*The Plant Journal* (1999) **18**(2), 163–171

# A minor form of starch branching enzyme in potato (*Solanum tuberosum* L.) tubers has a major effect on starch structure: cloning and characterisation of multiple forms of *SBE A*

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**Summary**

**Full length cDNAs encoding a second starch branching enzyme (SBE A) isoform have been isolated from potato tubers. The predicted protein has a molecular mass of 101 kDa including a transit peptide of 48 amino acids. Multiple forms of the *SBE A* gene exist which differ mainly in the length of a polyglutamic acid repeat at the C-terminus of the protein. Expression of the mature protein in *Escherichia coli* demonstrates that the gene encodes an active SBE. Northern analysis demonstrates that *SBE A* mRNA is expressed at very low levels in tubers but is the predominant isoform in leaves. This expression pattern was confirmed by Western analysis using isoform specific polyclonal antibodies raised against *E. coli* expressed SBE A. SBE A protein is found predominantly in the soluble phase of tuber extracts, indicating a stromal location within the plastid. Transgenic potato plants expressing an antisense *SBE A* RNA were generated in which almost complete reductions in SBE A were observed. SBE activity in the leaves of these plants was severely reduced, but tuber activity was largely unaffected. Even so, the composition and structure of tuber starch from these plants was greatly altered. The proportion of linear chains was not significantly increased but the average chain length of amylopectin was greater, resulting in an increase in apparent amylose content as judged by iodine binding. In addition, the starch had much higher levels of phosphorous.**

Received 12 October 1998; revised 11 February 1999; accepted 23 February 1999.

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## Introduction

Two isoforms of starch branching enzyme have been identified in starch storing organs of maize, pea and rice (Boyer and Preiss, 1978; Nakamura *et al.*, 1992; Smith, 1988). These isoforms have been classified into two classes (A and B) based on amino acid sequence comparisons (Burton *et al.*, 1995). Maize SBE II, rice SBE III and pea SBE I belong to the A class, whereas maize SBE I, rice SBE I and pea SBE II belong to the B class. Full length cDNA clones have been isolated for all these isoforms (Baba *et al.*, 1991; Bhattacharyya *et al.*, 1990; Burton *et al.*, 1995; Fisher *et al.*, 1993; Kawasaki *et al.*, 1993; Mizuno *et al.*, 1992, 1993). In maize, two isoforms of SBE II exist (SBE IIa and SBE IIb) and it has recently been shown that these are encoded by separate genes (Gao *et al.*, 1997). The two classes also have distinct biochemical characteristics, for example in maize, the SBE II (class A) isoform preferentially transfers shorter chains and has a lower affinity for amylose than maize SBE I (class B) (Guan and Preiss, 1993; Takeda *et al.*, 1993). Based on these *in vitro* results and the correlation between the expression pattern of SBE isoforms during pea embryo development and the average chain length of amylopectin (Burton *et al.*, 1995), it has been suggested that the different isoforms of SBE play different roles in the synthesis of amylopectin (Preiss and Sivak, 1996). Mutants affecting starch branching enzymes are only known for the class A isoform, in pea at the *rugosus* (*r*) locus (Bhattacharyya *et al.*, 1990) and in maize, at the *amylose extender* (*ae*) locus (SBEIIb isoform, Stinard *et al.*, 1993). The starch of these mutants has an increased amylose content and the amylopectin has an increased average chain length, but no gross changes in the polymodal chain distribution (Baba and Arai, 1984; Colonna and Mercier, 1984; Lloyd *et al.*, 1997). Studies involving leaf starch synthesis in peas also suggest that SBE may not be the most important factor controlling the polymodal distribution of glucan chain lengths in amylopectin (Tomlinson *et al.*, 1997). Recently, mutations in starch synthases were identified in pea (*rug5*) and maize (*dull1*) and were shown to dramatically affect the amylopectin structure and chain length distribution suggesting that starch synthases may be more important than SBE in this respect (Craig *et al.*, 1998; Gao *et al.*, 1998).

In potato, only one SBE activity has been detected (Blennow and Johansson, 1991) and cloning of the corres-

ponding gene identified it as a B class enzyme (Koßmann *et al.*, 1991). Antisense inhibition of this gene reduces starch branching enzyme activity of the tubers almost completely without affecting the amylose content of the starch or the chain length distribution of the amylopectin (Koßmann *et al.*, 1997; Safford *et al.*, 1998). However, small but significant changes in the physical properties of the starch can be detected (Safford *et al.*, 1998). These studies suggested that if an active second isoform of SBE was expressed in potato tubers then it was likely to be of low abundance. Recently, however, a second form of SBE belonging to the A class has been partially characterised from potato tubers (Larsson *et al.*, 1996; Larsson *et al.*, 1998).

We now show here, by isolation of full length cDNA clones, that several forms of *SBE A* exist in potato and that they do encode enzymatically active proteins. We further characterise expression patterns of SBE in potato and show, in antisense experiments, that although SBE A is a very minor form in the tuber it plays a major role in the control of starch structure.

