et al., 1999) were used. Plants were grown in soil-based compost in a greenhouse at a minimum temperature of 15°C (day) and 10°C (night), fed weekly with a nitrogen-containing fertilizer, and provided with supplementary lighting (16 h d^{-1}) in winter.

Isolation of cDNA Clones

A λgt10 cDNA library prepared from wild-type pea leaves was screened with a 1-kb *Eco*RI/*Xho*I fragment of the Sus cDNA from *Lotus japonicus* (Skøt et al., 1996). After hybridization at 60°C, filters were washed twice for 30 min with 2× SSC and 5 g L^{-1} SDS (SSC is 0.15 M NaCl and 0.015 M Na citrate). Positive clones were either subcloned into pBluescript or amplified by PCR then subcloned into vector pCR2.1 (Invitrogen, Groningen, The Netherlands). Sequences were determined with a dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, Norwalk, CT) and an ABI sequencer (Applied Biosystems, Warrington, Cheshire, UK). Sequence data were analyzed using University of Wisconsin Genetics Computer Group computer programs (Devereux et al., 1984).

For the three mutant lines (*rug4-a*, *rug4-b*, and *rug4-c*) *sus1* cDNAs were isolated and sequenced as described for *rug4-b* by Craig et al. (1999). For each of the lines, three overlapping fragments were generated by PCR. The sequence of three clones containing each fragment was determined. In each case, the sequence of the three clones was identical.

Preparation of DNA and DNA Gel Blots

Genomic DNA was extracted from pea leaves, purified, and concentrated according to Ellis (1994). DNA digested with various restriction enzymes was separated on 8-g L^{-1} agarose gels and blotted onto Duralon-UV membranes (Stratagene, La Jolla, CA). The radioactive probe was a 1.37-kb *Hin*dIII fragment of the *Sus2* gene of pea. After hybridization at 55°C, blots were washed four times for 15 min at 55°C with 2× SSC and 5 g L^{-1} SDS.

Preparation of RNA and RNA Gel Blots

Plant material (1–5 g) was harvested and immediately frozen in liquid nitrogen, and total RNA was isolated ac-

cording to Edwards et al. (1995). RNA was analyzed by blotting as described by Martin et al. (1985) except that RNA was transferred to Duralon-UV membranes (Stratagene). Either 3 μg of poly(A^+) RNA [purified on oligo(dT)-cellulose columns; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK] or 25 μg of total RNA was loaded in each lane. Equal loading in each lane was verified by methylene blue staining of RNA (Wilkinson et al., 1991). A 1.5-kb *Eco*RI cDNA fragment was used as the *Sus1* probe, a 1.7-kb *Xho*I/*Nco*I cDNA fragment was used as the *Sus2* probe, and a 0.3-kb *Xho*I/*Eco*RI cDNA fragment was used as the *Sus3* probe. These probes were specific for each transcript.

Plasmid Construction

Full-length cDNAs for *Sus1* and *Sus3* were cloned in pCR2.1, and the full-length cDNA for *Sus2* was cloned in pBluescript. *Nco*I restriction sites and in-frame ATG initiation codons were introduced at the start of each clone using a Quikchange site-directed PCR mutagenesis kit (Stratagene). PCR mutagenesis was also used to remove an *Nco*I restriction site 220 bp downstream from the start codon in *Sus1* and a *Eco*RI restriction site 1,227 bp downstream from the start codon in *Sus2*. Both of these changes were achieved by changing the third base in the codon without altering the specified amino acid.

Plasmids for expression of mature, full-length Sus proteins were constructed via triple ligations. For Sus1, an *Nco*I/*Eco*RV 980-bp fragment and a *Eco*RV/*Bam*HI 1,652-bp fragment were inserted between the *Nco*I and *Bam*HI sites of the expression vector pET3d (Studier et al., 1990) to give the plasmid pETS1. For Sus2, an *Nco*I/*Eco*RI 1,104-bp fragment and an *Eco*RI/*Bam*HI 1,781-bp fragment were inserted between the *Nco*I and *Bam*HI sites of pET3d to give the plasmid pETS2. For Sus3, an *Nco*I/*Sal*I 290-bp fragment and an *Sal*I/*Xba*I 2,400-bp fragment were inserted between the *Nco*I and *Sal*I sites of pET3-d to give the plasmid pETS3.

