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Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato

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Summary

We have isolated cDNA clones to two isoforms of granule-bound starch synthase (GBSS) from pea embryos and potato tubers. The sequences of both isoforms are related to that of glycogen synthase from *E. coli* and one, GBSSI, is very similar to the waxy protein of maize and other species. In pea, GBSSII carries a novel 203-amino-acid domain at its N-terminus. Genes encoding both proteins are expressed during pea embryo development, but GBSSII is most highly expressed earlier in development than GBSSI. Similarly, GBSSI and GBSSII are differentially expressed in developing potato tubers. Expression of both isoforms is much lower in other organs of pea than in embryos. GBSSII is expressed in every organ tested while GBSSI is not expressed in roots, stipules or flowers. The possible consequences of this differential use of GBSS isoforms are discussed.

Introduction

Starch is used in plants as a primary store of carbon skeletons for metabolism and biosynthesis. Many plants also use starch as a major long-term reserve in their storage organs. The control of this reserve is of central importance in the process of storage organ development and much of the regulation of starch quantity and quality occurs through starch biosynthesis.

The synthesis of starch occurs in chloroplasts in photosynthetic tissues and in amyloplasts in non-photosynthetic tissues. Within the plastid, ADP-glucose (ADPG) is produced from glucose-1-phosphate by ADPG pyrophosphorylase. Linear chains of α(1–4)-linked glucose residues are built by starch synthase by the addition of glucose from ADPG on to the non-reducing end of pre-existing glucose chains or other primers. Branches are made in the starch by starch branching enzyme, which cuts α(1–4)-linked chains and joins them with other chains by α(1–6) linkages (reviewed by Preiss, 1988). Most plant starches consist of a mixture of predominantly unbranched polymers (amylose) and branched polymers (amylopectin), at an approximate ratio of 1:3 by weight. However, there is enormous genetic and developmental variation in the precise ratio of these components. There are also differences in their molecular sizes and the degree, size and frequency of amylopectin branching. These differences lead to variation in the chemical and physical properties of starches from different sources.

Starch synthase (EC 2.4.1.11) extends starch molecules, and, *in vitro*, it acts on both amylose and amylopectin. Starch synthase activity is found both associated with the starch grain (granule-bound starch synthase) and in the stroma of the plastid (soluble starch synthase). The genetic relationship between these biochemically defined activities is not completely understood, but is best characterized in the endosperm of maize where mutant analysis has greatly facilitated the understanding of the role of starch synthase isoforms in starch biosynthesis. In the *waxy* mutations of maize (analogous mutants are also found in rice, *Amaranthus*, barley, potato and sorghum) (Echt and Schwartz, 1981; Hovenkamp-Hermelink *et al.*, 1987; Hseih, 1988; Konishi *et al.*, 1985; Sano, 1984; Tsai, 1974) there is a dramatic reduction in the amylose content of the starch grains and a concomitant loss of activity of granule-bound starch synthase (GBSS). The *waxy* starch grains do not contain a 59-kDa polypeptide present on *Waxy* grains, and antisera raised against this polypeptide have been used to isolate the cDNA and, in turn, the gene (Klösgen *et al.*, 1986; Shure *et al.*, 1983). Some *waxy* lines are phenotypically unstable because of the presence of genetically characterized transposable elements at the locus. Confirmation that the gene isolated is the *waxy* gene was obtained by the demonstration of insertions of DNA (the first described was a *Ds* transposable element) in *waxy* lines (Shure *et al.*, 1983). The derived amino acid sequence of the waxy protein resembles that of glycogen synthase from *E. coli* (an enzyme that catalyses the same

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reaction) (Kumar *et al.*, 1986). These data established the role of the waxy GBSS in amylose synthesis, and the existence of *waxy* mutants with similar phenotypes in other species confirms the general role of an abundant GBSS of about 60 kDa in amylose biosynthesis in storage organs. The overall amount of starch synthesized in *waxy* lines is the same as in *Waxy* lines (Shannon and Garwood, 1984), suggesting that the waxy GBSS is not required for amylopectin synthesis. The *waxy* mutation does not reduce amylose biosynthesis in parts of the maize plant other than the endosperm, pollen and embryo sac, suggesting that other GBSS isoforms might be active in other tissues (Echt and Schwartz, 1981). The soluble starch synthase (SSS) activity is thought to be the product of another gene(s) (Tsai, 1974).

Nelson *et al.* (1978) demonstrated that in *waxy* maize endosperm a GBSS enzyme could be detected which was most active in younger kernels, and which had a much lower K_m for ADPG than the GBSS detected in older *Waxy* kernels. This implied that even within the endosperm different isoforms of GBSS were active at different times during development. MacDonald and Preiss (1985) elaborated this analysis of maize starch synthases further, and, by releasing the proteins from the granules, described four isoforms of GBSS in addition to two of SSS.

We have demonstrated that the developing pea embryo, like the maize endosperm, has two distinct isoforms of GBSS (Smith, 1990). A protein corresponding to the maize waxy protein has been purified from pea starch granules. This has a similar molecular mass (59 kDa) to the waxy protein from other species; antibodies raised to it cross-react with the waxy protein of potato; and it is present in large amounts on the starch granule. However, starch synthase activity associated with this protein, when solubilized from the granule, has been difficult to demonstrate. In addition to this protein, a highly active form of GBSS, of 77 kDa, has been isolated from pea starch granules. This GBSS is immunologically distinct from the 59 kDa GBSS, and its activity is sufficient to account for the observed rate of starch synthesis in developing peas.