## Results

### *Isolation of a full length cDNA for* SBE A

A PCR-based approach was used to isolate *SBE A* cDNA clones from potato. A highly conserved amino acid sequence (GLYNFMGNEFGHPEWIDFR), present in the carboxy terminal one-third of all plant SBE, was used to design a degenerate PCR primer to amplify SBE sequences from a potato tuber cDNA library. Positive clones were used to re-screen the potato tuber cDNA library and the 5′ end of the gene was isolated by 5′RACE (Frohman *et al.*, 1988). A consensus sequence of the overlapping cDNA clones was compiled which predicted a full length cDNA of approximately 3050 bp excluding the polyA tail. A single large open reading frame encoding a protein of 882 amino acids or 101 kDa was identified. The potato SBE A protein showed very strong similarity (71%, data not shown) to the pea SBE A protein over the entire coding region. This sequence conservation extended to the extreme N-termini of both proteins (Figure 1a) and indicated that the potato SBE A protein has a putative plastid targeting sequence of 48 amino acids.

### *Multiple forms of* SBE A *exist*

During isolation of the *SBE A* cDNAs, a number of sequence differences were consistently detected indicating that more than one form of the *SBE A* gene may exist in potato. An alignment of the deduced amino acid sequences at the C-terminus of these clones was compiled (Figure 1b). The most noticeable difference between the clones was in the length of a polyglutamic acid stretch close to the C-terminus of the protein. Isoforms were identified that had either 1, 5, 6, 7, 10 or 11 contiguous E residues and were designated SBE A-1 to A-6, respectively. Other differences were apparent and the SBE A-1 isoform showed several unique changes including significant deletions in the 3′ untranslated region (data not shown) which indicated that it was distinct from the others. Multiple independent clones were isolated for all except SBE A-2 and SBE A-6 isoforms. Full length tuber-derived cDNAs were isolated corresponding to the *SBE A*-1, A-4, A-5 and A-6 isoforms and these were

(a)

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                                        ↓
        v10       v20       v30       v40       v50       v60       v70       v80
MVYTLSGVRFPTVPSVYKSNGFSSNGDRRNANVSVFLKKH--SLSRKILAEKSSYNSEFRPSTVAASGKVLVPGTQSDSSSSSTDQ  potato SBE A
MVYT SG:RFP :PS:.KS   :  :DRR::::S FLK:   S SR  L :K S::SE   ST:A S KVL:P::Q D:S:S :DQ
MVYTISGIRFPVLPSLHKS---TLRCDRRASSHSFFLKNNSSSFSRTSLYAKFSRDSETKSSTIAESDKVLIPEDQ-DNSVSLADQ  pea SBE A
        ^10       ^20       ^30       ^40       ^50       ^60       ^70       ^80
                                        ↑
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(b)

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LKPGKYKVALDSDDPLFGGFGRIDHNAEYFTFEGWYDDRPRSIMVYAPCKTAVVYALVDKEEEEEEEEEEEVAAVEEVVVEEE        SBE A-6
..............................................R.....................-..V.........        SBE A-5
..............................................SR.................----..V.........        SBE A-4
..........................C...................SR................-----..V.........        SBE A-3
..........................C...................SR..............------..V.........        SBE A-2
........V.....................S..S.....C.......SR..........L.---------..V...PI--..        SBE A-1
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**Figure 1.** Sequence alignment of SBE

(a) Comparison of N-terminal sequences of potato SBE A and pea SBE A. Identical amino acids are shown between the two sequences and similar or related amino acids are represented by a colon or a full stop, respectively. Dashes indicate gaps introduced to maximise the alignment. The arrows indicate the N-terminus of the mature proteins. The potato SBE A sequence is translated from full length clone *SBE A-6* and pea SBE A is from accession number X80009.

(b) Comparison of the C-terminal regions of potato SBE A proteins. Amino acids identical to SBE A-6 are indicated by dots and dashes indicate gaps introduced to maximise the alignment. Underlined amino acids may be PCR artefacts as only a single cDNA was isolated. Proteins were translated from the following sequences (accession numbers in brackets); full length cDNAs *SBE A-1* (AJ011885), *SBE A-4* (AJ011888), *SBE A-6* (AJ011890), *SBE A-5* (AJ011889), partial cDNA's *SBE A-2* (AJ011886), *SBE A-3* (AJ011887).

95–97% identical over the entire sequence including the untranslated regions (data not shown).

### *The* SBE A *cDNA encodes an active starch branching enzyme*

It should be noted that great difficulties were encountered in cloning full length *SBE A* cDNA clones containing an intact coding region. Only the *SBE A-6* cDNA had an intact open reading frame (ORF), the others had small deletions or nucleotide substitutions which caused a frameshift or premature termination of the *SBE A* ORF. There was no evidence that any of these changes were present in the endogenous sequences as determined by direct sequencing of amplified uncloned cDNAs or partial length cloned cDNAs (data not shown). It is probable that the SBE A protein is toxic to *E. coli* cells and that the observed changes arise from a negative selection pressure during cloning of full length cDNAs. Colonies containing an intact *SBE A* ORF did in fact grow slower than those with a disrupted ORF and on two independent occasions we observed insertion of transposon Tn10 at the same position in the *SBE A* gene which restored the growth rate of the cells. For these reasons, it is also probable that the sequences of the full length cDNAs contain other single base nucleotide changes.

