The Plant Journal (1995) 7(1), 3–15

Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development

Rachel A. Burton[1], J. Derek Bewley[1,†], Alison M. Smith[1], Madan K. Bhattacharyya[1,‡], Helma Tatge[1], Steve Ring[2], Vicky Bull[2], William D.O. Hamilton[3] and Cathie Martin[1,*]
[1]*Department of Genetics and Applied Genetics, John Innes Institute, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK,*
[2]*Department of Food Molecular Biochemistry, Institute of Food Research, Colney Lane, Norwich NR4 7UA, UK, and*
[3]*Axis Genetics Ltd, Babraham, Cambridge CB2 4AZ, UK*

Summary

cDNA clones for two isoforms of starch branching enzyme (SBEI and SBEII) have been isolated from pea embryos and sequenced. The deduced amino acid sequences of pea SBEI and SBEII are closely related to starch branching enzymes of maize, rice, potato and cassava and a number of glycogen branching enzymes from yeast, mammals and several prokaryotic species. In comparison with SBEI, the deduced amino acid sequence of SBEII lacks a flexible domain at the N-terminus of the mature protein. This domain is also present in maize SBEII and rice SBEIII and resembles one previously reported for pea granule-bound starch synthase II (GBSSII). However, in each case it is missing from the other isoform of SBE from the same species. On the basis of this structural feature (which exists in some isoforms from both monocots and dicots) and other differences in sequence, SBEs from plants may be divided into two distinct enzyme families. There is strong evidence from our own and other work that the amylopectin products of the enzymes from these two families are qualitatively different. Pea SBEI and SBEII are differentially expressed during embryo development. SBEI is relatively highly expressed in young embryos whilst maximum expression of SBEII occurs in older embryos. The differential expression of isoforms which have distinct catalytic properties means that the contribution of each SBE isoform to starch biosynthesis changes during embryo development. Qualitative measurement of amylopectin from developing and maturing embryos confirms that the nature of amylopectin changes during pea embryo development and that this correlates with the differential expression of SBE isoforms.

Received 15 July 1994; revised 13 September 1994; accepted 15 September 1994.
*For correspondence (fax +44 603 56844).
†Permanent address: Department of Botany, University of Guelph, Guelph, Ontario N1G 2W1, Canada.
‡Present address: Samuel Roberts Nobel Foundation, Ardmore, OK 73402, USA.

Introduction

Starch is the primary form in which carbon is stored in plants, and it makes up 50% or more of the dry weight of many storage organs. Starch occurs as partly crystalline granules in plastids, and is composed of two types of glucan polymer, amylose and amylopectin. Amylose consists of predominantly linear chains of α-1,4-linked glucose residues and amylopectin consists of shorter α-1,4-linked chains connected by α-1,6 linkages. Many of the physical and chemical properties of starches are determined by the relative amounts, size, chain length and branch frequency of these two types of polymer. These parameters vary significantly between different plant species giving rise to an enormous variety of starch types (reviewed in Smith and Martin, 1993).

Amylose is synthesized in plastids from ADP-glucose by starch synthase, which adds glucose residues to the non-reducing ends of α-1,4-linked polymers. Amylopectin is synthesized from linear chains by starch branching enzyme which hydrolyses an α-1,4 linkage within a chain and joins the reducing end created to an α-1,4-linked chain by an α-1,6 linkage. Starch synthases can extend branched chains which may then be further branched by branching enzymes. Starch synthases and starch branching enzymes therefore act together and sequentially during the biosynthesis of amylopectin.

Multiple isoforms of starch branching enzyme have been described biochemically in developing storage organs of maize, rice and pea (Boyer and Preiss, 1978; Boyer and Fisher, 1981; Hodges *et al.*, 1969; Matters and Boyer, 1981; Nakamura *et al.*, 1992; Smith, 1988). Although biochemical analysis is generally not sufficient to distinguish definitively between isoforms encoded by different genes and isoforms derived by post-translational modification of a common peptide precursor, the biochemical data are persuasive that the products of at least two distinct branching enzyme genes are active in the storage organs of these species. Genetic data support this view. In pea, a gene at the *rugosus* (*r*) locus encodes one isoform of starch branching enzyme (SBEI) (Bhattacharyya *et al.*, 1990). Wrinkled (*rr*) peas carry a transposon-like insertion in the *r* locus which results in a complete absence of SBEI activity (Bhattach-

aryya *et al.*, 1990; Smith, 1988). Whereas branching enzyme activity shows an early rise during the development of round peas, it is almost absent early in the development of wrinkled peas, although it later rises (Smith, 1988). This shows that SBEI activity is significant early in development, while later the activity of the second isoform (SBEII), rises. In wrinkled peas total starch content is reduced by about 40% when compared with round peas and the amylopectin content is reduced from about 70% of total starch to about 30%. This emphasizes that absence of SBEI can limit the rate of synthesis of starch in developing pea embryos in a way that cannot be compensated for by SBEII. It also indicates that SBEI may be responsible for the synthesis of about 75% of the amylopectin found in the starch granules of mature embryos.

In wrinkled peas the morphology of the starch granules is also altered. Instead of the 'simple' starch granules observed in round peas, deeply fissured starch granules are formed, showing that the early contribution of SBEI to amylopectin production is an essential component in the normal process of granule formation for which the later activity of SBEII cannot substitute.

Branching enzyme isoforms SBEI and SBEII from pea also have distinct biochemical properties with respect to the products they synthesize (Smith, 1988). SBEI makes a less soluble polymer in conjunction with phosphorylase a than SBEII and is more active in phosphorylase stimulation relative to amylose-branching assays than SBEII. This indicates that SBEI has a lower affinity for amylose than SBEII (Smith, 1988). This same distinction has been reported for maize SBEII and SBEI, respectively, and it has been demonstrated that maize SBEII preferentially catalyses formation of shorter branches than maize SBEI (Takeda *et al.*, 1993).

In this paper we report the isolation of cDNA clones encoding the second isoform of starch branching enzyme (SBEII) active in developing pea embryos. Through comparison of the predicted amino acid sequence of SBEII with that predicted by the SBEI cDNA and with those of SBEs from other species we show that both cDNAs encode proteins homologous to other branching enzymes, but that the two proteins are distinctly different.

Comparisons with other branching enzyme sequences suggest that SBEI and SBEII are representatives of two discrete families of branching enzyme found in both monocots and dicots. There are greater similarities between members of each group from different species than between members from different groups from within the same species. These structural differences probably reflect differences in the physical and catalytic properties of the enzymes. This may determine, in part, the contribution of each type to amylopectin biosynthesis in storage organs. The genes encoding the two isoforms are also differentially expressed during embryo development and this can be correlated to qualitative differences in the amylopectin formed during embryo development. This emphasizes that developmental control of gene expression is an important factor determining the contribution of branching enzyme isoforms to starch biosynthesis in storage organs.

Results

Isolation and characterization of cDNA clones encoding SBEII from pea

An antibody raised against SBEII (Denyer *et al.*, 1993) was used to screen an expression library made in λgt11 from cDNA to mRNA isolated from relatively mature pea embryos (300–600 mg fresh weight, *RR* embryos). From a screen of 3×10^5 p.f.u., four positive clones were isolated, the largest of which contained a 1.4 kb insert. Sequencing showed that it contained an open reading frame encoding 355 amino acids which bore considerable similarity to starch and glycogen branching enzymes (for example, 68% similarity, 50% identity to SBEI from pea). A new cDNA library of 10^6 p.f.u. was prepared in λgt10 from mRNA isolated from 300–600 mg embryos. This library was screened with the 1.4 kb insert from the λgt11 library and three positive clones were identified. The largest of these contained a 2.9 kb cDNA insert. This insert was isolated, subcloned into pBluescript and M13mp18/M13mp19, named pRS4A and sequenced.

Isolation and characterization of cDNA clones encoding SBEI from pea

The isolation of cDNA clones encoding part of SBEI has been described (Bhattacharyya *et al.*, 1990). These partial cDNA clones were used to screen a cDNA library in λgt10 made from mRNA from embryos of less than 300 mg fresh weight. One clone containing a 3.55 kb insert was isolated, subcloned into pUC1813, named pJAM425 and sequenced.

Determination of N-terminal sequence of SBEI and SBEII

SBEI and SBEII were purified as described previously (Smith, 1988). Following SDS–polyacrylamide gel electro-

Figure 1. Comparison of amino acid sequences of SBEI and SBEII from pea.
(a) Comparison of amino acid sequences of SBEI and SBEII from pea deduced from the nucleotide sequences of their cDNA clones. Identical amino acids are indicated by a vertical line, whilst similar amino acids are denoted by a colon and related amino acids by a dot.
(b) Transit peptide sequence of SBEI and partial sequence of the transit peptide of SBEII. The first amino acid of the mature protein is underlined.
(c) Predicted flexibility of N-terminal domains of SBEI and SBEII from pea derived using the Chou–Fasman algorithm (PEPTIDESTRUCTURE, Devereux *et al.*, 1984). Ovals indicate residues giving potential flexibility to the domain.