In this paper we describe the cloning of cDNAs encoding the 59 kDa (GBSSI) and 77 kDa (GBSSII) proteins from pea. The derived amino acid sequences of both proteins show homology to glycogen synthase from *E. coli* (Kumar *et al.*, 1986) indicating that they are isoforms of starch synthase. GBSSI is very similar to the waxy protein of maize and potato (Klösgen *et al.*, 1986; van der Leij *et al.*, 1991), while GBSSII would appear to be a novel type of granule-bound starch synthase. GBSSII is expressed earlier in seed development, while GBSSI is most highly expressed in more mature embryos. cDNA clones encoding homologous proteins to both GBSS isoforms were isolated from developing potato tubers, where differences in expression during development are also observed.

This complex pattern of expression of GBSS isoforms emphasizes that the character of plant starch is a function of changes occurring during development as well as the combined action of different biosynthetic enzymes.

Results

An expression library prepared from developing pea embryos in λgt11 produced two full-length cDNA clones of GBSSI and one of GBSSII after screening with antibodies. GBSSI and GBSSII clones had quite distinct DNA sequences, and cross-hybridized only very weakly at low stringency (3 × SSC, 55°C).

The derived amino acid sequences of GBSSI and GBSSII were used to search the EMBL database. GBSSI was very similar to the waxy protein of maize (Klösgen *et al.*, 1986) (73% similarity, 59% identity on GAP; gap weight 3.0, length weight 0.1: Devereux *et al.*, 1984) and glycogen synthase from *E. coli* (Kumar *et al.*, 1986) (55% similarity, 31% identity). GBSSII was less similar to the maize waxy protein (59% similarity, 31% identity), although the homology was significant. It was also homologous to glycogen synthase from *E. coli* (56% similarity, 33% identity) (Figure 1).

The N-termini of the mature proteins were determined by isolation from polyacrylamide gels of granule-bound proteins (Smith, 1990) and N-terminal sequencing. They were found to be GMXLFVGAEVGP for GBSSI and AVHKSFGADENGDGSE for GBSSII (where X was an amino acid that could not be identified). This confirmed that both cDNAs encoded their respective proteins and showed that there was a 75 amino acid pre-sequence on the GBSSI translation product and a 57 amino acid pre-sequence on GBSSII (Figure 2). Presumably both are cleaved upon targeting to the amyloplast. These sequences showed no significant homology to the maize waxy transit peptide (TP) or the TP of other chloroplast genes, although the pre-sequence of GBSSI conforms to the consensus for chloroplast transit peptides (cTP) suggested by Gavel and von Heijne (1990). It starts with MA and contains the sequence IVC ↓ G at the cleavage site (compared to the consensus ^{I}/V-X-^{A}/C ↓ A for cTPs). It also contains the basic residue K in the region −6 to −10, although R, which is found more commonly in this position, is not present. The high proportion of A and R, commented upon by Klösgen *et al.* (1986) in the waxy TP, is not present in the GBSSI pre-sequence.

The GBSSII pre-sequence does not conform particularly well to these conserved features. It starts MM and the sequence at the cleavage site is KQHVR ↓ A. R has been found at position −1 in the TP of other imported plastid proteins, including the PSI 10.8 kDa protein from barley (Okkels *et al.*, 1988) and nitrite reductase from spinach (Back *et al.*, 1988). V also occupies position −2 in a high