To confirm that the isolated *SBE A* gene encodes an active starch branching enzyme, a complementation test was performed in the glycogen branching enzyme-deficient strain of *Escherichia coli* KV832 (Kiel *et al.*, 1987). Wild type cells synthesise a glycogen-like polymer which stains a red/brown colour with iodine, whereas the KV832 cells make only linear chain glucans which stain a blueish-green. When a plasmid expressing the mature SBE A protein was transformed into the KV832 strain the cells stained a brown colour with iodine, similar to wild type *E. coli* cells or KV832 cells transformed with a plasmid containing the full length pea *SBE A* gene, whereas control cells, containing only the pQE32 vector, continued to stain blue-green (data not shown). This indicates that the potato *SBE A* gene was able to complement the glycogen branching enzyme mutation and that the cloned gene does indeed code for a protein with branching enzyme activity.

To conclusively demonstrate SBE activity, an alternative more tightly regulated T7 RNA polymerase-based expression system (pET29 series) was used to overexpress the mature potato SBE A protein coding sequence. In this system, a protein of the predicted size accumulated to more than 10% of the total cellular protein within a few hours of induction of expression (data not shown). Although most of the SBE A protein was present in inclusion bodies, SBE activity could easily be detected in sonicated cell free extracts (2.2 U $mg^{-1}$ versus 0.14 U $mg^{-1}$ for the control).

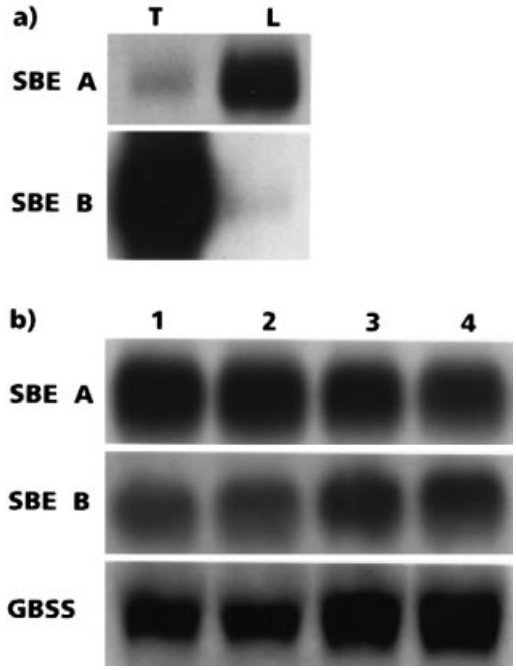


**Figure 2.** Northern analysis of SBE expression.
(a) Tissue specificity. Total RNA from tubers (T) or leaves (L) was electrophoresed in duplicate, blotted and probed with full length SBE A and B radiolabelled probes of the same specific activity. Blots were exposed to film for 16 h.
(b) Tuber development. Total RNA from tubers of various sizes harvested from a single plant at maturity was electrophoresed, blotted and probed with full length *SBE A* cDNA. The blot was then stripped and reprobed successively with *SBE B* or granule bound starch synthase (*GBSS*) cDNAs. Lane 1 (< 1 g), lane 2 (5 g), lane 3 (20 g) and lane 4 (40 g). Exposure times were adjusted to produce images of the same approximate intensity (*SBE A*, 72 h; *SBE B*, 4 h and *GBSS*, 1 h).

### SBE A *is differentially expressed*

To determine the expression pattern of the *SBE A* gene, a Northern blot analysis was performed with total RNA from the mature tubers and source leaves (> 10 cm) of greenhouse grown plants (Figure 2a). A transcript of the expected size for *SBE A* was detected in low amounts in tubers but was present at much higher levels in leaves. In contrast, *SBE B* was expressed at very high levels in tubers and at much lower levels in leaves. Quantitative analysis performed on a phosphorimager indicated that the *SBE A* RNA level was approximately 2% that of *SBE B* RNA in tubers.

The expression pattern of *SBE B* and *SBE A* was further analysed in tubers of different sizes, as shown in Figure 2b. *SBE A* expression was highest in the smallest tubers (< 1 g) and declined with increasing tuber size. In contrast, *SBE B* expression increased with tuber size and was greatest in the largest tubers, a pattern that was very similar to the expression of *GBSS I.*

Expression of SBE was also monitored at the protein level by Western analysis of crude soluble extracts of leaf and tuber tissue, using polyclonal antibodies generated

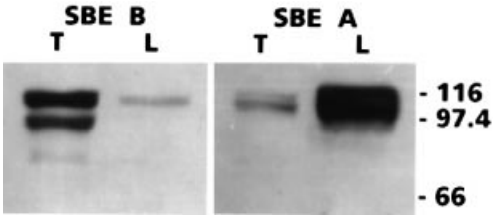


**Figure 3.** Western analysis of SBE expression.
Western blot showing expression pattern of SBE A and B in tubers (T) or leaves (L). Approximately 20 μg of protein from crude soluble extracts of tubers or 40 μg from leaves (equal amounts on a fresh weight basis) was analysed. Molecular mass markers are indicated on the right-hand side.

against the *E. coli*-expressed proteins. This analysis confirmed that SBE A expression was greatest in leaf tissue and that SBE B was most abundant in tubers (Figure 3). The SBE A protein appeared as a doublet of approximately 107 and 103 kDa in both tissues, whereas SBE B was detected as major band of 103 kDa in leaves and additional proteins of 93 and 80 kDa were detected in tubers. This multiple banding pattern for potato SBE B has been reported previously (Khoshnoodi *et al.*, 1996) and is probably due to proteolytic processing. Two bands (protein) have also been reported for SBE A from pea (Smtih, 1988) which are both lost in the *r* mutant line (Bhattacharyya *et al.,* 1990).