(a) PEASBEI.PEP X PEASBEII.PEP

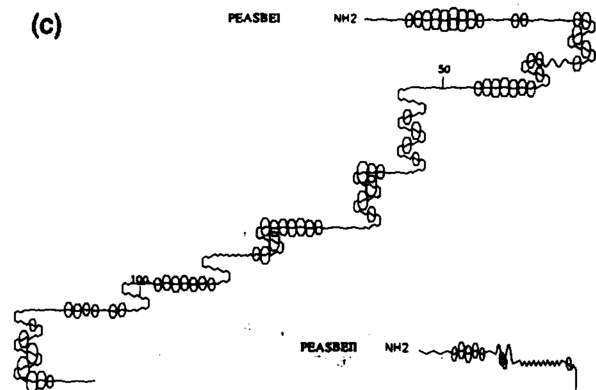
```
  1  AKFSRDSETKSSTIAESDKVLIPEDQDNSVSLADQLENPDITSEDAQNLE  50

     ..................................................

 51  DLTMKDGNKYNIDESTSSYREVGDEKGSVTSSSLVDVNTDTQAKKTSVHS  100

  1  ................................................VM  2

101  DKKVKVDKPKIIPPPGTGQKIYEIDPLLQAHRQHLDFRYGQYKRIREEID  150
     ... .. ..   .    ..  |.::|. |:: ::|:.:|. .| : :. |:
  3  TDDKSTMPSVEEDF..ENIGILNVDSSLEPFKDHFKYRLKRYLHQKKLIE  50

151  KYEGGLDAFSRGYEKFGFTRSATGITYREWAPGAKSAALVGDFNNWNPNA  200
     .|||||:.|.:||  ||||.|...||.||||||:|..|.::||||.:|...
 51  EYEGGLQEFAKGYLKFGFNREEDGISYREWAPAAQEAQIIGDFNGRNGSN  100

201  DVMTKDAFGVNEIFLPNNADGSPPIPHGSRVKIHMDTPSGIKD.SIPAWI  249
       |.||.|||  .| :|: |||.|:|||.||||:::.  ..|:.  .|||||
101  LHMEKDQFGVWSIQIPD.ADGNPAIPHNSRVKFRFKHSDGVWVDRIPAWI  149

250  KFSVQAPG..EIPYNGIYYDPPEEEKYVFKHPQPKRPQSIRIYESHIGMS  297
     |:..  .|.   . ||:|:|:||| .|:| ||||.|.:|.. ||||.|:|||
150  KYATVDPTRFAAPYDGVYWDPPLSERYQFKHPRPPKPKAPRIYEAHVGMS  199

298  SPEPKINTYANFRDDVLPRIKYLGYNAVQIMAIQEHSYYASFGYHVTN.F  346
     |.||:||.| :| |||||||:   .||.||:||: |||||||| ||||. |
200  SSEPRINSYREFADDVLPRIRENNYNTVQLMAVMEHSYYASFWYHVTKPF  249

347  FAASSRFGTPEELKSLIDRAHELGLLVLMDIVHSHSSNNTLDGLNMFD..  394
     ||.||| |.||:|| |||:||.||| ||||::|||.|||. |||| ||
250  FAVSSRSGSPEDLKYLIDKAHSLGLNVLMDVIHSHASNNVTDGLNGFDVG  299

395  .GTDGHYFHPGSRGYHWMWDSRLFNYGSWEVLRYLLSNARWWLDEYKFDG  443
      :.:. |||:|.||||.:||||||||:.|.  .:|||| ||||:||||||
300  QSSQQSYFHAGDRGYHKLWDSRLFNYANWKS.SFLLSNLRWWLEEYKFDG  348

444  FRFDGVTSMMYTHHGLQVSFTGNYSEYFGLATDVEAVVYMMLVNDLIHGL  493
     ||||||||||:| |||:.:.|||:|.|||: .|||:||||:||.|.|:|::
349  FRFDGVTSMLYHHHGINMAFTGDYNEYFSEETDVDAVVYLMLANSLVHDI  398

494  FPEAVSIGEDVSGMPTFCLPTQDGGIGFNYRLHMAVADKWIELLKK.QDE  542
     :|:|..|:|||||||.:. |. :.||||:||| ||::|||||:.||. .|.
399  LPDATDIAEDVSGMPGLGRPVSEVGIGFDYRLAMAIPDKWIDYLKNKKDS  448

543  DWRMGDIVHTLTNRRWLEKCVVYAESHDQALVGDKTLAFWLMDKDMYDFM  592
     :|.| :|  .|||||: |||| |||||||.:||||||:||:|||.:||. |
449  EWSMKEISLNLTNRRYTEKCVSYAESHDQSIVGDKTIAFLLMDEEMYSSM  498

593  ALDRPSTPLIDRGIALHKMIRLITMGLGGEGYLNFMGNEFGHPEWIDFPR  642
     .     .| |:|||.|||||::||::||||||||||||||||||||||||||
499  SCLTMLSPTIERGISLHKMIHFITLALGGEGYLNFMGNEFGHPEWIDFPR  548

643  GEQHLPNGKIVPGNNNSYDKCR.RRFDLGDADYLRYHGMQEFDRAMQHLE  691
     :              ||. ||:|||  .::|.|.:.|||. |..||||||. |:
549  E...........GNGWSYEKCRLTQWNLVDTNHLRYKFMNAFDRAMNLLD  587

692  ERYGFMTSEHQYISRKNEGDRVIIFERDNLVFVFNFHWTNSYSDYKVGCL  741
     ::::::.|..|.:|..|::|:||:|||::||||||||  .|.|.:||||||
588  DKFSILASTKQIVSSTNNEDKVIVFERGDLVFVFNFHPENTYEGYKVGCD  637

742  KPGKYKIVLDSDDTLFGGFNRLNHTAEYFTS........EGWYDDRPRSF  783
      ||||::.||||.| ||| .|:.|.|: |||          |. :::||.||
638  LPGKYRVALDSDATEFGGHGRVGHDADQFTSPEGIPGIPETNFNNRPNSF  687

784  LVYAPSRTAVVYALADGVESEPIELSDGVESEPIELSVGVESEPIELSVE  833
      |..|.:|.|||  .|: :.|. : .     :. |  .:.:.:   :.
688  KVLSPPHTCVVYYRVDERQEESNNPN...LGSVEETFAAADTDVARIPDV  734

834  EAESEPIERSVEEVESETTQQSVEVESETTQQSVEVESETTQ         875
     . |||. : .  | :||.. :.. :. | .  : :
735  SMESEDSNLDRIEDNSEDAVDAGILKVEREVVGDN                769
```

(b)

PEASBEI MVYTISGIRFPVLPSLHKSTLRCDRRASSHSFFLKNNSSSFSRTSLYA

PEASBEII ATTTTTTHNSKNKQYLAKQKPVELTLGYQNPNGCKVCSFGSKGSIY QKVSSGFKGVSV

(c)



phoresis, peptides (15 pmol of each sample) were blotted on to PTFE membrane and their N-terminal sequences were determined as described in Dry *et al.* (1992). The N-terminal sequence for SBEI was AKFS$^{R}/_{E}$$^{D}/_{T}$SETK$^{S}/_{I}$-ATIAESD and the N-terminal sequence for SBEII was VMTD$^{D}/_{E}$KS$^{T}/_{E}$MPSVEXD (where X indicates an uncharacterized amino acid and split residues represent indistinguishable alternatives). The deduced amino acid sequence from the cDNA clone of SBEI showed 100% identity to the N-terminal sequence of SBEI, 47 amino acids downstream from the initiating ATG, and the deduced amino acid sequence for SBEII showed 100% identity to its N-terminal sequence at a point 58 amino acids into the open reading frame (Figure 1a).

The sequence of the 47 amino acid transit peptide of SBEI conforms reasonably well to the sequences of other chloroplast transit peptides, having a high serine/threonine and lysine/arginine content, and only a single acidic residue. The sequence at the cleavage site RTSLY↓A also conforms to others reported and bears some similarities to the consensus proposed R$^{I}/_{V}$X$^{A}/_{C}$↓A (Gavel and von Heijne, 1990) (Figure 1b). The SBEII cDNA clone is not quite full length, in that it lacks an initiating ATG. Its transit peptide must consist of at least 57 amino acids. From the sequence available this transit peptide is also serine/threonine and lysine/arginine rich and contains very few acidic residues. The sequence at the cleavage site (KGVS↓V) also conforms to others reported (Gavel and von Heijne, 1990) (Figure 1b).