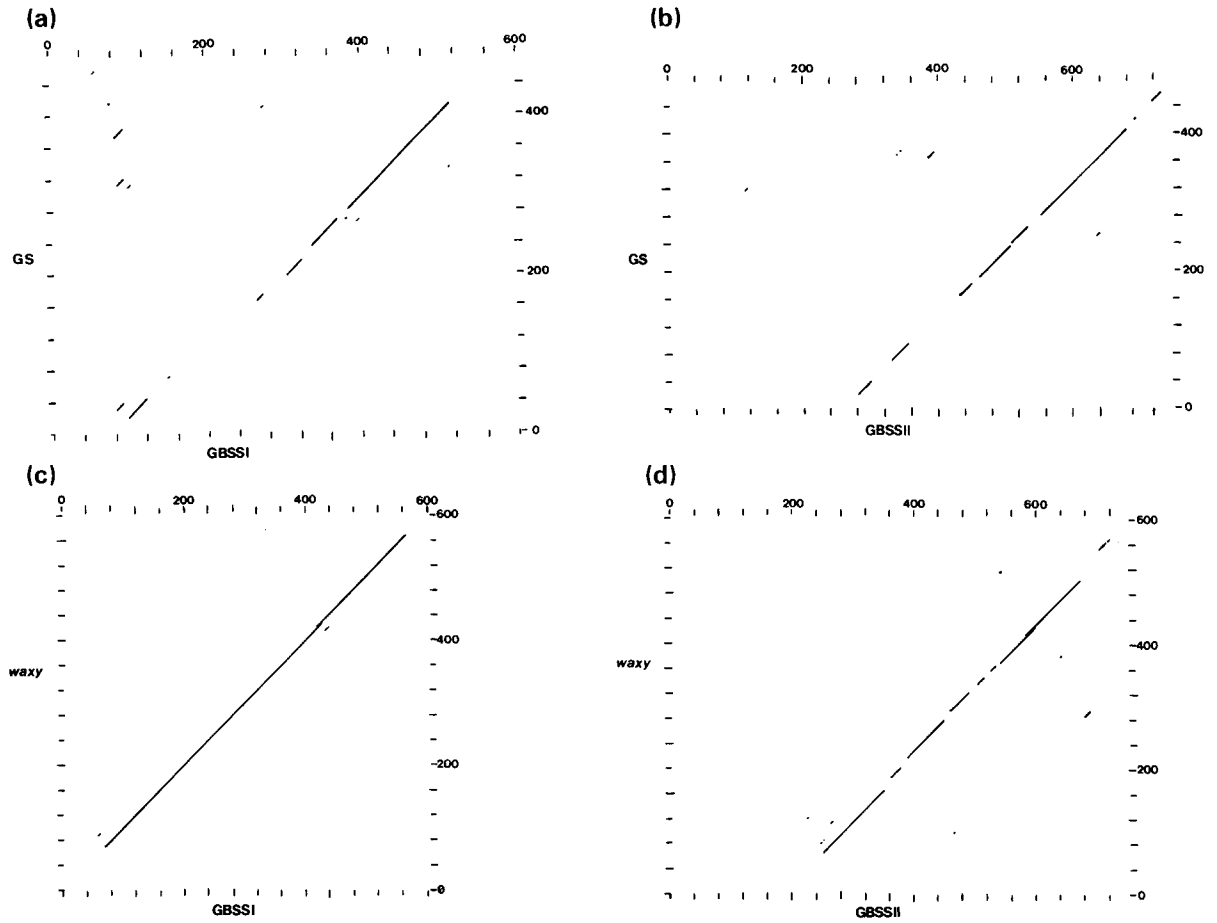

Figure 1. Comparison of derived amino acid sequences of (a) pea GBSSI to *E. coli* glycogen synthase (GS) (Kumar *et al.*, 1986); (b) pea GBSSII to *E. coli* glycogen synthase (GS); (c) pea GBSSI to the *waxy* gene of maize (Klösgen *et al.*, 1986); (d) GBSSII to the *waxy* gene of maize.
Data were produced using the University of Wisconsin sequencing programs COMPARE and DOTPLOT with a window of 40 amino acids and a stringency of 19 (Devereux *et al.*, 1984) and include the pre-sequences of GBSSI and GBSSII.

proportion of cTPs (Gavel and von Heijne, 1990). The GBSSII pre-sequence also contains R in the region −6 to −10.

Perhaps more significant is the resemblance between the cleavage site of GBSSII (RLNHKQHVR) and a sequence that lies within the GBSSI pre-sequence (RSLNKLHVR, Figure 2). Because the N-terminal sequencing of GBSSII demonstrates that this site can be cleaved, it is likely that the equivalent site within the GBSSI pre-sequence represents an alternative cleavage site used *in vivo*. This would imply that import of GBSSI either involves alternative cleavage sites or two sequential cleavages. The biological significance of these homologies and their roles in transport into amyloplasts and chloroplasts remain to be investigated fully.

The sequence of mature GBSSI (Figure 3) did not reveal any obvious reason why starch synthase activity had not been readily detected for this protein, when isolated (Smith, 1990). The motif containing the binding site for ADPG or ADP (KTGG) (Furukawa *et al.*, 1990), thought to be part of the active site of the enzyme, was present close to the N-terminus of the mature GBSSI protein. Several

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.MATITGSSMPTRTACFNYQGRSAESKLNL.PQI.HFNNNQAFP.VLGL.......RSLN.KLHVRTARATSGSSDTSEKSLGKIVC   GBSSI
 |.:||.:|  .:::::   .|.:|.:  :|.:||.|||:        | || | |||
MMLSL.GSD.AT...VLPFHAK...NLKFTPKLSTLNGDLAFSKGLGVGRLNCGSVR.LNHKQHVR                        GBSSII
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Figure 2. Comparison of amino acid pre-sequences of GBSSI and GBSSII.
A line indicates identical amino acids, a colon similar amino acids, and a point related amino acids as calculated by GAP (Devereux *et al.*, 1984). The cleavage sites are indicated by arrows. The pre-sequences were determined by comparison of the amino acid sequences derived from the cDNA nucleic acid sequences and the N-terminal sequence of the mature proteins.

Figure 3. Comparison of the derived amino acid sequences of the mature forms of GBSSI and GBSSII from pea aligned by GAP (gap weight 3.0, length weight 0.1) (Devereux *et al.*, 1984).
The motif KTGG, thought to interact with the polyphosphate of ADPG or ADP, is boxed. *Represents a site altered in *waxy* mutants of maize: *1, site of two and three amino acid insertions in revertants of *wx-ml* which reduce GBSS activity to 53% and 32%, respectively (Wessler *et al.*, 1986); *2, site of *Ac* insertion in *wx-m9* and *Ds* insertion in derivative lines. Additional amino acids here cause some reduction but not complete loss of GBSS activity (Wessler *et al.*, 1987); *3, site of waxy *B4* mutation; additional amino acids here cause complete loss of amylose GBSS activity (Wessler *et al.*, 1987). Annotations as in Figure 2.

amino acids differ between the maize waxy protein and pea GBSSI, but these differences are largely conservative changes. Comparison of the GBSSI sequence to the sequence of GBSS from maize and potato (Klösgen *et al.*, 1986; van der Leij *et al.*, 1991) revealed nine amino acids that were the same or functionally similar in maize and potato GBSS but different (by a non-conservative amino acid change) in pea GBSSI. Not enough is known of the functional domains of GBSS to predict if these differences would cause loss of activity. However, GBSSII or *E. coli* glycogen synthase, or both, have an amino acid similar to that in pea GBSSI in each of the nine positions. At the C-terminus, the GBSSI sequence is not very similar to that of the waxy protein from maize. However, it is extremely similar to the sequence from potato in this region, again suggesting that it does not contain a point mutation which renders it inactive. Interestingly, the other major difference between the GBSSI and maize waxy peptide sequences is amino acids at positions 422 and 423 in the maize protein (Klösgen *et al.*, 1986) absent at position 346 (*2 in Figure 3) in GBSSI. In maize this represents the site of *Ac/Ds* insertion in the *wx-m9* allele and its derivatives. Novel splicing of *Ds* from this site, which would allow for the introduction of new amino acids in the peptide, does not cause complete loss of GBSS activity or amylose production, again suggesting that this difference between the waxy sequence and the pea GBSSI sequence does not influence GBSSI activity (Wessler *et al.*, 1987). Significantly, other amino acids (*1 and *3 in Figure 3), known from mutational analysis to influence waxy GBSS activity and amylose production, are conserved in pea GBSSI (Wessler *et al.*, 1986, 1987). Overall, it seems more likely that the lack of activity for GBSSI from pea is the result of some sort of inactivation during isolation from the granule. Others have also reported difficulties in purification of active waxy protein from starch granules (Shure *et al.*, 1983).