### *Generation of plants with reduced* SBE A *levels*

To determine the role of SBE A in starch biosynthesis, antisense constructs were designed using a 1.2 kb partial length *SBE A* cDNA under control of either the constitutive 2×35S CaMV promoter or the developmentally regulated potato GBSS I promoter. Transformed and control plants were grown to maturity in the greenhouse. Approximately 10% of the antisense lines driven by the CaMV promoter had significantly reduced expression of SBE A in both leaf and tuber. A representative Western blot of the four most severely affected lines (152, 244, 249 and 280) and transformed and wild-type controls are shown in Figure 4(a,b). The SBE B protein in tubers was comparatively unaffected although lines 249 and 280 showed some reductions (Figure 4c). This pattern was reflected when the total SBE activity in tubers was determined (Table 1). With the GBSS promoter, approximately 15% of the antisense lines showed significantly reduced tuber SBE A expression, and an analysis of the most severely affected lines (0368, 0386 and 03106) again showed that total SBE activity in the tubers was not significantly altered (Table 1). In contrast, leaves of the SBE A down-regulated plants showed significantly reduced SBE activity and, in the most extreme line (249), only 7% of activity remained. The reduction was much greater when the *SBE A* antisense was expressed from the 2×35S promoter than from the GBSS promoter and in these lines the SBE A protein was still detectable

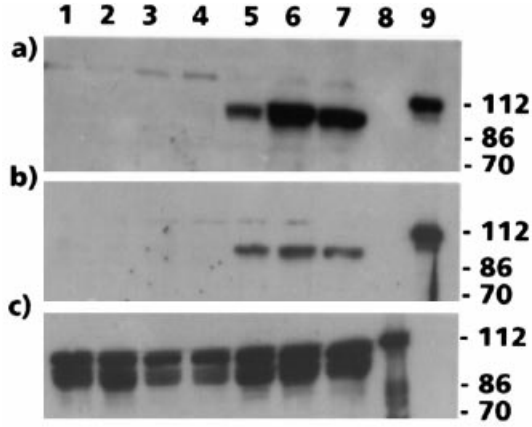


**Figure 4.** Western analysis of SBE A expression in transgenic plants lines.
(a) SBE A in leaves.
(b) SBE A in tuber.
(c) SBE B in tuber.
Lanes 1–5 antisense SBE A lines 152, 244, 249, 280, 360, respectively; lane 6 transformed control 359; lane 7 wild type control; lane 8 recombinant SBE B protein (40 ng); lane 9 recombinant SBE A protein (10 ng). Tubers were extracted in native buffer and leaves in SDS sample buffer. Approximately 20 μg of protein from tubers or 40 μg from leaves (equal amounts on a fresh weight basis) was analysed. Molecular mass markers are indicated on the right-hand side.

by Western analysis (data not shown). The transformed plants with reduced levels of SBE A were phenotypically indistinguishable from control plants and had similar yields of tubers and starch (data not shown).

### *Reduction in* SBE A *increases apparent amylose content and phosphorous levels of tuber starch*

The effect that reduction of SBE A in tubers had on the composition and structure of the starch was determined. Light microscopic examination showed that the gross morphology of starch granules was unchanged (data not shown). The amylose content of the starch was determined by spectrometric iodine binding and showed that those plants with reduced SBE A expression had significantly increased apparent amylose levels (up to 38.5% compared to 30% in the controls, Table 1). This is the first description of potato starches with significantly increased amylose levels. The phosphorous content of the starches was also determined since previous work has shown that alteration of several starch biosynthetic enzymes in potato tubers affects starch phosphorylation (Abel *et al.*, 1996; Safford *et al.*, 1998). The phosphorous content of control starch was between 500 and 680 p.p.m., whereas starches from lines with reduced SBE A expression had significantly increased levels up to 2300 p.p.m. (Table 1).

### *Amylopectin structure rather than amylose is greatly altered*

The molecular structure of the starches was further analysed. Two major fractions were resolved when starch was

