Discrete Forms of Amylose Are Synthesized by Isoforms of GBSSI in Pea

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Amyloses with distinct molecular masses are found in the starch of pea embryos compared with the starch of pea leaves. In pea embryos, a granule-bound starch synthase protein (GBSSIa) is required for the synthesis of a significant portion of the amylose. However, this protein seems to be insignificant in the synthesis of amylose in pea leaves. cDNA clones encoding a second isoform of GBSSI, GBSSIb, have been isolated from pea leaves. Comparison of GBSSIa and GBSSIb activities shows them to have distinct properties. These differences have been confirmed by the expression of GBSSIa and GBSSIb in the amylose-free mutant of potato. GBSSIa and GBSSIb make distinct forms of amylose that differ in their molecular mass. These differences in product specificity, coupled with differences in the tissues in which GBSSIa and GBSSIb are most active, explain the distinct forms of amylose found in different tissues of pea. The shorter form of amylose formed by GBSSIa confers less susceptibility to the retrogradation of starch pastes than the amylose formed by GBSSIb. The product specificity of GBSSIa could provide beneficial attributes to starches for food and non-food uses.

INTRODUCTION

Starch is composed of two glucan polymers: amylose, a predominantly linear $\alpha$-$1,4$-linked glucan, and amyllopectin, in which the $\alpha$-$1,4$-linked chains are branched extensively by $1,6$-linkages. In higher plants, starch is made both in chloroplasts (“transitory” starch) in leaves and in amyloplasts (“reserve” or “storage” starch) in nonphotosynthetic storage organs. The small, disc-shaped granules of transitory starch show little variation between different plant species (French, 1984). By contrast, the size and shape of storage starch, which forms relatively long-term reserves and is designed to be compact and stable but is metabolized readily on demand, is extremely diverse and species specific (for review, see Wang et al., 1998).

Both the ratio of amylose to amyllopectin in storage starch and the average size of the two polymers show significant variation between different plant species. These parameters also may differ between transitory and storage starch within one species and can vary with the developmental age of the plant organ and even within the granules themselves (Jane and Shen, 1992; Yun and Matheson, 1992). These parameters are important in determining the physical characteristics of starch, such as swelling, solubility, pasting, viscosity, and retrogradation.

In storage starch, amylose content can vary between 11 and 37%, but on average, it constitutes 30% of the total glucan (Shannon and Garwood, 1984). In transitory starch, its contribution is generally lower. The starch from rice and pea leaves contains $<15\%$ amylose (Taira et al., 1991; Tomlinson et al., 1997), and the starch of young tobacco leaves contains 15 to 17% amylose (Matheson and Wheatley, 1962).

The molecular mass of amylose varies between species. For example, wheat amylose has an average degree of polymerization of 570 Glc units (equivalent to a molecular mass of $\sim 100$ kD) compared with potato amylose, which is reported to have an average degree of polymerization of between 1500 and 6000 (equivalent to a molecular mass of between 250 kD and 1 MD) (Hizukuri and Takagi, 1984; Jane and Shen, 1992). It does contain some branches, but the frequency and length of these branches differ between the storage starches of different species (Shannon and Garwood, 1984).

Both amylose and amyllopectin are synthesized by starch synthases, which catalyze the transfer of Glc from ADP-Glc.
to the nonreducing end of glucan chains via an α-1,4-linkage. One isoform of starch synthase is bound exclusively to starch granules (granule-bound starch synthase I [GBSSI]). The other isoforms of starch synthase, which are both located in the plastid stroma and to varying extents bound to starch granules, synthesize amylopectin (soluble starch synthases). There is considerable evidence from mutation and antisense studies to suggest that specific starch synthase isoforms play different qualitative roles in determining amylopectin branch length distribution (Craig et al., 1998; Edwards et al., 1999b; Lloyd et al., 1999). However, the soluble starch synthases do not appear to be involved in amyllose synthesis.

GBSSI is bound tightly to the starch granule. The sequence of GBSSI is known for many plant species (Mason-Gamer et al., 1998). In contrast to the more diverse soluble isoforms, it is a very highly conserved protein of ~60 kDa. Amylose synthesis is associated with GBSSI activity. The waxy mutants of cereals (Weatherwax, 1922; Murata et al., 1965; Ishikawa et al., 1994; Nakamura et al., 1995), the amf mutant of potato (Hovenkamp-Hermelink et al., 1987), the lam mutants of pea (Denyer et al., 1995), and GBSSI antisense lines of potato (Visser et al., 1991) all involve the reduction or elimination of GBSSI activity and a specific reduction or elimination of amylose in the starch of the storage organs of these species.