Comparison of the sequences of GBSSI, GBSSII and glycogen synthase from *E. coli* revealed extensive similarity between all three proteins along the entire length of the GBSSI and glycogen synthase proteins and most of the GBSSII protein (Figures 1 and 3). In particular, there were highly conserved sequences around the N-terminal KTGG

motif identified as the ADPG/ADP binding site in glycogen synthase (Furukawa *et al.*, 1990). There was also a highly conserved domain SRFEPCGLXQLXXMXYGTXXXXXX-GGLXDT at the C-terminus of all three proteins, also noted as conserved between waxy-type GBSS sequences from maize, barley, potato and rice (van der Leij *et al.*, 1991). Interestingly, part of this domain (GGLXD) is similar to the domain around the conserved polyphosphate binding site (KTGGLXD), although it lacks the lysine residue that is thought to interact with the polyphosphate group of ADPG and ADP (Furukawa *et al.*, 1990).

At the N-terminus of GBSSII there is a domain of 203 amino acids which shows no sequence similarity to other sequences within the GBSSI or glycogen synthase proteins (Figures 1 and 3). This sequence is present in the mature protein and accounts for the larger size of GBSSII (77 kDa) compared to GBSSI (59 kDa). This unique domain was not significantly similar to any other sequences in the EMBL database.

The extra N-terminal domain is the most outstanding feature of the novel GBSSII isoform. Overall it is hydrophilic, carries a net positive charge and is rather serine-rich. It might represent a domain subject to phosphorylation and involved in regulating enzyme activity or it might influence the binding of the enzyme to the starch granule. The C-terminal end of this novel domain includes three consecutive proline residues (amino acids 195–197) which mark the end of the N-terminal extension as also observed in human glutathione reductase (Karplus and Schulz, 1987). Overall, the entire N-terminal domain is very flexible, as estimated by the method of Karplus and Schulz (1987).

Expression of GBSS isoforms within the pea plant

One possible functional explanation for the existence of multiple isoforms of particular enzymes is that they are active at different times during development or in different organs. Such differential expression of GBSS isoforms could have profound consequences on the quantity and quality of starch produced by the plant. Expression of the GBSS isoforms was examined in RNA isolated from pods, embryos, stipules, leaves, flowers and roots of pea. Pods, stipules and leaves are photosynthetic in pea, whereas the other organs are not. Organs of different ages were examined to determine if there were differences in expression according to whether the organs were active sources or sinks.

High levels of transcripts of both GBSSI and GBSSII were detected in developing embryos (Figure 4a and b). Some expression was also detected in pods, and GBSSII, but not GBSSI, was detected in roots (Figure 4c). It was extremely difficult to detect any expression in flowers, or stipules and leaves of any age (Figure 4b). A more sensitive assay for transcripts that involved amplification by polymerase chain reaction (PCR) indicated that there was expression of GBSSII in all organs (Figure 4d). Using this sensitive assay, GBSSI transcript was observed in pods and leaves, but was not detected in flowers or stipules (Figure 4b and d).

Thus, although the genes encoding the GBSS isoforms show similar patterns of expression in some plant organs, they are not always co-ordinately expressed. The low levels of expression of both genes in leaves was surprising because this is a site of photosynthetic starch biosynthesis. Similarly, no difference in levels of GBSS transcripts was detected between light- and dark-grown leaves. Proteins of the same size as GBSSI and GBSSII can be readily detected in leaves as well as in embryos using specific antisera (A. Smith, unpublished results). The detection of high levels of GBSS transcripts in embryos but not in leaves implies that there may be different turnover rates of GBSS in developing storage organs and in leaves. There may also be other isoforms of GBSS active in leaves: a small transcript homologous to GBSSII was observed in leaves and roots (Figure 4b). This could be a transcript encoding another GBSS isoform. No significant changes in expression of either GBSSI or GBSSII were detected with increasing age of these organs, indicating that large changes in expression are not associated with changing source/sink function.

Expression of GBSS isoforms during embryo development

The expression of the two starch synthase genes was examined during pea embryo development by extraction of poly(A)$^+$ RNA from embryos at six stages during development. Expression of the gene encoding GBSSII was highest in young embryos (100–200 mg fresh weight) and steady-state levels declined subsequently (Figure 5). In contrast, the expression of the GBSSI gene was relatively high in all stages of development, but increased about fourfold after the peas reached 300 mg fresh weight.

The peak of GBSSII expression occurred considerably before the highest levels of legumin mRNA. The later increase in GBSSI transcript levels occurred at the same time as the increase in legumin mRNA levels. Thus the expression of the genes encoding the two GBSS isoforms is not co-ordinate: the early expression of GBSSII is reciprocal to the expression of GBSSI. These differences in expression pattern suggest that the two GBSS genes may serve complementary functions in starch biosynthesis in developing embryos.