The open reading frame of the SBEI cDNA is 2.766 kb long, which, after removal of the transit peptide encodes a mature protein of 875 amino acids (predicted size 99.8 kDa). The open reading frame of the SBEII cDNA is 2.355 kb, which after removal of the transit peptide encodes a mature protein of 769 amino acids (predicted size 87.7 kDa). Both SBEI and SBEII proteins have predicted sizes significantly smaller than their estimated sizes on SDS–PAGE of approximately 112 kDa and 100 kDa, respectively (Smith, 1988). This could be a result of post-translational modification, but more likely reflects the fact that both proteins have high negative charges (–53 for SBEI and –41 for SBEII at pH 7.0 derived by the PEPSTATS program, Devereux *et al.* (1984)) which may cause them to run aberrantly on SDS–PAGE.

Structural comparison of SBEI and SBEII from pea

The deduced amino acid sequences of SBEI and SBEII from pea were aligned (Figure 1a). The most obvious difference between the two sequences was an additional section of 121 amino acids at the N-terminus of the mature SBEI protein which was not present in the SBEII protein. This extra domain is notable because of three adjacent prolines towards its C-terminus (amino acids 113–115 in the mature SBEI protein) and the large number of single or grouped serine residues within the domain. The sections of sequence from the start of the mature protein to the point at which homology between SBEI and SBEII starts (Ile 121 in SBEI and Ile 21 in SBEII) were analysed to determine their predicted flexibility according to the Chou–Fasman algorithm (PEPTIDEPLOT; Chou and Fasman, 1978; Devereux *et al.*, 1984). The N-terminal domain in SBEI was predicted to be very flexible (Figure 1c).

Four other differences between the predicted amino acid sequences of pea SBEI and SBEII are noteworthy; one is the 11 extra amino acids in SBEI (644–654) compared with SBEII, which lie in a region of high sequence conservation overall. A second is three extra amino acids (VGQ) in SBEII (298–300) compared with SBEI, which also lie in a well-conserved region. The third is a break in the homology between the two sequences between 590 and 599 for SBEI and 496 and 505 for SBEII in an otherwise highly conserved stretch of sequence. The fourth is eight extra amino acids in SBEII (669–676) not present in SBEI and lying in the most C-terminal region of homology between the two proteins.

SBEI and SBEII belong to different families of branching enzymes

The predicted amino acid sequences of pea SBEI and pea SBEII were compared with the available sequences of starch branching enzymes from plants and with glycogen branching enzymes from yeast, mammals and bacteria. Pea SBEI was most similar to maize SBEII (77% identity) and pea SBEII was 72% identical to SBEI from maize. (This is compared with 53% overall identity between pea SBEI and pea SBEII.) From a structural viewpoint, therefore, the branching enzyme isoforms fall into two distinct families, with members of each family being more similar to each other than they are to the other starch branching enzyme isoform from the same species despite the evolutionary distance between maize or rice and pea. This point is most clearly seen when the structural relationships between different enzymes are plotted as a dendrogram (Figure 2, PILEUP, Devereux *et al.*, 1984). We suggest that SBEI from pea, SBEIII from rice and SBEII from maize belong to one starch branching enzyme family (family A). The other family (family B) contains all but one of the other starch branching enzymes reported, including SBEII from pea and SBEI from maize. The glycogen branching enzymes from yeast and human are most closely related to family A starch branching enzymes. The bacterial branching enzymes form another distinct group with one extra member, a partial sequence of a branching enzyme from rice (SBEII) for which no protein or biochemical activity has yet been defined (Nakamura and Yamanouchi, 1992).

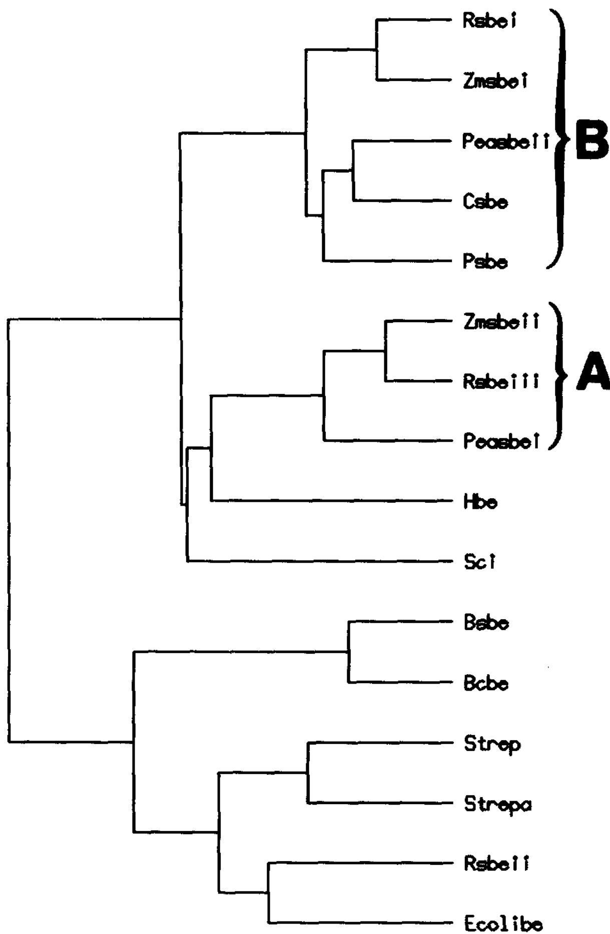


Figure 2. Dendrogram to illustrate the degree of relatedness between branching enzymes.
This figure was generated using the PILEUP program (Devereux *et al.*, 1984) The identity of the proteins is Rsbei, ii and iii, rice starch branching enzymes I, II and III, respectively (Mizuno *et al.*, 1993; Nakamura and Yamanouchi, 1992); Zmsbei and ii, maize starch branching enzymes I and II, respectively (Baba *et al.*, 1991; Fisher *et al.*, 1993); Peasbei and ii, pea starch branching enzymes I and II, respectively; Csbe, cassava starch branching enzyme (Salehuzzaman *et al.*, 1992). Psbe, potato starch branching enzyme (Poulsen and Kreiberg, 1993); Hbe, human glycogen branching enzyme (Thon *et al.*, 1993); Sci, *Saccharomyces cerevisiae* branching enzyme (Thon *et al.*, 1992); Bsbe, *Bacillus stearothermophilus* glycogen branching enzyme (Kiel *et al.*, 1991); Bcbe, *Bacillus caldolyticus* glycogen branching enzyme (Kiel *et al.*, 1992); Strep, *Streptomyces coelicolor* glycogen branching enzyme (Bruton, personal communication); Ecolibe, *Escherichia coli* glycogen branching enzyme (Baecker *et al.*, 1986). The distances along the branch lengths are proportional to the similarity between the sequences as calculated using an unweighted pair-group method based on arithmetic averages specified in the PILEUP program. The plant starch branching enzymes fall into two distinct families, A and B.

Numbers of branching enzyme genes in pea

The cross-hybridization between pea SBEI and pea SBEII cDNA clones was tested empirically (Figure 3a). Despite significant sequence similarity no cross-hybridization was found between the two cDNA clones at either high (0.5 × SSC, 65°C) or low stringency (2 × SSC, 55°C). The cDNA clones were therefore used to probe genomic DNA from pea to estimate the total number of branching enzyme genes from each family in this species.

At high stringency pea SBEI detected three bands in pea genomic DNA cut with *Bgl*II and two bands in DNA cut with *Sal*I and *Eco*RV as predicted from the sites in the cDNA sequence. In the *Sal*I + *Eco*RV digest, a polymorphism could be observed in the size of one of the bands between *r* and *R* lines due to the insertion of the small transposon-like element that causes the *r* mutation (Bhattacharyya *et al.*, 1990). At low stringency no additional hybridization was observed. These data suggested that a unique gene encodes the SBEI isoform in pea (Figure 3b). Similarly, pea SBEII only detected one band in *Sac*I digests and no additional bands upon washing at low stringency. The cDNA sequence predicted one fragment for *Sph*I and *Bgl*I digests and two for *Nco*I digests. The extra band in each case probably results from extra sites within introns in the genomic DNA. These data support the view that SBEI and SBEII isoforms are each encoded by a single gene; if there are any additional branching enzyme isoforms they must be encoded by radically divergent sequences.