**Table 1.** Starch branching enzyme activity and tuber starch composition (amylose and phosphorous content) of antisense SBE A plants and controls

| Transgenic plant SBE A antisense | Line | SBE activity (U $g^{-1}$ fw) | | Tuber starch | |
|---|---|---|---|---|---|
| | | Tuber | Leaf | % amylose | P (p.p.m.) |
| 2 × 35S promoter | 152 | 31.30 ± 2.09 | 0.59 ± 0.07 | 37.5 ± 0.2 | 890 |
| | 244 | 30.97 ± 1.32 | 1.05 ± 0.16 | 36.4 ± 0.3 | n.d. |
| | 249 | 17.90 ± 1.90 | 0.43 ± 0.03 | 38.5 ± 1.6 | 1480 |
| | 280 | 24.85 ± 0.83 | 1.01 ± 0.03 | 36.7 ± 0.0 | n.d. |
| | 360 | 34.84 ± 1.65 | 1.80 ± 0.14 | 30.7 ± 0.3 | n.d. |
| Controls | 359 | 36.06 ± 1.98 | 5.89 ± 0.58 | n.d. | 680 |
| | 242 | 36.54 ± 1.66 | 6.12 ± 0.25 | 30.2 ± 0.8 | 500 |
| GBSS promoter | 0368 | 32.21 ± 0.71 | 3.22 ± 0.08 | 36.4 ± 0.3 | 2300 |
| | 0386 | 39.71 ± 0.76 | 2.32 ± 0.11 | 36.5 ± 0.3 | 1010 |
| | 03106 | 34.66 ± 0.65 | n.d. | 36.9 ± 0.3 | 1400 |
| Controls | 0301 | 31.52 ± 0.34 | 7.85 ± 0.19 | n.d. | n.d. |
| | 0302 | 46.06 ± 1.76 | 8.79 ± 0.19 | 29.8 ± 0.3 | 650 |

Individual lines are identified according to the promoter used to drive expression of the antisense RNA. SBE activities are means of two assays performed in duplicate. Amylose content was measured in quadruplicate. Elemental phosphorous (P) is shown in parts per million (p.p.m., μg $g^{-1}$). Standard deviations are shown. n.d. not determined.

solubilised in DMSO and separated by gel permeation chromatography (Figure 5). In the control potato starch (line 0302), the major component eluted as a sharp symmetrical peak with a dextran-equivalent molecular size of about $10^7$. This peak contained the amylopectin fraction as it completely disappeared following debranching of the starch with isoamylase. The second fraction eluted as a broader, flatter peak with a molecular size range of $10^5$–$10^6$. Not all of this material is amylose as integration of the area under this peak estimated that it represented 40% of the total glucan whereas the amylose content determined by iodine binding was only 30%. A significant amount of material with a molecular size of approximately $1 \times 10^6$ is derived from amylopectin. Waxy (amylose-free) maize and potato starches also show a similar quantity of material (about 15%) at this position and this disappears completely upon debranching (data not shown). After debranching with isoamylase, the amylopectin containing material was completely converted into smaller glucan chains which had a bimodal distribution (molecular size $10^3$–$10^4$). The lower molecular size amylose-containing component also showed a significant decrease in size and quantity after debranching, again indicating the presence of a significant amount of branched material in the original broad peak. The decrease in the size of potato amylose after debranching has been reported before and it is known that potato amylose has significantly more branches than amylose from other sources (Murugesan *et al.*, 1993).

Large changes were observed in the composition of the starch from lines with reduced SBE A expression. The three most affected lines all showed very similar GPC profiles so only the data from line 03106 are shown (Figure 5). The amylopectin peak decreased compared to

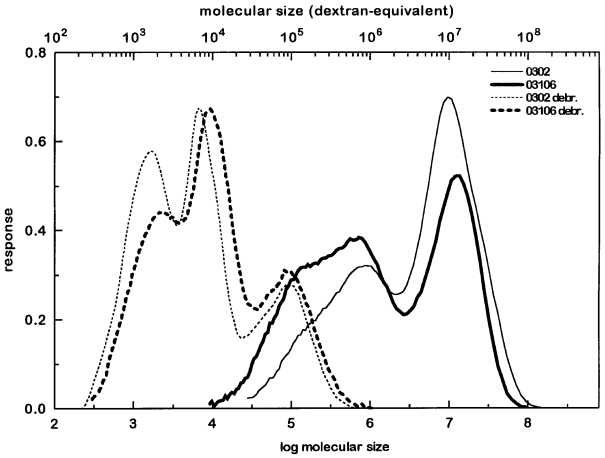


**Figure 5.** Gel permeation chromatogram of potato starches.
Starch from a control (0302) and a representative antisense *SBE A* line (03106) are shown before (solid lines) and after debranching (broken lines). The detector response (refractive index, arbitrary scale) is plotted against molecular size, based on dextran standards. Due to differences in hydrodynamic volume between amylose, amylopectin and dextran, absolute molecular size values are not accurate for starch components.

the control and the amylose-containing peak increased, although this increase was larger than expected from the amylose values determined by iodine binding measurements. Some of this material may be equivalent to the 'intermediate material' which is found in other high amylose starches (e.g. Baba and Arai, 1984; Shi *et al.*, 1998). After debranching, the proportion of long linear chains (dextran-equivalent molecular size approximately $10^5$) was very similar to the control, indicating that the true amylose content of the starch had not in fact increased significantly. There were, however, considerable differences in the short chains of the amylopectin. There was a

large decrease in the proportion of small chains and a significant overall increase in the chain length in both peaks. Analysis of the debranched starches by high performance anion exchange chromatography confirmed that there were fewer short chains (DP 6–23) and more long chains (DP 23–60, data not shown) in the amylopectin. It seems likely that the apparent increases in amylose content (determined iodometrically, Table 1) reflect elevated levels of relatively long branches (dextran-equivalent molecular size approximately $10^4$) rather than strictly linear chains.