Isoforms of GBSS in potato

A full-length cDNA clone encoding GBSSI and several partial cDNA clones homologous to GBSSII were isolated

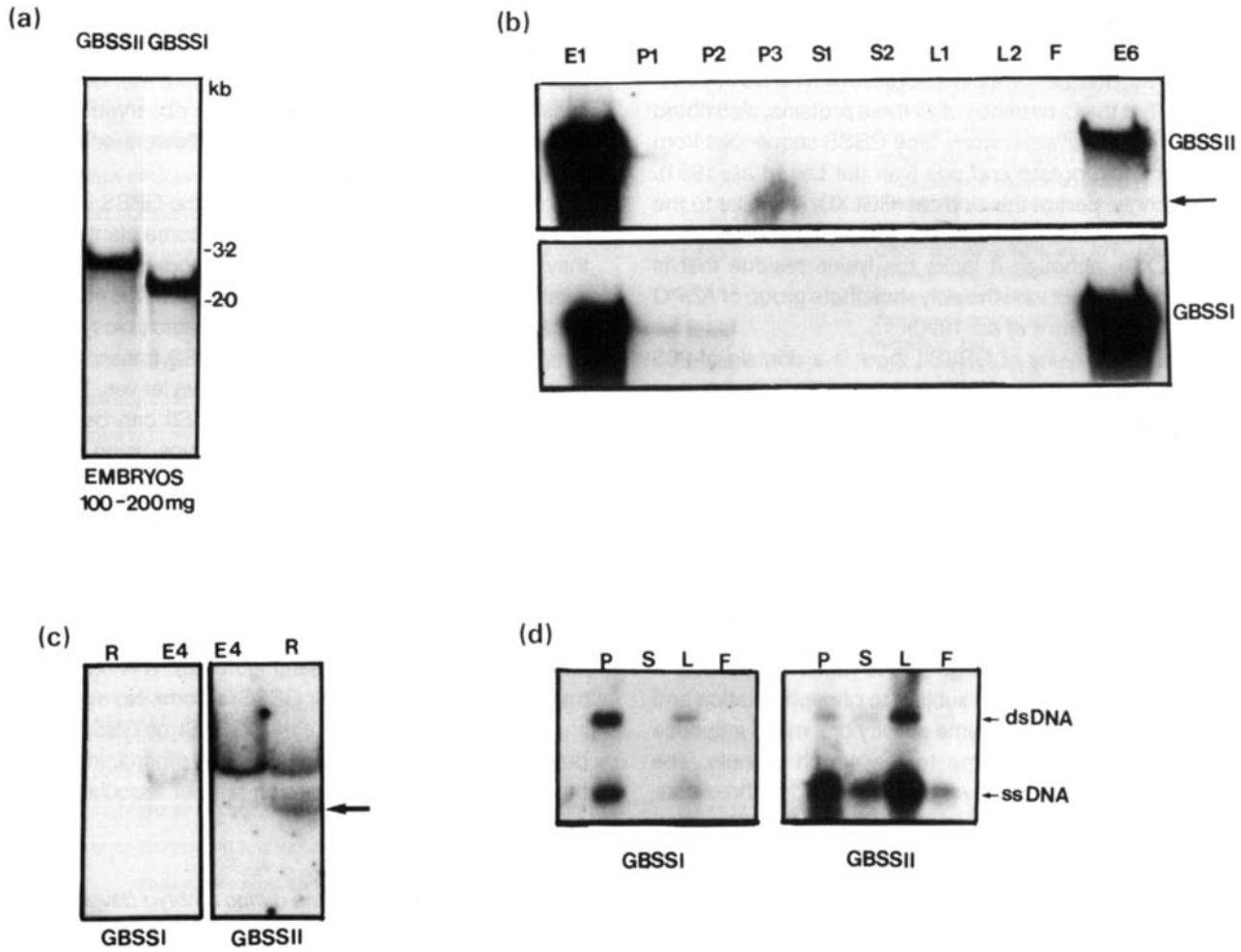

Figure 4. Expression of GBSSI and GBSSII in different organs of pea.
(a) Northern blot probed with GBSSII cDNA, then stripped and reprobed with GBSSI cDNA. Poly(A)$^+$ RNA from embryos of fresh weight 100–200 mg was used.
(b) Northern blots of poly(A)$^+$ RNA (5 μg) from young, 100 mg embryos (E1) and mature, 600 mg embryos (E6), pods (P1, young, containing 120 mg embryos; P2, mature, containing 600 mg embryos; P3, mature, senescent pods), stipules (S1, from first fully open leaf on 66-day-old plant; S2, from fourth oldest leaf), leaves (L1, from first fully open leaf; L2, fourth oldest leaf), and flowers probed with cDNA clones of GBSSI and GBSSII.
(c) Total RNA (25 μg) from roots (R) and 300–400 mg embryos (E4) was used for gels (arrows indicate novel band hybridizing to GBSSII in poly(A)$^+$ RNA from leaves and roots).
(d) Southern blots of cDNA from different organs amplified by PCR. cDNA was made to poly(A)$^+$ RNA from young pods (P), young stipules (S), young leaves (L) and flowers (F), and probed with GBSSI and GBSSII cDNA. Both double-stranded (ds) and single-stranded (ss) DNA bands were amplified as indicated, as a result of primer imbalance. Since high concentrations 5′ primers were used to give greater sensitivity to transcript amplification. The method was used to determine the presence or absence of GBSS transcripts but is, at best, only semi-quantitative (Jackson *et al.*, 1991).

from developing potato tubers. The nucleic acid sequence and derived amino acid sequence of the GBSSI clone was identical to that previously published for GBSS from potato (van der Leij *et al.*, 1991). The sequence for the longest clone homologous to GBSSII (1.9 kb) was not identical to the potato GBSS sequences previously published. The open reading frame (ORF) of this clone corresponded to amino acids 199–753 of pea GBSSII and contained a KTGG motif diagnostic of the ADPG/ADP-binding site. The similarity in the amino acid sequence of this ORF to GBSSII extended into the N-terminal 203 amino acids, unique to GBSSII (Figure 6). We therefore conclude that these cDNA clones encode part of the protein equivalent to GBSSII in potato. Such a protein has also been identified antigenically (Smith, 1990). These data support the view that mulitple isoforms of GBSS exist in species other than pea.