Expression of branching enzyme isoforms in developing storage organs

The expression of SBEI and SBEII was compared during the development of pea embryos (Figure 4). In young embryos (<200 mg fresh weight) SBEI was relatively highly expressed and the steady-state levels of SBEI transcripts declined at embryo fresh weights above 200 mg. SBEII expression was very low in young embryos and maximum transcript levels of SBEII were attained in embryos of about 400–500 mg fresh weight. The transcripts of the two branching enzyme isoforms were therefore present at approximately reciprocal levels during development, SBEI being most abundant early in development and SBEII being most abundant later in development. SBEII transcript increased coordinately with the transcript for a legumin storage protein gene (pRC943). These data support the biochemical evidence that SBEI is significantly more active than SBEII early in pea embryo development, and that SBEII activity increases later in embryo development (Smith, 1988) and indicates that the basis for these developmental changes is changing gene expression.

Expression of SBEI and SBEII in other organs of pea

Expression of SBEI and SBEII in other organs of pea was tested firstly by Northern blots (Figure 4). The expression in leaves, stipules, pods and flowers was much lower than in embryos, despite the fact that leaves, at least, contain detectable amounts of protein of both isoforms (Tomlinson and Smith, unpublished results). Expression of the genes

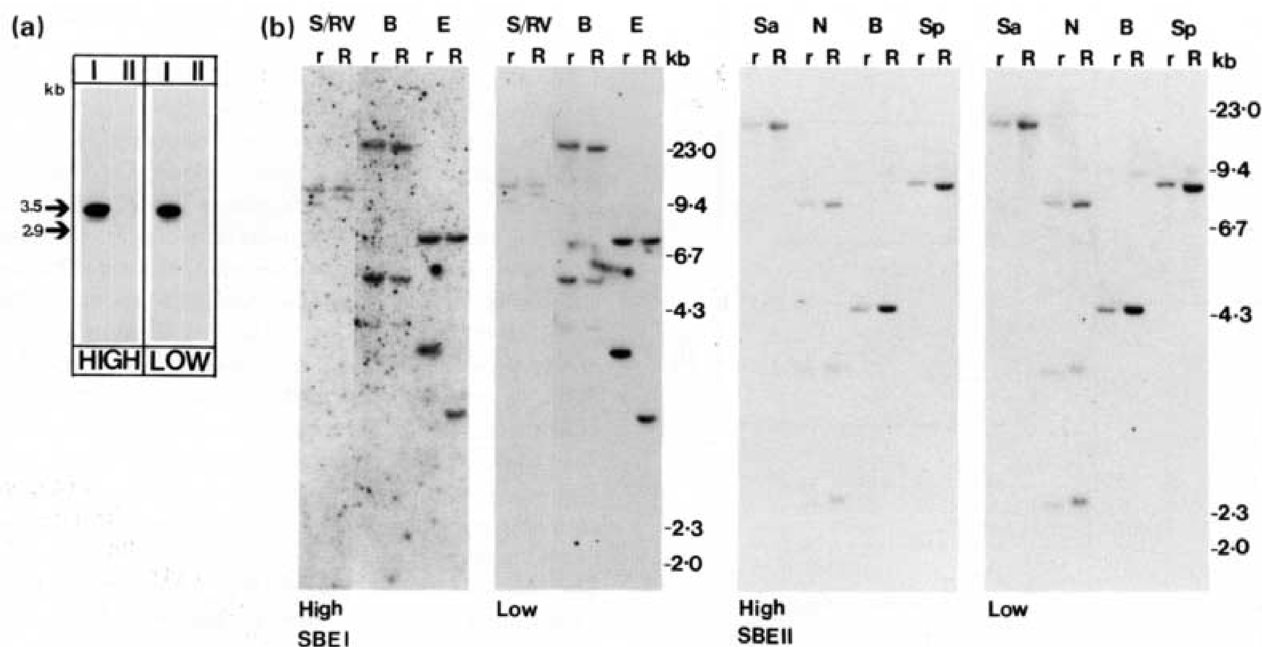


Figure 3. Estimation of the number of branching enzymes in pea.
(a) Southern blot of cDNA clones of SBEI and SBEII from pea to show the amount of cross-hybridization between the two pea SBE sequences. The blots were probed with the pea SBEI cDNA and washed at high (HIGH) (0.5 × SSC, 0.5% SDS, 65°C) and low (LOW) (2 × SSC, 0.5% SDS, 55°C) stringency. I, pea SBEI; II, pea SBEII; kb, kilobase.
(b) Southern blots showing 10 µg of genomic DNA from pea cut with *Sal*I+*Eco*RV (S/RV), *Bgl*II (B) and *Eco*RI (E) for SBEI, *Sac*I (Sa), *Nco*I (N), *Bgl*I (B) and *Sph*I (Sp) for SBEII and probed with cDNA clones of SBEI and SBEII from pea. Blots were washed at high (0.5 × SSC, 0.5% SDS, 65°C) and low (2 × SSC, 0.5% SDS, 55°C) stringency. Sizes are shown in kilobases. From the cDNA sequence *Sal*I and *Eco*RV would be predicted to cut the SBEI gene into two parts if there were no sites for these enzymes in introns, *Bgl*II into three fragments and *Eco*RI into three fragments. The *Sal*I/*Eco*RV and *Bgl*II digests indicate that SBEI is encoded by a single copy gene. The third band in the *Eco*RI digest is very small and ran off the gel. Following similar considerations *Sac*I, *Bgl*I and *Sph*I would be predicted to give single bands for SBEII and *Nco*I would give two bands. The *Sac*I digest shows a single band for SBEII arguing that the second band seen with *Bgl*I and *Sph*I digests and the third for *Nco*I are due to additional sites for these enzymes in the introns of the SBEII gene. Washing at lower stringency did not give any evidence for other closely related SBE genes in pea.

encoding both isoforms was therefore tested in a more sensitive manner by PCR amplification of cDNA made to mRNA from leaves grown in light or held for 48 h in darkness, stipules, pods, roots and flowers (Figure 5). Both SBEI and SBEII cDNAs were amplified from RNA in all these organs. Although there were differences in the amounts of cDNA amplified for SBEI and SBEII in different organs this cannot be related directly to differences in the activity of each isoform, since their turnover times vary greatly between different organs. However, we found no organ in which only one of the two isoforms was expressed.

Qualitative analysis of starch formed during embryo development

To discover the impact of the different temporal expression patterns of the SBE isoforms on starch structure during embryo development, we purified starch from young (150–250 mg fresh weight) and maturing (>400 mg fresh weight) embryos, isolated the amylose and amylopectin fractions by gel-filtration chromatography, and measured the average branch length in the amylopectin by assaying the extent of iodine binding. The iodine binding, and hence the wavelength of maximum absorbance of the complex, is dependent on the average branch length of the amylopectin (Banks and Greenwood, 1975). As branch length increases greater amounts of iodine are bound and the wavelength of maximum absorbance (λ max) increases.

There was a clear difference between the amylopectin fractions of young and mature embryos. The wavelengths of maximum absorbance of the iodine–amylopectin complexes for two independently prepared batches of starch from maturing embryos (when SBEII is most highly expressed) were 570 nm (same value obtained from two determinations) and 568 ± 1.7 nm (mean ± SE of three determinations). Values for two, independent batches of starch from young embryos when SBEI is most highly expressed were 540 ± 0.3 nm (mean ± SE of three determinations), and 545 nm (four determinations giving identical values). These results indicate strongly that the average branch length of amylopectin molecules increases through embryo development. From comparison with iodine binding to standards this change in λ max would involve a change of about twofold in average branch length (Banks and Greenwood, 1975).

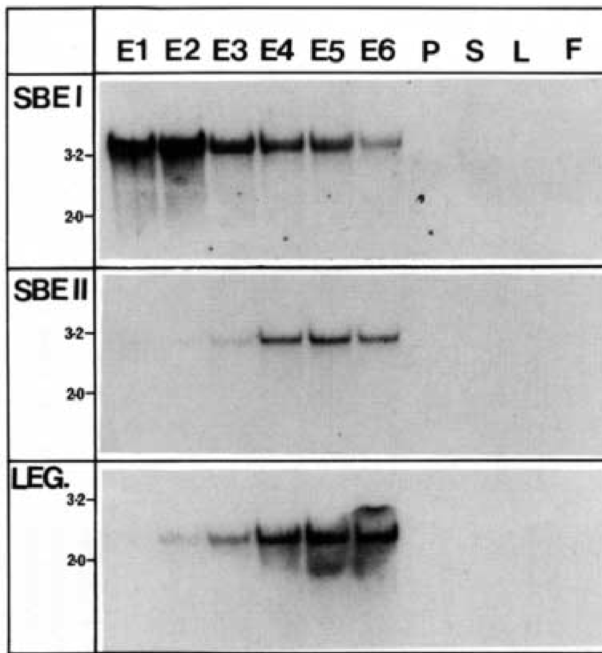


Figure 4. RNA gel blots of mRNA of SBEI, SBEII and legumin in developing pea embryos and in different organs.