Expression in *Escherichia coli*

pETS1, pETS2, and pETS3 were each transformed into the *E. coli* expression vector BL21(DE3; Studier and Moffat, 1986). Single transformed colonies were used to inoculate 10 mL of Luria broth containing 200 μg mL^{-1} ampicillin and incubated at 30°C overnight with shaking. One milliliter of the overnight culture was subcultured into 50 mL of the same medium containing 100 μg mL^{-1} ampicillin and grown for about 3 h at 30°C, until the A_{600} reached 0.6. Expression of the recombinant protein was induced by adding isopropyl β-D-thiogalactoside at a final concentration of 1 mM, then shaking at 350 rpm at 20°C for 20 h prior to harvest.

Purification of Recombinant Sus Protein

E. coli cells were harvested by centrifugation. Cells from 10 50-mL cultures were resuspended in 10 mL of 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.5, 1 mM EDTA, 50 mL L^{-1} glycerol, and 0.1 mM dithiothreitol (DTT), and incubated at 4°C for 30 min with 1 mg mL^{-1} lysozyme. Cells were then disrupted by two passages through a French pressure cell and centrifuged for 20 min at 30,000*g*. The supernatant was further centrifuged for 1 h at 40,000*g*. The final supernatant was brought to 65% saturation with ammonium sulfate. Precipitated proteins were collected by centrifugation, suspended in 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 50 mL L^{-1} glycerol, and 2 mM DTT, and centrifuged for 30 min at 40,000*g*. The supernatant was applied to a HiLoad Superdex 200 column attached to a FPLC (Amersham Pharmacia Biotech), equilibrated with the suspension buffer. Fractions of highest Sus activity (assayed as the cleavage reaction by an indirect method; Ross and Davies, 1992) were combined and applied to a MonoQ HR5/5 column (Amersham Pharmacia Biotech) equilibrated with the suspension buffer. Sus was eluted with a 50-mL linear gradient from 0 to 300 mM KCl in this buffer and 1-mL fractions were collected. The fraction of highest activity was used for kinetic analysis. The purity of the preparation was assessed with SDS-PAGE. Protein concentrations were measured using the Bio-Rad (Hemel Hempstead, Herts, UK) Protein Assay, with bovine serum albumin as the standard.

Preparation of Membrane Fractions

Embryos from seeds of 300 to 400 mg fresh weight were ground to a fine powder in liquid nitrogen and homogenized in 1 mL of extraction medium containing 50 mM HEPES-NaOH, pH 7.5, 2 mM EDTA, 1 mM EGTA, 5 mM ascorbic acid, 2 mM DTT, 10 g L^{-1} polyvinylpolypyrrolidone, and 10 mL L^{-1} phosphatase inhibitor cocktail I (Sigma, Poole, Dorset, UK). The homogenate was centrifuged for 10 min at 10,000*g* and the resultant supernatant separated into soluble and microsomal membrane fractions by centrifugation for 1 h at 100,000*g*. The microsomal membrane fraction was washed twice by resuspension in extraction medium, then centrifugation as above. The soluble and membrane fractions were analyzed by SDS-PAGE and immunoblotting.

PAGE and Immunoblotting

SDS-PAGE was performed according to Laemmli (1970) with a 10% (w/v) acrylamide resolving gel (7 cm long, 1 mm thick) and a 3% (w/v) acrylamide stacking gel (37.5:1 [w/w] acrylamide:bis-acrylamide) in a vertical electrophoresis cell. Proteins were visualized by staining with Brilliant Blue G. Native gels were prepared and run as for SDS gels except that SDS was omitted throughout and a 7% (w/v) acrylamide resolving gel was used. Gels were run at 4°C. Immunoblotting was performed as described by Barratt et al. (1989) using an overnight incubation of the filter at 4°C with a 1:25,000 dilution of rat antiserum against Sus3. Blots were developed with Fast BCIP/NBT (Sigma) following incubation with alkaline-phosphatase-conjugated rabbit anti-rat serum.

Preparation of Antisera

Recombinant Sus3 was overexpressed by induction with 1 mM isopropyl β-D-thiogalactoside for 2 h at 37°C with shaking at 350 rpm. This induction results in aggregation and accumulation of the recombinant protein in inclusion bodies. Following disruption by passage through a French pressure cell, the cell lysate was centrifuged and the pelleted fraction containing the inclusion bodies washed to remove adhering proteins as described by Nguyen et al. (1993). The washed inclusion bodies were solubilized with SDS solution and SDS-PAGE performed as described by Schagger et al. (1988). Strips containing Sus3 were excised from the gels and the proteins were electroeluted, dialyzed extensively against water, and freeze dried. Samples (100 μg) were dissolved in phosphate-buffered saline, 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. Dilutions of crude serum from the rat were used in immunoblot experiments. The specificity of the preimmune serum and the antiserum was checked by immunoblotting against purified recombinant Sus1, Sus2, and Sus3. The antiserum reacted with all three isoforms, and the pre-immune serum showed no reaction with any of the isoforms.