## Discussion

We have cloned and characterised a second form of starch branching enzyme (SBE A) from potato and have shown, by complementation and overexpression in *E. coli*, that the *SBE A* gene encodes an active enzyme. Multiple forms of *SBE A* exist; at least six different cDNAs were identified, differing mainly in the length of a polyglutamic acid repeat at the C-terminus of the protein. Full length tuber-derived cDNAs were isolated for a number of these isoforms, one of which (SBE A-4) corresponds to the previously isolated protein coding sequence (Larsson *et al.*, 1998). The full length cDNAs were very similar over the entire sequence, including the untranslated regions. In contrast, in maize and *Arabidopsis,* where two forms of the *SBE A* gene are known, these sequences are much more divergent at the ends (Fisher *et al.*, 1996; Gao *et al.*, 1997). The function of the individual genes in Arabidopsis is not known whereas, in maize, *SBE 2b* is involved in the synthesis of amylopectin in the starch-storing endosperm and *SBE 2a* is expressed in leaf and vegetative tissues where transitory starch is synthesised (Gao *et al.*, 1997). It is not known if all potato *SBE A* genes are functionally equivalent or equally expressed, but cDNAs for all of them have been isolated from both leaves and tubers (data not shown).

We have determined by both Northern and Western analysis that starch branching enzymes are expressed differentially in the tubers and leaves of potato, thus expanding on the work of Larsson *et al.* (1998). In tubers, SBE A is expressed at very low but clearly detectable levels and SBE B is the major isoform. Potato seems to be unique among starch-storing organs in having such a biased expression pattern, as in most other plants both enzymes are present in similar amounts. Potato SBE A is expressed earlier in tuber development than SBE B, which is similar to the expression pattern of the SBE A in pea embryos (Burton *et al.*, 1995). In the leaves of potato. SBE A is the major form and SBE B the minor form which is also very similar to the expression of the corresponding SBE in pea leaves (Burton *et al.*, 1995).

In previous studies potato SBE A has only been detected in association with the starch granule (Larsson *et al.*, 1996; Larsson *et al.*, 1998), however, our Western analysis shows that potato SBE A is predominantly a soluble enzyme. We could not detect any SBE A protein in purified starch (5 mg, data not shown) and its previous detection in the granule may have been specific to the waxy potato variety studied.

Although potato SBE A represents less than 2% of the SBE activity of the tuber, antisense inhibition experiments show that it has a major effect on starch structure. Starches from plants with reduced SBE A have an apparent increased amylose content (38% versus 30% in controls) as determined by iodine binding. Gel permeation chromatography showed that the lower molecular size amylose-containing fraction had increased but analysis of the debranched starch showed that this fraction actually contained significantly branched or 'intermediate' material and that the true amylose content (i.e. the long linear chain fraction) was not significantly increased. The structure of the amylopectin was greatly altered, however, as there were many fewer short chains and the average chain length was significantly increased which accounted for the increased iodine binding observed.

These changes in starch structure are similar, in some respects, to those seen in the storage starch of the *ae* and *r* mutants of maize and pea which have reduced expression of SBE A isoform. The amylopectin from these starches has an increased average chain length and there is a significant amount of intermediate material (Shi *et al.*, 1998; Tomlinson *et al.*, 1997). However, the increase in amylose is much greater in these mutants. This could be due to the much larger decrease in SBE activity in these mutants since the SBE A isoform is much more abundant in these organs than in potato tubers. The granule location of the isoforms may also be relevant; a significant proportion of SBE A in maize is associated with the starch granule (Mu-Forster *et al.*, 1996), whereas the majority of potato SBE A appears to be stroma located.

An additional change in the starch composition of plants with reduced SBE A was a large increase in the phosphorous content. Increased phosphate has also been found in starches from antisense *SBE B* potato (Safford *et al.*, 1998) and *SBE A* (*ae*) mutant maize plants (Takeda *et al.*, 1993). The majority of the phosphate groups are found in the longer chains of amylopectin (Blennow *et al.*, 1998) and since the average chain length of the amylopectin is increased in the *SBE A* mutants it is perhaps not surprising that they have higher phosphate contents. This correlation does not hold for SBE B in potato since the chain length distribution of the amylopectin was not altered (Safford *et al.*, 1998) and the mechanism of phosphate incorporation into starch remains a mystery. The *SBE A* antisense lines generated in this study may prove useful in elucidating this mechanism.

Our results show that SBE A, the minor form of SBE in potato tubers, has a major impact on starch structure. The role of the major form of SBE (SBE B) in potato tubers

remains to be determined and this will be addressed by generating plants with reduced levels of both SBE A and B.

## Experimental procedures

### *Plant material*

Potato (*Solanum tuberosum*) cultivar Desiree was used. Plants were grown in compost in 25 cm pots in the greenhouse with additional illumination in winter.