Poly(A)$^+$ RNA was extracted from pea embryos at different stages of development; E1, 0–100 mg fresh weight (fwt); E2, 100–200 mg fwt; E3, 200–300 mg fwt; E4; 300–400 mg fwt; E5, 400–500 mg fwt; E6, greater than 600 mg fwt; and from P, pods; S, stipule; L, leaf; F, flower. Aliquots of poly(A)$^+$ RNA (7 μg) were run on gels, blotted and probed with pea SBEI, SBEII and legumin cDNA fragments. Sizes are indicated in kilobases.

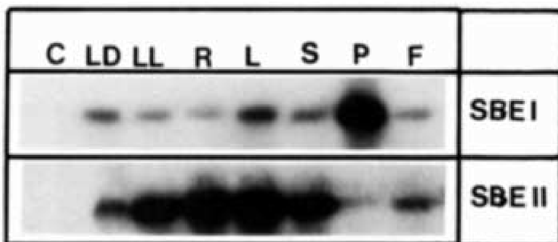


Figure 5. Expression of pea SBEI and SBEII in different organs of pea as detected by PCR.

First strand cDNA was made to mRNA extracted from: LD, leaves held in darkness for 48 hours; LL, comparable leaves grown in light; R, roots; L, leaves; S, stipules; P, pods; and F, flowers (Frohman *et al.*, 1989). C, control amplification of water instead of cDNA. cDNA was amplified using primers specific for SBEI and SBEII, and an adapter sequence for the 3' primer described by Frohman *et al.* (1989). cDNA was probed with fragments from SBEI and SBEII cDNA clones to confirm the identity of the amplified transcripts. Blots were washed at high stringency (0.1× SSC, 0.5% SDS, 65°C).

Discussion

We have cloned and sequenced cDNAs representing two genes encoding isoforms of SBE in pea. From previous evidence, we are confident that these two isoforms account for all of the SBE activity in the developing pea embryo (Bhattacharyya *et al.*, 1990; Smith, 1988). Our Southern blot analysis supports the view that there are no other closely related branching enzyme genes in pea. The derived amino acid sequences of the two isoforms are very similar structurally but differ primarily through the presence of an extra domain of high predicted flexibility at the N-terminus of SBEI.

This structural distinction extends to isoforms of branching enzymes from other species. In maize, although SBEII is no larger than maize SBEI, it also shows additional sequence at its N-terminus which includes three consecutive proline residues lying just before the start of the region of homology between the two isoforms (Figure 6). There is also an extra N-terminal domain in rice SBEIII (compared with rice SBEI) which includes two consecutive proline residues towards the carboxyl end. These extra N-terminal domains are not particularly serine rich in maize or rice but they are predicted to be flexible (PEPTIDEPLOT, Devereux *et al.*, 1984), and may therefore be equivalent to the additional N-terminal domain in pea SBEI.

The existence of a highly flexible N-terminal domain in members of one of the two families of SBE isoforms is strikingly reminiscent of the difference between the two isoforms of starch synthase in pea embryos (Dry *et al.*, 1992). The major 77 kDa isoform (GBSSII), which is both granule-bound and soluble (Denyer *et al.*, 1993; Smith, 1990), differs from the exclusively granule-bound GBSSI primarily in that it has an extra domain of high predicted flexibility at the N-terminus, which also has three consecutive proline residues at its C-terminal end. It seems unlikely that these domains are involved in the catalytic functions of starch synthase and SBE isoforms. They may be important in interactions between enzymes (for example, between starch synthases and SBEs) or in the interactions between the proteins and starch itself, or in determining the type of glucan chain the enzyme can use as a substrate. It is interesting in this context that SBEI of maize and SBEII of pea (without the domain) have higher affinities for amylose than SBEII of maize and SBEI of pea, respectively (Smith, 1988; Takeda *et al.*, 1993;), and starch synthases of the same class as GBSSI (without the domain) are thought to be involved exclusively in the synthesis of amylose.

Other sequence differences between SBEI and SBEII from pea gain significance when a number of branching enzyme sequences are aligned (Figure 6). The structure of branching enzymes has recently been related to that of α-amylases (Jespersen *et al.*, 1993). Most glycosylases belong to the structurally related superfamily of amylolytic proteins. Crystal structures of α-amylases reveal that these enzymes contain a catalytic $(\beta/\alpha)_8$ barrel domain comprised of eight regions of parallel β-strands that form a central cylinder. This acts as a scaffold for substrate binding and catalysis and is surrounded by eight parallel regions of α-helix (Buisson *et al.*, 1987; Klein *et al.*, 1992; Matsuura *et al.*, 1984). Sites for specific substrate binding and catalytic