The mechanisms by which different isoforms of starch synthase catalyze the same reaction and yet generate polymer variation are not fully understood, but there is increasing evidence to suggest that isoform localization within the plastid and fundamental differences in kinetics and reaction mechanisms are important determinants of product specificity (Smith et al., 1997; Denyer et al., 1999; Edwards et al., 1999a; Imparl-Radosevich et al., 1999; Commuri and Keeling, 2001). Furthermore, recent work has suggested that GBSSI, like the soluble starch synthases, may exist as more than one isoform.

The leaves and stem of waxy rice, the leaves and pericarp of waxy maize, the pericarp of waxy wheat, and the pods, leaves, and nodules of lam pea all contain amylose, implying that another gene (or genes) controls amyllose production in the organs of these plants (Shannon and Garwood, 1984; Taira et al., 1991; Denyer et al., 1997; Nakamura et al., 1998). A protein of similar molecular mass, antigenic properties, and N-terminal sequence to GBSSI has been detected in the leaves of lam pea (Denyer et al., 1997), and a cDNA encoding a GBSSI-like starch synthase has been isolated from waxy wheat and shown to be expressed in nonstorage tissues (Fujita and Taira, 1998; Nakamura et al., 1998; Vrinten and Nakamura, 2000).

The existence of two different forms of GBSSI, expressed in different parts of the plant in which the starches have a different polymer composition, suggests that GBSSI isoforms might have distinct characteristics that make specific contributions to amyllose synthesis, although the structural similarities between GBSSI proteins are so high that this is not an obvious inference. In potato, by contrast, only one isoform of GBSSI has been described, and mutation of the gene encoding this protein (AMF) results in the complete elimination of amyllose from tubers, leaves, and roots (Hovenkamp-Hermelink et al., 1987). This finding suggests that there is only one isoform of GBSSI active in the major starch-synthesizing tissues of potato.

In this article, we compare the molecular mass of amyllose partially purified from the starch of pea leaves and embryos by gel-permeation chromatography and show that pea leaves contain an amyllose that is distinct in its average molecular mass from that in embryos. A cDNA clone encoding a second isoform of pea GBSSI, designated GBSSIb (Denyer et al., 1997), is expressed in the leaves of pea and at a low level in pea embryos, where the first isoform, GBSSIa, is very highly expressed (Dry et al., 1992). Characterization of the in vitro activities of the two isoforms after the expression of both in Escherichia coli shows them to have distinct properties.

We analyzed the nature of their products in vivo by expression of each in an amyllose-free (amf; a GBSSI mutant) potato and show that both produce amyllose but that their products are distinct. The molecular mass of amyllose synthesized by GBSSIb in amf potato was the same as that of pea leaf amyllose, but the amyllose synthesized by GBSSIa was smaller. A difference in the physical properties of the starches synthesized by GBSSIa and GBSSIb also was observed. The significance of this difference with respect to the functional behavior of the polymers is discussed.

RESULTS

Pea Leaf Amylose Has a Greater Molecular Mass Than Pea Embryo Amylose

Amylose was partially purified from pea leaf and pea embryo starch by butanol precipitation, and its molecular mass distribution was analyzed by gel filtration chromatography. The results are shown in Figure 1. For both leaf and embryo, an initial contaminating amylepectin-containing peak (wavelength of maximal absorbance [λmax] of 550 nm) was eluted in fractions 18 to 23. Pea leaf amyllose (λmax of 610 nm) appeared to have a higher molecular mass (peak elution fractions 38 to 41, corresponding to a dextran equivalent peak molecular mass of 655,390 ± 10 D) (Figure 1A) than pea embryo amyllose (λmax of 620 nm; peak elution fractions 41 and 42, corresponding to a dextran equivalent peak molecular mass of 470,210 ± 10 D) (Figure 1B). The high λmax values indicate that the amyllose fractions had little or no contamination with branched glucans.

A cDNA encoding the major GBSSI protein in pea embryos, GBSSIa, has been cloned and characterized (Dry et
To determine whether the difference in molecular mass of the amyloses in leaf and embryo could be attributed to the activity of different GBSSI isoforms, we isolated a cDNA clone encoding a GBSSI that was highly expressed in pea leaves (GBSSIb) and compared its properties in vitro and in vivo with those of GBSSIa.

**Isolation and Characterization of a Full-Length cDNA Clone for Pea GBSSIb**

cDNA clones encoding the GBSSI isoform expressed highly in pea leaves were identified from a cDNA library made to mRNA from pea leaves using the potato GBSSI cDNA as a probe and washing at low stringency. The three positive clones isolated were identical over regions of shared sequence. The longest GBSSIb cDNA was 2100 bp in length, which included a 68-bp poly(A)\(^{+}\) tail and an open reading frame of 1839 bp flanked by 5‘ and 3‘ untranslated regions of 5 and 87 bp, respectively.