The expression of both GBSS isoforms was examined in developing potato tubers. The steady-state levels of GBSSII transcript were highest in very young tubers and declined in larger ones. In contrast, expression of GBSSI increased slightly with the size of the tubers up to about 24 g fresh weight and was correlated with expression of the storage protein, patatin (Figure 7). These data support the view that there is differential expression of GBSS isoforms in developing potato tubers as well as pea

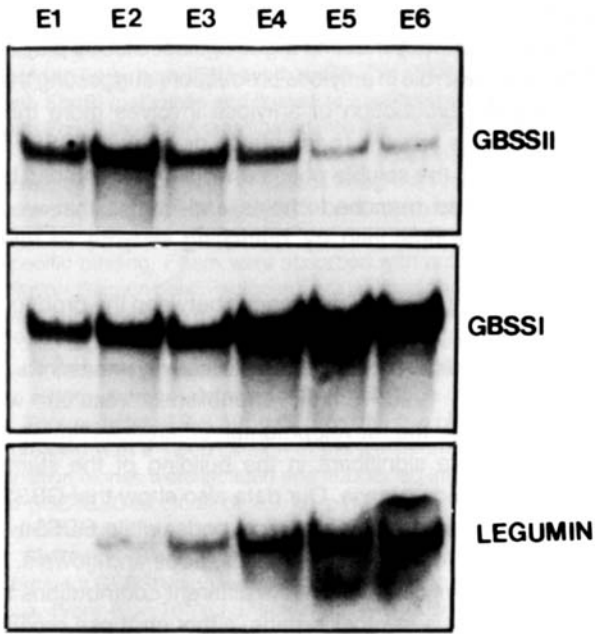

Figure 5. Expression of GBSSI and GBSSII in developing pea embryos. Northern blot of poly(A)$^+$ RNA (5 μg) extracted from embryos: E1, less than 100 mg fresh weight; E2, 100–200 mg; E3, 200–300 mg; E4, 300–400 mg: E5, 400–500 mg; E6, greater than 600 mg, probed with GBSSII, then stripped and probed with GBSSI, then stripped again and probed with legumin cDNA.

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POTGBSSI                    GMNLIPV GTEVGPWSKT GGLGDVLGGL PPVLAGNGHR
                            ||.|:|| |.|||||||| ||||||||||  ||||||||||
PEAGBSSI                    GMSLVFV GAEVGPWSKT GGLGDVLGGL PPVLAGNGHR

PEAGBSSII SGGEKPPPLA GTNVMNIILV SAECAPWSKT GGLGDVAGSL PKALARRGHR
          . :|||||||| |||||||||| ..|||||||| ||||||||.| ||||||||||
POTGBSSII NIDEKPPPLA GTNVMNIILV ASECAPWSKT GGLGDVAGAL PKALARRGHR
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Figure 6. Comparison of part of the deduced amino acid sequence from cDNA clones of GBSS isoforms from developing potato tubers.
The sequences contain the KTGG motif (boxed) thought to be involved in polyphosphate binding (Furukawa *et al.*, 1990). The sequence of GBSSI from potato is identical to that previously published. The sequence of GBSSII from potato includes amino acids in the N-terminal domain unique to GBSSI from pea. Annotation of sequence as in Figure 2.

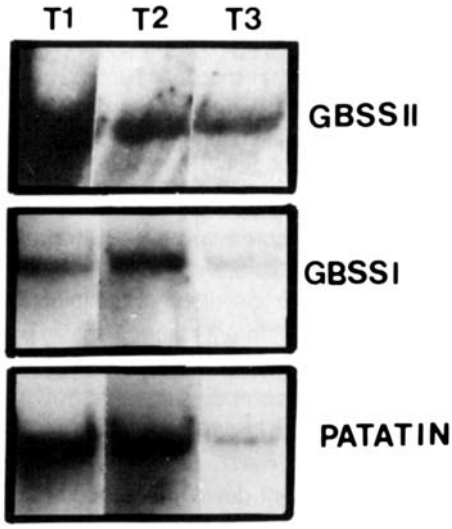

Figure 7. Expression of GBSSI and GBSSII in developing potato tubers. Northern blot of poly(A)$^+$ RNA (7 μg) from: T1, tubers less than 3 g; T2, tubers of 12–24 g; T3, tubers greater than 40 g, probed with a cDNA clone encoding GBSSII from potato, or one encoding GBSSI from potato, or one encoding patatin (pGM403).

embryos. These similar patterns of gene expression reflect the similar function of these organs rather than their developmental origins (which are very different). They therefore suggest that gene expression may be responding primarily to parameters associated with organ function rather than developmental identity.

Discussion

Isoforms of granule-bound starch synthases

We have demonstrated the existence of genes encoding two isoforms of granule-bound starch synthase in developing pea embryos. One isoform (GBSSI) is very similar to the waxy protein of maize and GBSS proteins isolated from other species. The other (GBSSII) is novel. Both proteins are considered to be granule-bound because they remain associated with granules after extensive washing but can be solubilized by amylase digestion of the granule (Smith, 1990). However, a protein of 77 kDa, antigenetically closely related to GBSSII, is also observed in the soluble fraction of amylopasts (Denyer and Smith, 1992). It is possible, therefore, that GBSSII may be bound to the granule and also active in the amyloplast stroma where it may represent a significant component of the soluble starch synthase activity.