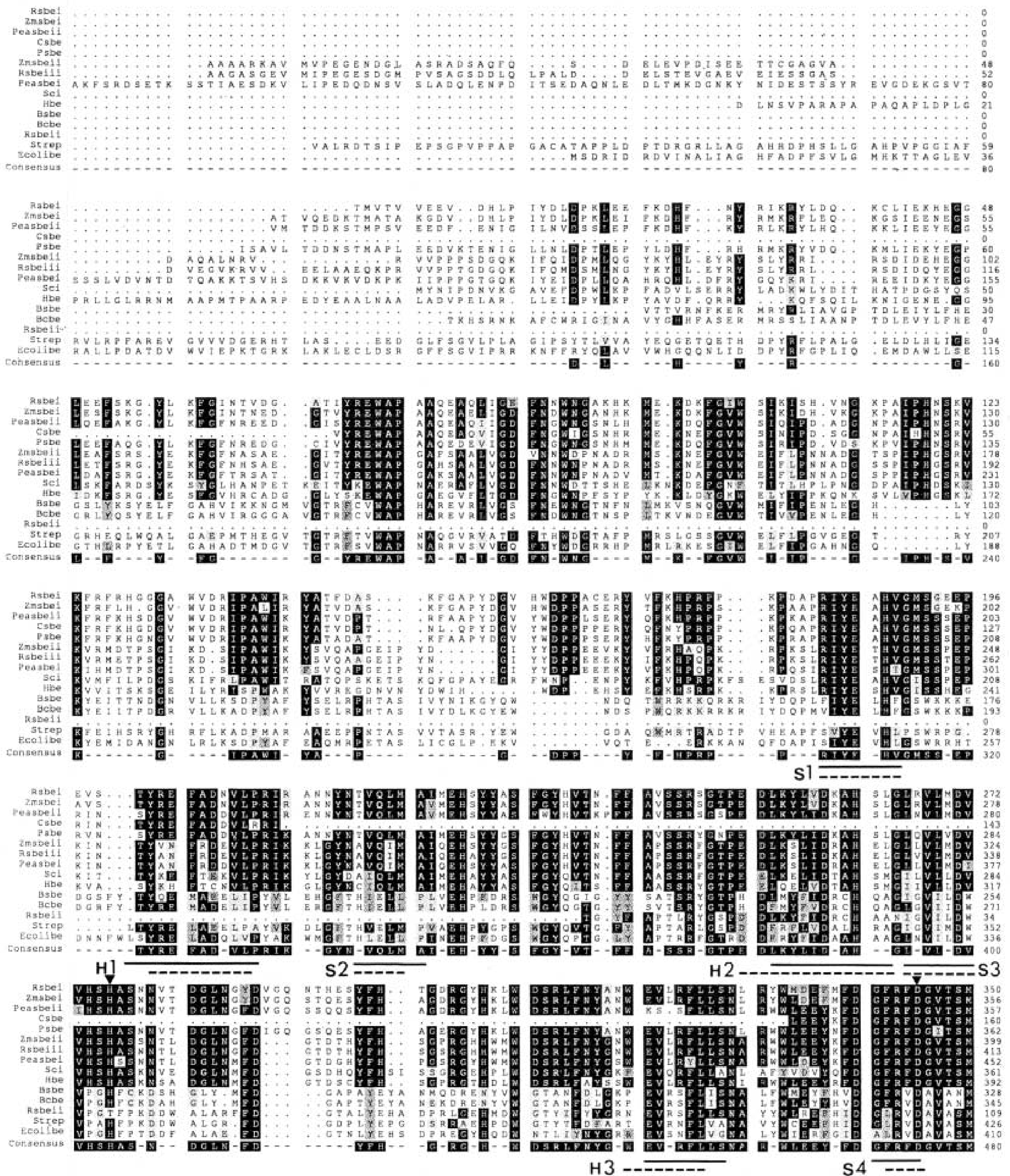
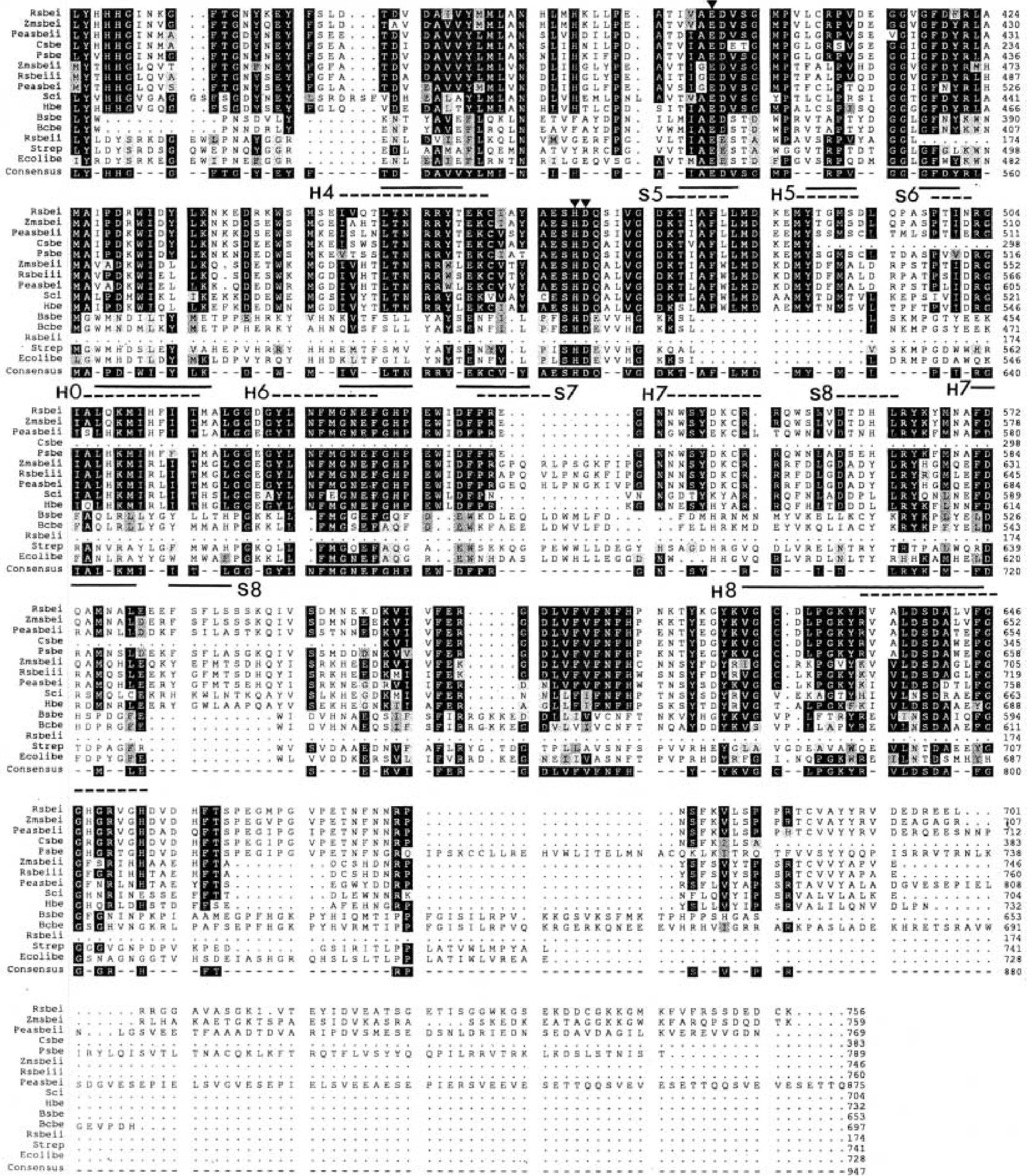


Figure 6. Alignments of amino acid sequences of starch branching enzymes and glycogen branching enzymes.
Fifteen branching enzymes are aligned using the PILEUP and PRETTYBOX programs (Devereux *et al.*, 1984) to produce a consensus sequence, shown on the bottom line. The consensus is calculated to a plurality of 7.9. Solid black boxes indicate identical amino acids, heavily hatched boxes indicate similar amino acids and lightly hatched boxes related amino acids. The identity of the sequences is given in the legend to Figure 2. The predicted positions of the β strands (S1–S8)



and α-helices (H1–H8) of the $(\beta/\alpha)_8$ barrel domain common to the amylopectic superfamily of proteins are underlined, with a solid line for the prokaryotic sequences and a broken line for the plant sequences (Jespersen *et al.*, 1993). HO is an additional region predicted to form an α-helix. The conserved residues of the active site are arrowed above their positions.

amino acid side-chains reside on the C-terminal ends of the β-strands or in the loops between the β-strands and α-helices. Structural predictions indicate that similar structures exist in other glucanases including those hydrolysing α-1,4 linkages such as cyclodextrin glucano-transferase and α-glucosidases and those catalysing formation and cleavage of α-1,6 linkages, such as branching enzymes and pullulanases. These enzymes therefore also belong to the α-amylase superfamily. The locations of the eight β-strand and α-helical regions have been predicted for *Escherichia coli* glycogen branching enzyme and maize SBEI (Jespersen *et al.*, 1993). Our alignment (Figure 6) shows the primary sequence of all branching enzymes to be conserved in those regions of predicted β-strand (labelled S1–S8 in Figure 6) and α-helix (labelled H1–H8 in Figure 6) except in the most C-terminal region (α-helix 6, α-helix 7, β-strand 8 and α-helix 8). The α-helical region 6 is in a similar position within all the proteins but there is no conservation of amino acid sequence in this region between the enzymes from prokaryotes and those from eukaryotes. There is also no conservation of sequence in α-helix 7 or in β-strand 8 between prokaryotic and eukaryotic enzymes although amongst the eukaryotic enzymes there is strong conservation of primary sequence in all these regions, and especially in α-helix region 7.

The residues participating in the active site of glucanases are conserved in all the branching enzyme proteins (arrowed in Figure 2) including Asp 474, Glu 536 and Asp 605 (numbering according to consensus sequence) which are involved in catalysis of α-1,4 bond hydrolysis and His 404 and His 604 which bind to the glucosyl residue on the non-reducing side of the glucan bond to be cleaved in the substrate. All these amino acid residues lie in regions highly conserved between all branching enzymes, including members of plant families A and B. In amylolytic enzymes active on branched substrates, the branch of the glucan chain is thought to be bound by the loops between β-strand 1/α-helix 1, β-strand 2/α-helix 2; β-strand 3/α-helix 3; β-strand 7/α-helix 7 and β-strand 8/α-helix 8. Bacterial glycogen branching enzyme and maize SBEI (family B) differ in the lengths of the loops between β-strand 7/α-helix 7 and β-strand 8/α-helix 8 and this difference has been proposed to account for differences in the branch length of the product of these two enzymes (Jespersen *et al.*, 1993). There is also a difference in the loop size between β-strand 8 and α-helix 8 between members of starch branching enzyme families A and B involving 11 extra amino acids which are well conserved ($^{P}/_{E}$QXLP$^{S}/_{N}$-GK$^{F}/_{I}{}^{I}/_{V}$P) in family A members and which are absent in family B members. This difference is likely to be significant since family A and family B members are different in the branching reactions they catalyse (Smith, 1988; Takeda *et al.*, 1993) and in the case of maize have been shown to transfer branches of different lengths *in vitro* (Takeda *et al.*, 1993). The particular roles of these domains in affecting enzyme specificity must await further analysis of mutants.

In summary, the presence of an additional flexible N-terminal domain in SBE family A members may affect physical properties of the enzyme while a number of other differences between family A and family B members may determine differences in enzyme specificity.