### *Library screening*

The initial isolation of a potato *SBE A* cDNA clone was from an amplified potato tuber cDNA library in the λZap vector (Stratagene). One half μl of a potato cDNA library (titre $2.3 \times 10^9$ Pfu $ml^{-1}$) was used as a template in a 50 μl reaction containing 100 pmol of a 16-fold degenerate SBE primer (AATTTc/tATGGGIAAc/tGAa/gTTc/tGG) and 25 pmol of a T7 promoter primer (present in the Zap vector 3′ to the cDNA sequences), 100 μM dNTPs, 2.5 U Taq polymerase and the buffer supplied with the Taq polymerase (Stratagene). Hot start PCR was then performed by incubating for 1 min at 94°C, 1 min at 58°C and 3 min at 72°C, for 35 cycles. The PCR products were extracted with phenol/chloroform, ethanol precipitated and resuspended in TE pH 8.0 before cloning into the T/A cloning vector pT7Blue (Invitrogen). Two clones were sequenced and designated *SBE A-2* (AJ011886) and *SBE A-3* (AJ011887).

### *Rapid amplification of cDNA ends (RACE) and PCR conditions*

RACE was performed essentially according to Frohman *et al.* (1988). Two μg of total RNA from mature potato tubers was reverse transcribed using M-MLV reverse transcriptase and random hexamers (Pharmacia) as primer according to the manufacturer's (BRL) instructions. Excess primers were removed on a Centricon 100 column and cDNA was recovered and precipitated with isopropanol. cDNA was A-tailed with terminal deoxynucleotide transferase according to the manufacturer's instructions (BRL) then diluted to 0.5 ml with TE pH 8. Ten μl of the cDNA pool was amplified by 35 cycles of 94°C for 45 sec, 50°C for 25 sec, 72°C for 1.5 min and a final incubation at 72°C for 10 min. PCR products in the range of 600–800 bp fragments were re-amplified in a second PCR amplification using nested primers. Products were purified by chloroform extraction and cloned into pT7Blue. A second round of RACE was performed with a new A-tailed cDNA library constructed using an SBE A specific oligo as primer. A clone of 1500 bp was found to contain the 5′ end of the gene (accession number AJ011891, data not shown).

To amplify full length *SBE A* clones (*SBE A-1, SBE A-4* and *SBE A-6*), tuber cDNA was prepared according to the RACE protocol using an oligodT primer (5 pmol) and PCR was performed using a proof-reading polymerase (Ultma, Perkin-Elmer) with 2 μl of the cDNA in a 50 μl PCR reaction with 25 pmol of *SBE A* specific primers 5′; GGGGCCTTGAACTCAGCAAT and 3′; CGTCCCAGCATTCGACATAA and 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 3 min. PCR products were phosphorylated and cloned into the Eco RV site of pBSKSP (Stratagene).

### *Complementation of* E. coli *mutant KV832*

A full length cDNA clone (*SBE A-5*) was amplified by PCR with Vent polymerase (New England Biolabs) using tuber cDNA as template and CTTGGATCCTTGAACTCAGCAATTTG and TAACTCGAGCAACGCGATCACAAGTTCGT as 5′ and 3′ primers, respectively. This PCR product was cut with *Bgl*II and *Xho*I and cloned into the *Bam*HI and *Sal*I sites of the expression vector pQE32 (QIAGEN). This plasmid (pSJ90) was transformed into the *E. coli* mutant KV832 (Kiel *et al.*, 1987) and cells were grown overnight on solid PYG media (0.85% $KH_2PO_4$, 1.1% $K_2HPO_4$, 0.6% yeast extract) containing 1.0% glucose. To test for complementation, a loop of cells was scraped off and resuspended in 150 μl water to which was added 15 μl of Lugol's solution (2 g KI and 1 g $I_2$ per 300 ml water).

### *Expression of SBE in* E. coli *and generation of polyclonal antibodies*

A T7 RNA polymerase expression construct for *SBE A* was constructed by cloning the *Nco*I (Klenow)-*Xho*I fragment of pSJ90 into the *Eco*RV-*Xho*I sites of pET29a (Novagen). The acidic tail of the SBE A protein was deleted by cutting this clone with *Spe*I and *Xho*I, repairing the ends with Klenow polymerase and religation to create pSJ93. This fused a 6× histidine tag to the C-terminus of the protein (...VVYALVEHHHHHH). A 250 ml culture of pSJ93 in the *E. coli* strain BL21(DE3) was grown in 2YT medium to an optical density ($A_{600}$) of approximately 1.0, expression was induced by adding IPTG to 0.1 mM and growth continued for 90 min. Cells were collected by centrifugation and inclusion bodies (approximately 10 mg) were purified according to the manufacturer's instructions (Novagen). Rabbit polyclonal antibodies were generated using 1 mg of the inclusion bodies as the first inoculum and a booster injection of 1 mg of electrophoretically purified protein was given 1 month later.

An *E. coli* expression vector containing the mature potato SBE B protein with an N-terminal histidine tag was constructed by cloning a *Nsp*BII–*Xho*I fragment of a full length SBE B (Safford *et al.*, 1998) into the *Bam*HI (Klenow)–*Sal*I sites of pQE31 (Qiagen). Expression and purification of the guanidine-denatured protein on an Ni-NTA column was performed according to the manufacturer's instructions (Qiagen). Approximately 100–200 μg of protein was used to generate rabbit polyclonal antibodies.