Structurally, the relationship between GBSSI and GBSSII is very interesting. The major difference between the two proteins is an additional 203 amino acids at the N-terminus of the mature GBSSII compared to the mature GBSSI. This region is very hydrophilic and serine-rich. It could be involved in determining the partitioning of the GBSSII protein between soluble and granule-bound phases or it could represent a domain subject to phosphorylation through its high serine content.

Differential expression of GBSS genes

While GBSSI is expressed at all stages of embryo development, there is an increase in transcript levels later in development, coincident with the increase in storage protein transcript levels. GBSSII, on the other hand, is most highly expressed in younger embryos and transcript levels decline coincidently with the rise in GBSSI transcripts later in development. Thus, genes involved in starch biosynthesis are not all co-ordinately regulated and there are significant differences in gene expression during the development of the storage organ which may have profound effects on the quality of starch synthesized at any one time. Although starch biosynthesis increases relatively linearly during development (100–600 mg fresh weight)

(Smith, 1988), analysis of branching enzyme activity has shown an early rise in activity during pea seed development followed by a second later increase (Smith, 1988). From mutant analysis, these two phases of activity seem to result from differential expression of at least two isoforms of branching enzyme in pea (Bhattacharyya *et al.*, 1990). Thus, both branching enzyme and GBSS activities may consist of isoforms expressed early and others expressed later in development. We propose that the biological significance of this differential expression lies in the quality of the starch made early in development, compared to that made later. A developmental dimension to starch production may be important in the construction of the starch grain, as illustrated by the abnormal compound starch grains present in wrinkled (*rr*) peas which lack isoform I of starch branching enzyme, that is active early in embryo development (Bhattacharyya *et al.*, 1990; Smith, 1988).

Two similar GBSS isoforms were also found in potato and differences in expression of these genes were observed during tuber development. Multiple isoforms appear to be active at different stages in maize endosperm development. Nelson *et al.* (1978) demonstrated GBSS activity early in endosperm development in *waxy* kernels subsequently shown to produce no 59 kDa GBSS polypeptide (Echt and Schwartz, 1981). Therefore in storage organs of three different developmental origins (embryo, tuber and endosperm), a similar pattern of granule-bound starch synthase isoform production seems to operate, suggesting that these changes in gene expression are governed by parameters common to storage organ formation; there may be a functional significance to these changes relating to the formation of starch granules in storage organs.

Role of GBSS isoforms in amylose biosynthesis

The waxy protein of maize (and other species) is intimately involved in the synthesis of amylose, and there is little evidence that, *in vivo*, it is involved in extension of amylopectin chains. The finding that the amylose component of starch increases during development (Shannon and Garwood, 1984) is compatible with the idea that in a range of species this GBSS isoform (GBSSI) may be most highly expressed later in storage organ development. Because mutations in GBSSII, or in its cousins in other species, have yet to be found, it is not clear whether this isoform is involved in amylose biosynthesis to any extent.

The waxy GBSS of maize can extend amylopectin *in vitro* (Leloir *et al.*, 1961; MacDonald and Preiss, 1985) and so its predominant effect on amylose production is thought to result from its binding to the starch grain. Binding may allow extension of starch chains to form amylose, within the starch grain, which remain unbranched because of inaccessibility to branching enzyme. If so, there is no reason, *a priori*, why GBSSII should not also predominantly synthesize amylose when bound to the granule. However, the data from *waxy* mutants argue against GBSSII playing any significant role in amylose production, suggesting that the specific production of amylose involves more than binding of the enzyme to the starch granule. If GBSSII is also active in the soluble phase it would, presumably, be able to extend branched chains and chains that were subsequently branched by branching enzyme to form amylopectin.

Given these potential differences between the products of the two starch synthase isoforms, and the direct evidence of differences in specific activity measured *in vitro* (Smith, 1990; Smith, unpublished results), we conclude that the developmental differences in gene expression are significant in the building of the starch grains in storage organs. Our data also show that GBSSI is expressed in roots, leaves and pods, while GBSSII is expressed in roots, leaves, stipules, pods and flowers. It is therefore likely that they make different contributions to starch synthesis in other organs, although these organs may also contain other novel isoforms of GBSS, complicating the overall processes of starch biosynthesis in plants.

Experimental procedures

Plant material

Freshly harvested developing embryos (25–500 mg fresh weight) of a round-seeded line of *Pisum sativum* (BC1/9 RR) derived from J1430 (John Innes germplasm collection) by Hedley *et al.* (1986) were harvested from plants grown in a greenhouse at a minimum temperature of 12°C. For analysis of mRNA, flowers, leaves, stipules and pods were harvested from 66-day-old pea plants. Roots were obtained from seeds germinated for 4 days in the dark at 25°C, and the first 1 cm of root was harvested. mRNA was also extracted from leaves of 42-day-old plants grown in the light or placed in darkness for 24 h.

Potato microtubers var. Desirée were a generous gift from Dr Howard Davies, Scottish Crop Research Insititute, Invergowrie, Dundee. For mRNA analysis, developing tubers were harvested from Desirée plants grown at 15°C, 16 h light.

Antibodies to GBSSI and GBSSII

Antibodies were those described by Smith (1990).

Preparation of mRNA

Developing embryos or developing tubers were divided into similar weight classes. All plant material was frozen in liquid N_2. Polyadenylated RNA (poly(A)$^+$ RNA) was extracted according to Prescott and Martin (1987), Ward *et al.* (1989) and Martin *et al.* (1985). RNA for cDNA libraries was passed twice over the oligo (dT) cellulose column.