Our data show SBEI and SBEII genes to be differentially expressed during embryo development. From analysis of mutation of the *r* locus we knew that SBEI is active earlier in development than SBEII (Bhattacharyya *et al.*, 1990; Smith, 1988). Our new data show that there are non-coordinated changes in transcript levels of the two isoforms which determine the differences in the activity of the two isoforms during development; SBEI being the 'early' form and SBEII the 'later' form (Smith, 1988). No differential expression of rice branching enzyme isoforms has been reported (Mizuno *et al.*, 1993). This could be taken to mean that branching of starch in endosperm (unlike pea embryo) does not undergo changes in the nature of its branching enzyme activity through development. However, measurement of developmental age in terms of time (as has been done in rice) rather than fresh weight may obscure differences in gene expression that occur over a period of very rapid growth and rapid starch biosynthesis. The change from SBEI activity to a mixture of SBEI and II activity during pea embryo development is accompanied by a change in the structure of amylopectin molecules, suggesting that the two isoforms transfer chains of different lengths *in vivo*. The existence of a causal relationship between changes in SBE isoforms and changes in amylopectin structure is strongly supported by recent measurements of the properties of highly purified SBEI and II from maize (Takeda *et al.*, 1993). These show that the latter transfers shorter branches than the former when branching amylose *in vivo*. If this represents a general difference between family B and family A isoforms, amylopectin of young pea embryos would be expected to have a shorter average branch length than amylopectin of older embryos. Our measurements of the absorbance of iodine-amylopectin complexes are entirely consistent with this expectation.

Comparison of the amylopectin of wild-type and *r* mutant embryos might be expected to yield further information about the roles of SBEI and II. However, about 20% of the starch of mutant embryos is made up of material intermediate in molecular weight between the amylose and amylopectin fractions of wild-type embryos. As such it has a branching pattern different from either of these fractions, although the average content of longer chains is higher in this intermediate fraction than in normal amylopectin, supporting the view that SBEII, the isoform active in wrinkled peas, preferentially transfers longer chains during branching (Colonna and Mercier, 1984). However, the timing of appearance of SBE activity and the

ratio of SBE activity to starch synthase activity are also radically altered in the mutant embryos and may be as important as the alteration in the nature of the SBE activity in determining differences in starch structure between mutant and wild-type embryos.

In screens for new chemically generated mutations of pea, using the wrinkle-seeded character to select for lesions in starch biosynthesis, no mutations in the SBEII gene were found despite multiple alleles of the SBEI being isolated (Hedley and Wang, personal communication). This suggests that mutations of the SBEII gene do not lead to a wrinkle-seeded phenotype. The biochemical basis for this may be that absence of SBEII activity does not limit starch biosynthesis to the extent that a wrinkle-seeded phenotype is produced. Because of the lower contribution of SBEII to amylopectin biosynthesis and its relatively late activity during embryo development its absence is unlikely to have a serious effect on the overall rate of starch synthesis and hence the shape of the mature seed.

Taken as a whole, our results suggest strongly that SBEs of families A and B play different roles in determining the structure of amylopectin in storage organs. Differences in the balance of the two isoforms, both in overall activity and contributions during development, could also determine qualitative differences in starch between different plant species.

Experimental procedures

Plant material

A round-seeded line of *Pisum sativum* L. derived from JI430 (Hedley *et al.*, 1986) was the source of all pea material. It was grown in a greenhouse with minimum temperatures of 12°C. For preparation of mRNA, embryos were harvested, the testas were removed and the material was frozen in liquid nitrogen and stored at –80°C. For analysis of mRNA from flowers, leaves, stipules and pods, 66-day-old plants were used. Dark-grown plants were kept in complete darkness for 48 h prior to leaf harvest. mRNA was extracted from the apical 10 mm of root tips from peas germinated on wet filter paper for 4 days in darkness at 25°C.

Preparation of mRNA

Total RNA was extracted as described by Prescott and Martin (1987) and poly(A)$^+$ mRNA was isolated by passage over oligo(dT)-cellulose. mRNA for cDNA libraries was passed twice over oligo-(dT)cellulose.

Northern blots and Southern blots

Steady-state levels of mRNA (7 μg poly(A)$^+$ RNA) were analysed on Northern blots, as described by Martin *et al.* (1985). Genomic DNA extracted from pea (*RR* and *rr* isolines) leaves was digested with different restriction enzymes and separated on 0.8% agarose gels. Southern blotting was according to Southern (1975) and Wahl *et al.* (1979).

Northern blots and Southern blots were probed with radioactive DNA fragments labelled by nick translation (Rigby *et al.*, 1977). The full-length cDNA clone of SBEI (pJAM425) and the 2.9 kb fragment of SBEII (RS4A) were used for all hybridizations. A legumin cDNA clone, pRC943, (a gift from Dr R. Casey) was used to assay legumin gene expression during embryo development. Hybridization of probes was performed according to Martin *et al.* (1985) and Northern filters were washed in 2 × SSC, 0.5% SDS at 65°C. Southern blots were washed in either 0.5 × SSC, 0.5% SDS at 65°C (high stringency) or 2 × SSC, 0.5% SDS, 55°C (low stringency).

Construction and immunological screening of cDNA libraries in λgt11

cDNA was prepared from mRNA isolated from embryos of 300–600 mg fresh weight. Double-stranded blunt-ended cDNA was synthesized according to the instructions in the Amersham (Amersham International, Amersham, UK) cDNA synthesis kit. Adaptors were ligated on to the cDNA and it was cloned into the *Eco*RI site of λgt11 according to the instructions in the Amersham λgt11 cloning kit. Approximately 3 × 10^5 p.f.u. were screened using the anti-SBEII antiserum. The antiserum was pre-absorbed with *Escherichia coli* lysate (10 mg ml^{-1}) at a 1:50 dilution to block non-specific binding. Filters were probed with the antiserum at a dilution of 1:500.

Construction and screening of libraries in λgt10

cDNA was synthesized from mRNA from embryos of less than 300 mg for isolation of SBEI-cDNA clones and from 300-600 mg embryos for isolation of SBEII cDNA clones, and prepared as described for the Amersham λgt10 cloning kit (Amersham International, Amersham, UK). For the isolation of cDNA clones of SBEI and SBEII, approximately 5 × 10^5 p.f.u. were screened in each case.

cDNA sequencing

Sequences were determined according to Sanger *et al.* (1977) using Sequenase (United States Biochemical Corporation) following subcloning into M13, pUC1813, pUC118 or pBluescript. Both strands were sequenced and the sites used for cloning were confirmed by sequencing. The nucleotide sequence data for pea SBEI and SBEII will appear in the EMBL database under the accession numbers X80009 and X80010, respectively.

N-terminal sequencing

Protein for N-terminal sequencing was prepared as described by Dry *et al.* (1992).

PCR method

The presence or absence of SBEI and SBEII transcripts was tested by 3′ RACE PCR amplification (Frohman *et al.*, 1989). First-strand cDNA was prepared by reverse transcription of 10 μg of total RNA

primed with the dT17 adaptor sequence (Frohman *et al.*, 1989) which hybridizes to the poly(A) tail of mRNA. The 5′ primer used for amplification of SBEI cDNA, was 5′-GGAAGTACTAAGGT ACCTACTTTCACTTTCAAATGC-3′ and that for SBEII was 5′-CTA-GCCATGGCTATCCCTGACAAG-3′. The dT17 adaptor sequence was used as the 3′ primer (Frohman *et al.*, 1989). Amplification consisted of 40 cycles of denaturation (94°C; 40 sec), annealing (55°C; 2 min) and extension (72°C; 3 min). The primers used amplify partial cDNAs of 1.5 kb each, to avoid limitation of amplification by the length of the transcript. Amplified cDNA was blotted on to nitrocellulose and probed with SBEI and SBEII cDNA clones to confirm the identity of the amplified products. No cross-hybridization was observed between products amplified with the SBEI primer and probed with the SBEII probe or vice versa. Control amplifications without added cDNA showed no amplified products.

Starch analysis

Starch was purified from developing pea embryos according to Smith (1990). After drying the starch was solubilized in 0.1 M NaOH at a concentration of 5 mg ml^{-1} and subjected to gel permeation chromatography on a sepharose CL2B column (790 mm × 15 mm) over a period of 9 h with 0.01 M NaOH as the eluting medium.

An aliquot of 1.5 ml from each 2.5 ml fraction was neutralized with 15 μl 1 M acetic acid to form an acetate buffer, diluted with 2.5 ml 0.05 M KI and treated with 7.5 μl iodine solution. The λ max of the iodine–glucan complex of fractions containing either amylose or amylopectin was measured on a scanning spectrophotometer in the range 550–640 nm.

Acknowledgements

It is a pleasure to thank Chris Sidebottom for N-terminal sequencing, James Lloyd for help with starch analysis, Rudiger Simon for patient advice and help with computer analysis of peptide sequences, David Hopwood and Kay Denyer for helpful commentary on the manuscript and Peter Scott, Barrie Allen, Andrew Davies and Nigel Hannet for photography. We also thank Peter Poulsen, Maribo Seeds, and Celia Bruton, Department of Genetics, John Innes Institute, for making available potato and *Streptomyces coelicolor* branching enzyme sequences in advance of publication and Rod Casey, John Innes Institute, for the gift of the legumin cDNA clone, pRC943. We acknowledge a financial contribution from the Axis Genetics Company Limited (Cambridge, UK) for work on SBEI, and the assistance of Jacqui Timors and Debbie Harrison in sequencing the SBEI gene. During the course of this research R.A.B. was supported by the Plant Molecular Biology Initiative from the Agriculture and Food Research Council and J.D.B. by NSERC grant A2210.