The derived amino acid sequence of GBSSIb predicts a 613-amino acid polypeptide of 67.6 kD, which shows significant similarity (68.8% identity and 75.6% similarity) to the predicted sequence of pea GBSSIa (Figure 2A). The N-terminal region of full-length GBSSIb contains the amino acid sequence 5‘-GMNLIFVGTAPWLSKTGGLGDVL-3‘, which is identical to that obtained from protein sequencing of GBSSIb extracted from lam pod starch (Denyer et al., 1997).
Figure 2. Comparative Analysis of Pea GBSSI Isoforms.

(A) Comparison of the derived amino acid sequences of pea GBSSib (top) and pea GBSSIa (bottom) aligned by GAP (gap weight of 3.0, length weight of 0.1; Devereux et al., 1984). The first amino acid of the mature GBSSIa and GBSSIb proteins is boxed in dark gray. The N-terminal KTGGL motif thought to interact with ADP/ADP-Glc and the C-terminal KTGGL look-alike motif thought to influence kinetic properties are boxed in light gray.
This sequence corresponds to the N terminus of mature GBSSIb, which is predicted to have an 85- amino acid transit peptide.

Mature GBSSIb is a protein of 58.4 kD (528 amino acids) and has a predicted pl of 6.44. Mature GBSSIa is a protein of 58.3 kD (528 amino acids) with a predicted pl of 6.22. The two mature peptide sequences are 73% identical (80% similar). Both possess the N-terminal KTGGL motif thought to be responsible for binding ADP/ADP-Glc and the C-terminal KTGGL “look-alike” motif that has been shown to influence the kinetic properties of potato GBSSI in vitro (Edwards et al., 1999a).

However, a database search showed that mature GBSSIb is more similar (76.5% identity and 83.5% similarity) to mature potato GBSSI than it is to GBSSIa from pea. A phylogenetic tree of GBSSI from several species is shown in Figure 2B. There appear to be two major classes. One class consists entirely of GBSSI from monocots. The second class includes all of the GBSSI forms from dicots and one isoform from wheat, GBSSII, which is expressed in leaves (Vrinten and Nakamura, 2000). This second class is subdivided, with the pea embryo GBSSIa clustering with the GBSSI from another legume, bean.

**Differential Expression of GBSSI Isoforms in Pea**

The expression of GBSSIa and GBSSIb in pea leaf and pea embryo was examined by quantitative reverse transcriptase–mediated PCR, which indicated that the two isoforms were expressed differentially. GBSSIa was found to be highly expressed in embryos. The GBSSIa transcript also was detected in leaves. These results confirmed data previously reported by Dry et al. (1992). Low levels of the GBSSIb transcript were detected in embryo. GBSSIb was most highly expressed in leaves, particularly young leaves (Figure 3).

**Expression of GBSSIa and GBSSIb in E. coli**

To determine whether the different isoforms of GBSSI from pea had different enzyme specificities, both proteins were expressed in *E. coli* for analysis of their kinetic properties. For all expression analyses, GBSSI proteins with an N-terminal S-tag fusion peptide were used. Both GBSSIa and GBSSIb were synthesized in approximately the same amounts in the soluble phase of *E. coli* (0.024 to 0.026 µg/µL, as measured by the S-tag assay), and both had the correct molecular mass of ~60 kD, as judged by protein gel blot analysis (Figure 4).

No significant starch synthase activity was found associated with GBSSIa under any of the conditions tested. This agrees with the results obtained when GBSSIa was solubilized from pea embryos (Smith, 1990) and suggests that this isoform is inactive when not bound to starch granules and that the appropriate conditions for assay in solution have not yet been established.

In contrast, GBSSIb had activity that was very similar to that of GBSSI from potato tuber (Edwards et al., 1999a). The
The kinetics of pea GBSSIb and potato GBSSI (both expressed in *E. coli*) are compared in Tables 1 and 2. GBSSIb is a more active enzyme than potato GBSSI. Its specific activity of \(0.6 \text{ mol ADP-Glc·min}^{-1}\text{·mg}^{-1}\) at 5 mg/mL amylopectin and 1 mM ADP-Glc was somewhat higher than that of the potato enzyme (0.2 \(\text{ mol ADP-Glc·min}^{-1}\text{·mg}^{-1}\)) under the same conditions, as was the activation by 50 mg/mL amylopectin and by 0.5 M citrate (Table 2). The specific activity of GBSSIb with maltooligosaccharides was approximately threefold greater than that of potato, although the activation in combination with amylopectin was approximately the same.