Construction and immunological screening of cDNA libraries in λgt11

cDNA was synthesized from a mixture of poly (A)$^+$ RNA from pea embryos of a range of sizes between 0 and 500 mg fresh weight.

cDNA from potatoes was synthesized from mRNA from microtubers. Double-stranded, blunt-ended cDNA was synthesized using an Amersham cDNA synthesis kit. The cDNA was methylated with *Eco*RI methylase and ligated to *Eco*RI linkers. After cutting with *Eco*RI the cDNA was ligated into the *Eco*RI site of λgt11.

Approximately 2.4×10^5 p.f.u. from the pea cDNA library were screened for expression of GBSSI using the Amersham Superscreen Immunoscreening System. Antiserum was preabsorbed with *E. coli* lysate (10 mg ml^{-1}) at 1:50 dilution to block non-specific binding. Filters were absorbed with antiserum at 1:250 dilution. Sixteen positive clones were isolated and the two largest were subcloned into the *Eco*RI site of pUC18. These clones were 2.0 kb long and contained poly(A) tracts at their 3′ ends. They were deduced to be full length by the presence of stop codons on all three frames upstream of the initial ATG.

Approximately 6.3×10^5 p.f.u. from the pea cDNA library were screened with a 1:500 dilution of the antiserum to GBSSII. Two positive clones were isolated and subcloned into the *Eco*RI site of pUC18. One clone of 2.9 kb proved to be full length and contained a poly(A) tract at the 3′ end.

Similar procedures were used to screen the potato cDNA library for GBSSI using the antiserum specific to this protein from pea. From 8×10^4 p.f.u. screened, two positive clones were isolated. No positive clones were isolated from the potato cDNA library using the antiserum to GBSSII. Instead the DNA in the plaques was probed with a 2 kb *Eco*RI fragment from the 5′ end of the GBSSII cDNA clone from pea. Following washing with 2 × SSC (0.3 M NaCl, 0.03 M Na-citrate pH 7.0) at 55°C, five positive clones were isolated. The longest was subcloned into the *Eco*RI site of pUC18.

Sequencing

Sequences were determined according to Sanger *et al.* (1977) using Sequenase (United States Biochemical Corporation), following subcloning into M13mp18 and M13mp19. Both strands were sequenced and the restriction sites used for cloning were confirmed by sequencing. GBSSI and GBSSII nucleic acid sequences have been submitted to the EMBL Database Library.

Northern blot analysis

RNA was analysed by Northern blots as described by Martin *et al.* (1985). Either 5 or 7 μg of poly(A)$^+$ RNA or 25 μg of total RNA was used per track.

PCR analysis of transcripts

Where transcript levels were very low, PCR was used to amplify cDNA to test for the presence or absence of a particular transcript. First strand cDNA was made to 2 μg poly(A)$^+$ RNA-primed with a dT(17)-adaptor (Frohman *et al.*, 1989). The PCR amplification used 24-mers as 5′ oligos from sequences covering the first ATG of the sequences of GBSSI and GBSSII cDNA clones. An adaptor sequence containing *Cla*I, *Sal*I and *Xho*I sites (homologous to the 3′ end of the dT(17) adaptor) was used as the 3′ primer. Following amplification of 0.01 volume of cDNA for 40 cycles as described by Frohman *et al.* (1989), the DNA was separated on 1.5% agarose gels and then blotted onto nitrocellulose filters as described by Southern (1975) and Wahl *et al.* (1979).

Transcripts on both Northern and Southern blots were identified by probing with cDNA fragments made radioactive by nick translation (Rigby *et al.*, 1977) and washing the filters at 0.1 × SSC, 65°C. PCR amplification was used to determine the presence or absence of particular transcripts but was, at best, semi-quantitative (Jackson *et al.*, 1991). Filters were also probed with cDNA clones of the pea storage protein, legumin, pRC943 (a gift from Rod Casey) and the potato storage protein, patatin, pGM203 (a gift from Mike Bevan).

Preparation of proteins from pea and potato starch

Pea or potato starch (2 g) was boiled for 5 min at a concentration of 75 mg ml^{-1} in 25 mM Tris-HCl pH 8.5, 2% (w/v) SDS. The boiled starch was centrifuged (10 000 ***g*** for 30 min) and the supernatant diluted five times with MilliQ water. The SDS concentration was reduced further by overnight dialysis at 4°C against 5 litres 5 mM Tris-HCl pH 8.5, 0.05% SDS using Spectra/Por® dialysis membrane (12 000 molecular weight cut-off). The samples were then concentrated using an Amicon ultrafiltration cell (150 ml, PM30 membrane) followed by Amicon centricon 30 microconcentrators to reduce the final volume of each to approximately 1 ml. Proteins were separated by SDS–PAGE (16 × 18 cm gels) using a 7.5% separating gel and 3% stacking gel, according to Laemmli (1970). Precautions to minimize N-terminal blocking during sample preparation and electrophoresis were as described by Matsudaira (1987). Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) in 10 mM 3-cyclohexylamino-1-propanesulphonic acid (CAPS), 10% methanol pH 11.0 for 1 h at 0.5 A. The membranes were washed for 5 min in MilliQ water and proteins visualized with 0.5% (w/v) Coomassie brilliant blue R (Serva) in 50% (v/v) methanol (Rathburns) and destained in 50% (v/v) methanol, 10% (v/v) acetic acid (BDH, AristaR). The relevant protein bands were excised, washed with water, air dried and stored at −20°C.

N-terminal sequencing

Sequential Edman degradation was performed on an Applied Biosystems (ABI) 475 pulse liquid protein sequencer. Phenylthiohydantoin amino acid derivatives were identified on a ABI 120 analyser. The excised PVDF bands were loaded on top of a polybrene-treated glass fibre disc and sequenced with the normal 'run' cycle following the manufacturer's instructions. N-terminal sequencing of proteins was performed by Dr Chris Sidebottom, Unilever Research, Colworth Laboratory.

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