References

Baba, T., Kimura, K., Mizuno, K., Etoh, H., Ishida, Y., Shida, O. and Arai, Y. (1991) Sequence conservation of the catalytic regions of amylolytic enzymes in maize branching enzyme I. *Biochem. Biophys. Res. Commun.* **181**, 87–94.

Baecker, P.A., Greenberg, E. and Preiss, J. (1986) Biosynthesis of bacterial glucan: primary structure of *Escherichia coli* 1,4-alpha-D-glucan 1,4-alpha-D-glucan 6-alpha-D-(1,4-alpha-D-glucano)-transferase as deduced from the nucleotide sequence of the *glgB* gene. *J. Biol. Chem.* **261**, 8738–8743.

Banks, W. and Greenwood, C.T. (1975) *Starch and its Components.* Edinburgh: Edinburgh University Press.

Bhattacharyya, M.K., Smith, A.M., Ellis, T.H.N., Hedley, C. and Martin, C. (1990) The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch branching enzyme. *Cell*, **60**, 115–121.

Boyer, C.D. and Preiss, J. (1978) Multiple forms of (1,4-alpha-D-glucan (1-4)-alpha-D-glucan-6-glucosyl transferase from developing *Zea mays* L. kernels. *Carbohydr. Res.* **61**, 321–334.

Boyer, C.D. and Fisher, M.P. (1981) Comparison of soluble starch synthases and branching enzymes from developing maize and teosinte seeds. *Phytochemistry*, **23**, 733–737.

Buisson, G., Duée, E., Haser, R. and Payan, F. (1987) Three dimensional structure of porcine pancreatic α-amylase at 2.9 A resolution. Role of calcium in structure and activity. *EMBO J.* **6**, 3909–3916.

Chou, P.Y. and Fasman, G.D. (1978) Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* **47**, 45–148.

Colonna, P. and Mercier, C. (1984) Macromolecular structure of wrinkled- and smooth pea starch components. *Carbohydr. Res.* **26**, 233–247.

Denyer, K., Sidebottom, C., Hylton, C.M. and Smith, A.M. (1993) Soluble isoforms of starch synthase and starch branching enzyme also occur within starch granules in developing pea embryos. *Plant J.* **4**, 191–198.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**, 387–395.

Dry, I., Smith, A., Edwards, A., Bhattacharyya, M., Dunn, P. and Martin, C. (1992) Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. *Plant J.* **2**, 193–202.

Fisher, D.K., Boyer, C.D. and Hannah, L.C. (1993) Starch branching enzyme II from maize endosperm. *Plant Physiol.* **102**, 1045–1046.

Frohman, M.A., Dush, M.K. and Martin, G.R. (1989) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl Acad. Sci. USA*, **85**, 8998–9002.

Gavel, Y. and von Heijne, G. (1990) A conserved cleavage-site motif in chloroplast transit peptides. *FEBS Lett.* **261**, 455–458.

Hedley, C.L., Smith, C.M., Ambrose, M.J., Cook, S. and Wang, T.L. (1986) An analysis of seed development in *Pisum sativum*, II. The effect of the *r* locus on growth and development of the seed. *Ann. Bot.* **58**, 371–379.

Hodges, H.F., Creech, R.G. and Loerch, J.D. (1969) Biosynthesis of phytoglycogen in maize endosperm. The branching enzymes. *Biochim. Biophys. Acta*, **185**, 70–79.

Jespersen, H.M., MacGregor, E.A., Heurissat, B., Sierks, M.R. and Svensson, B. (1993) Starch- and glycogen-debranching and branching enzymes: Prediction of structural features of the catalytic $(B/\alpha)_8$-barrel domain and evolutionary relationship to other amylolytic enzymes. *J. Protein Chem.* **12**, 791–805.

Kiel, J.A.K.W., Boels, J.M., Beldman, G. and Venema, G. (1991) Molecular cloning and nucleotide sequence of the glycogen branching enzyme gene (*glgB*) from *Bacillus stearothermophilus* and expression in *Escherichia coli* and *Bacillus subtilis*. *Mol. Gen. Genet.* **230**, 136–144.

Kiel, J.A.K.W., Boels, J., Beldman, G. and Venema, G. (1992) The *glgB* gene from the thermophile *Bacillus caldolyticus* encodes a thermolabile branching enzyme. *DNA Seq.* **3**, 221–232.

Klein, C., Hollender, J., Bender, H. and Schultz, G.E. (1992) Catalytic center of cyclodextrin glycosyltransferase derived from X-ray structure analysis combined with site-directed mutagenesis. *Biochemistry,* **31,** 8740–8746.

Martin, C., Carpenter, R., Sommer, H., Saedler, H. and Coen, E.S. (1985) Molecular analysis of instability in flower pigmentation in *Antirrhinum majus* following isolation of the *pallida* locus by transposon tagging. *EMBO J.* **4,** 1625–1630.

Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. (1984) Structure and possible catalytic residues of taka-amylase A. *J. Biochem.* **95,** 697–702.

Matters, G.L. and Boyer, C.D. (1981) Starch synthases and starch branching enzymes from *Pisum sativum. Phytochemistry,* **20,** 1805–1809.

Mizuno, K., Kawasaki, T., Shimada, H., Satoh, H., Kobayashi, E., Okumura, S., Arai, Y. and Baba, T. (1993) Alteration of the structural properties of starch components by the lack of an isoform of starch branching enzyme in rice seeds. *J. Biol. Chem.* **268,** 19 084–19 091.

Nakamura, Y. and Yamanouchi, H. (1992) Nucleotide sequence of a cDNA encoding starch branching enzyme, or Q enzyme I, from rice endosperm. *Plant Physiol.* **99,** 1265–1266.

Nakamura, Y., Takeichi, T., Kawaguchi, K. and Yamanouchi, H. (1992) Purification of two forms of starch branching enzyme (Q enzyme) from developing rice endosperm. *Physiol. Plant.* **84,** 329–335.

Poulsen, P. and Kreiberg, J. D. (1993) Starch branching enzyme cDNA from *Solanum tuberosum. Plant Physiol.* **102,** 1053–1054.

Prescott, A.G. and Martin, C. (1987) Rapid method for the quantitative assessment of levels of specific mRNAs in plants. *Plant Mol. Biol. Rep.* **4,** 219–224.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) Labelling of deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113,** 237–251.

Salehuzzaman, S.N.I.M., Jacobsen, E. and Visser, R.G.F. (1992) Cloning, partial sequencing and expression of a cDNA coding for branching enzyme in cassava. *Plant Mol. Biol.* **20,** 809–819.

Sanger, R., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl Acad. Sci. USA,* **74,** 5463–5467.

Smith, A.M. (1988) Major differences in isoforms of starch-branching enzyme between developing embryos of round- and wrinkled-seeded peas (*Pisum sativum* L). *Planta,* **175,** 270–279.

Smith, A.M. (1990) Evidence that the 'waxy' protein of pea is not the major starch granule-bound starch synthase. *Planta,* **182,** 599–604.

Smith, A.M. and Martin, C. (1993) Starch biosynthesis and the potential for its manipulation. In *Biosynthesis and Manipulation of Plant Products,* Volume 3, *Plant Biotechnology Series* (Grierson, D., ed.). London: Blackie A & P, pp. 1–54.

Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503–517.

Takeda, Y., Guan, H.-P. and Preiss, J. (1993) Branching of amylose by the branching isoenzymes of maize endosperm. *Carbohydr. Res.* **240,** 253–263.

Thon, V.J., Vigneron-Lesens, C., Marianne-Pepin, T., Montreuil, J., Decq, A., Rachez, C., Ball, S.G. and Cannon, S.F. (1992) Coordinate regulation of glycogen metabolism in the yeast *Saccharomyces cerevisiae.* Induction of the glycogen branching enzyme. *J. Biol. Chem.* **267,** 15 224–15 228.

Thon, V.J., Khalil, M. and Cannon, J.F. (1993) Isolation of human glycogen branching enzyme cDNAs by screening complementation in yeast. *J. Biol. Chem.* **268,** 7509–7513.

Wahl, G.M., Stern, M. and Stark, G.R. (1979) Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridisation using dextran sulphate. *Proc. Natl Acad. Sci. USA,* **76,** 3683–3687.

EMBL Data Library accession numbers X80009 (SBEI) and X80010 (SBEII).