### *RNA isolation and Northern analysis*

RNA was isolated from potato plants by the method of Logemann *et al.* (1987). For Northern analysis, 30 μg of total RNA was denatured in 40% formamide in 1× MOPS (0.02 M 3-N-Morpholinopropane sulphonic acid, 5 mM NaAcetate, 1 mM EDTA pH 7) buffer + bromphenol blue and electrophoresed on a 1.3% agarose gel in 1× MOPS buffer containing 15% (v/v) formaldehyde. Equal loading of RNA was checked by ethidium bromide staining. RNA was transferred to nylon membrane (HY-BOND N, Amersham) and UV crosslinked using the autosetting of the Stratalinker (Stratagene), air dried and baked at 80°C for 1 h before hybridisation as follows: the blot was pre-hybridised for approximately 1 h in a buffer containing 0.125 M $Na_2HPO_4$ pH 7.2, 50% formamide, 0.25 M NaCl, 7% SDS, 7% PEG 8000, 10 mM EDTA at 42°C before a random primed $^{32}$P-labelled DNA probe was added ($2\times 10^6$ cpm $ml^{-1}$) and hybridisation continued overnight. The blot was washed twice for 15 min at room temperature in 2× SSC, 0.1%

SDS, twice in the same buffer at 65°C and once at 65°C in 0.2× SSC, 0.1% SDS before exposing to X-ray film.

### *Transformation of potato*

Plant transformation vectors were constructed which placed an antisense 1.2 kb *SBE A-3* cDNA corresponding to the 3′ end of the gene between the double 35S promoter from Cauliflower mosaic virus (Guerineau *et al.*, 1988), or the potato GBSS promoter (Visser *et al.*, 1991), and the NOS poly(A) signal in the plant transformation vector pGPTV-HYG (Becker *et al.*, 1992). Plasmids were introduced into *Agrobacterium tumefaciens* LBA 4404 and used to transform tuber explants as described by Safford *et al.* (1998) except that the selection employed hygromycin B at 5 mg $l^{-1}$.

### *Preparation of plant extracts*

Plant material was extracted in buffer (4 ml $g^{-1}$ fresh weight) containing 100 mM Tris pH 7.5, 5% (v/v) glycerol, 2.5% (w/v) polyvinylpolypyrollidone, 0.1% (w/v) sodium metabisulfite, 2.5 mM 1,4-dithiothreitol, 5 mM EDTA, 2 mM Perfabloc-, 1 mM benzamidine. Leaf extracts required an additional gel filtration step to remove an inhibitor. Gel filtration was performed in Sephadex G-25 superfine (Pharmacia) spin columns. For some Western blot experiments, plant material was extracted directly in boiling SDS-sample buffer (4 ml $g^{-1}$ fw.).

### *Starch branching enzyme assay*

SBE was assayed using the phosphorylase a stimulation assay (Hawker *et al.*, 1974). The reaction was carried out at 30°C for 1 h in a final volume of 200 µl and contained 100 mM 2-(N-morpholino)ethanesulphonic acid (MES), 50 mM [U-$^{14}$C]glucose-1-phosphate (370 MBq $mole^{-1}$, ICN), 1.3 U rabbit muscle phosphorylase a (Sigma), 5–20 µl plant extract. Boiled plant extracts were used as controls. Glucose polymers were precipitated and washed as described by Hawker *et al.* (1974). The final pellet was dissolved in DMSO and counted with ReadySafe-scintillation cocktail (Beckmann). One unit of SBE activity is defined as the incorporation of 1 µmol glucose into a methanol insoluble polymer per minute.

### *Western blot analysis*

Extracts were separated by SDS-PAGE on 7.5% gels and blotted onto nitrocellulose using a semi-dry blotting apparatus (BioRad). Blotting was carried out over 1 h at 2 mA $cm^{-2}$ gel surface using a blotting buffer with 48 mM Tris, 0.035% SDS and 20% methanol. Blots were blocked with 5% skimmed milk powder in TBS-T (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h. Blots were incubated for at least 1 h in 1:1000 diluted rabbit anti-SBE B or II serum followed by three 15 min washes in TBS-T. The second incubation with 1:5000 diluted anti-rabbit-IgG horseradish peroxidase conjugate (Amersham) was carried out for 1 h. Following three further washes the blots were developed using an enhanced chemiluminescent kit (Amersham).

### *Starch extraction and analysis*

Starch was extracted and phosphorous levels determined as described by Safford *et al.* (1998). Starch was dissolved in DMSO and analysed by gel permeation chromatography (Shi *et al.*, 1998). Amylose content was determined by the iodine colourimetric method of Morrison and Laignelet (1983).

## Acknowledgements

The authors wish to thank Tina Sanders for help with plant transformation; Alice Belton and Gemma Smith for starch analysis; Yong-Cheng Shi for GPC analysis; Heidi Parsonage for expression of SBE B in *E. coli*; Bob Cowper for growth of the plants; and Jens Koßmann for the KV832 strain.

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EMBL database accession numbers *SBE A-1* (AJ011885), *SBE A-2* (AJ011886), *SBE A-3* (AJ011887), *SBE A-4* (AJ011888), *SBE A-5* (AJ011889), *SBE A-6* (AJ011890) and 5′RACE (AJ011891).