Kinetic Analyses

Continuous, coupled assays were performed at 30°C, monitored at 340 nm in a microtitre plate reader, and initiated by addition of purified enzyme. For both the Suc cleavage and the Suc synthesis reactions, the concentrations of components and the pH of the assay were optimized to give the maximum activity. The assay for the cleavage reaction contained, in 250 μL, 50 mM HEPES (pH 7.5), 1 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM NAD, 1.9 units hexokinase, 1.25 units Glc-6-P dehydrogenase (from *Leuconostoc mesenteroides*), 0.9 units P-Glc isomerase, various concentrations of Suc, and various concentrations of UDP. The synthetic reaction was assayed by a modification of the method of Huang et al. (1999). The assay contained, in 250 μL, 50 mM 3-[dimethyl(hydroxymethyl)-methylamino]-2-hydroxypropane sulfonic acid (AMPSO, pH 9.0), 20 mM KCl, 2 mM K_2HPO_4, 4 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.25 mM NADH, 5 units of pyruvate kinase, 5 units of lactate dehydrogenase, various concentrations of Fru, and various concentrations of UDP-Glc.

The kinetic constants were derived from initial rate analysis by systematically varying the concentration of one substrate at each of several different concentrations of the second substrate. In the direction of Suc cleavage, eight concentrations of UDP over the range 0.05 to 2 mM were used at each of eight concentrations of Suc over the range 10 to 200 mM. In the direction of Suc synthesis, eight concentrations of UDP-Glc over the range 0.05 to 1 mM were used at each of eight concentrations of Fru over the range 1 to 100 mM. All kinetic constants and corresponding asymptotic SEs were determined by nonlinear regression analysis of the untransformed data using the Marquardt-Levenberg algorithm (Marquardt, 1963). Data were fitted to appropriate kinetic equations describing a compulsorily ordered ternary complex reaction mechanism in which UDP and UDP-Glc bind first or are released last, depending on the direction of the reaction (Wolosiuk and Pontis, 1974; Doehlert, 1987) using SigmaPlot 2000 (SPSS, Chicago). At least 128 measurements were used in each determination. For each analysis, the multiple correlation coefficient was greater than 0.975. Kinetic constants are as defined by Cornish-Bowden (1995).

Kinetic constants for UDP and Suc were determined in the direction of Suc cleavage from the equation:

$$v = \frac{V_{max} \times [\text{UDP}] \times [\text{Suc}]}{{}^{\text{Suc}}K_m \times {}^{\text{UDP}}K_i + {}^{\text{Suc}}K_m \times [\text{UDP}] + {}^{\text{UDP}}K_m \times [\text{Suc}] + [\text{UDP}] \times [\text{Suc}]}$$

Kinetic constants for UDP-Glc and Fru were determined in the direction of Suc synthesis from the equation:

$$v = \frac{V_{max} \times [\text{UDP-Glc}] \times [\text{Fru}]}{{}^{\text{Fru}}K_m \times {}^{\text{UDP-Glc}}K_i + {}^{\text{Fru}}K_m \times [\text{UDP-Glc}] + {}^{\text{UDP-Glc}}K_m \times [\text{Fru}] + [\text{UDP-Glc}] \times [\text{Fru}] \times (1 + [\text{Fru}]/{}^{\text{Fru}}K_{si})}$$

Measurements of Cellulose Content

Measurements were made on mature embryos. Each sample was approximately 60 mg of material derived from a single embryo. Two such samples were taken per embryo. Tissue was finely homogenized, then boiled for 1.5 h in 10 mL of 5% (v/v) nitric acid and 15% (v/v) acetic acid. Insoluble material was washed three times with water and made up to 4 mL in water. Samples of 0.3 mL were mixed with 0.3 mL of 0.5% (w/v) aqueous phenol solution, then 3 mL of sulfuric acid. An optical density at 490 nm was measured after 1 h. Standards consisted of samples of 0.2 to 2.6 mg of pure cellulose, which were taken through the acid treatment in exactly the same way as the tissue samples prior to phenol-sulfuric acid assay.

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