Like potato GBSSI, pea GBSSIb exhibited a lag in initiating glucan synthesis of 1 to 4 min, depending on amylopectin concentration (data not shown). GBSSIb showed processive rather than distributive activity (the enzyme preferentially added successive Glc units to a single glucan chain rather than to different chains) when supplied with 100 mM maltotriose and 2.5 mg/mL amylopectin (data not shown), as does potato GBSSI (Denyer et al., 1999; Edwards et al., 1999a).

Table 1. Comparison of the Activities of GBSSIb from Pea and GBSSI from Potato Tuber

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>AP (mg/mL)</th>
<th>ADP-Glc (mM)</th>
<th>AP (K_m) (mg/mL)</th>
<th>ADP/Glc (K_m) (mM)</th>
<th>(V_{max}) ((\mu\text{mol·min}^{-1}·\text{mg}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea leaf GBSSIb</td>
<td>0–25</td>
<td>1</td>
<td>15.0 ± 7.0</td>
<td>0.9 ± 0.1</td>
<td>2.7 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0–2</td>
<td>13 ± 2</td>
<td>1.2 ± 0.2</td>
<td>19 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0–2</td>
<td>10.0 ± 1.0</td>
<td>1.3 ± 0.1</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>Potato GBSSI</td>
<td>0–25</td>
<td>1</td>
<td>10.0 ± 1.0</td>
<td>1.3 ± 0.1</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0–2</td>
<td>1.1 ± 0.2</td>
<td>2.0 ± 0.0</td>
<td></td>
</tr>
</tbody>
</table>

Expression of GBSSIa and GBSSIb in the *amf* Mutant of Potato

We were unable to detect any activity from solubilized GBSSIa from pea embryos or from GBSSIa expressed in *E. coli* and assayed in vitro. To determine whether the enzyme encoded by this cDNA was capable of synthesizing amyllose, we introduced it under the control of a strong promoter into the *amf* mutant of potato, which does not make amyllose (as a result of mutation of the GBSSI gene; Hovenkamp-Hermelink et al., 1987). We compared the activity of GBSSIa with that of GBSSIb from pea controlled by the same promoter in *amf*. Further controls included the *amf* mutant, a wild-type diploid line (which was formed by doubling up from the haploid progenitor of the *amf* line [Visser et al., 1989]; the *amf* line also is diploid after chromosome doubling of the haploid), and the *amf* mutant transformed with the genomic clone of potato GBSSI (BW101; van der Leij et al., 1991).

Starch from developing tubers of independent transformants was screened for the presence of GBSSI by Coomassie blue staining of polyacrylamide gels and by protein gel blot analysis (Figure 5). For the GBSSIa lines, 24 of 50 transformants were found to express substantial amounts of GBSSIa comparable to the amount of GBSSI found in wild-type potato (Figure 5A). Lines Ia.5, Ia.7, Ia.12, and Ia.3 (used as a control because no GBSSI protein was visible) were
used for further analysis. The transformation efficiency of plasmid pBinGBSSlb was approximately the same as that of plasmid pBinGBSSla, but the expression levels of GBSSlb generally were relatively low, especially compared with the amount of GBSSI in wild-type potato (Figure 5B).

This may have been because the gene construct had a rather short leader sequence (49 nucleotides), which could have limited the efficient translation of GBSSlb in transgenic potato, or because unusual codons at the start of the GBSSlb sequence limited translation in potato because the levels of GBSSla and GBSSlb transcript were equivalent in the transgenic lines used for further analysis (data not shown). The GBSSlb-expressing lines Ib.12 and Ib.4 were studied further, together with the nonexpressing line Ib.8.

Starch Synthase Activity and Protein Synthesis in amf Potato

The activities of granule-bound starch synthase from the transgenic lines are summarized in Table 3. These activities were assayed on the isolated and washed starch granules and did not involve solubilization of GBSSI. All of the lines expressing GBSSI protein exhibited significantly higher starch synthase activity on the granules than the amf control. When the transgenic plants were corrected for background activity measured in the amf control, Ia.12, with the highest level of GBSSla expression, had activity that was between 14.5 and 23.7% of that of the wild type or the fully complemented mutant (BW101), whereas Ib.12, with the highest level of GBSSlb expression, had activity between 5.7 and 9.3% of that of the wild type or the fully complemented mutant (BW101).

When these results were compared with the amounts of GBSSI made in each line (Table 4), it was clear that GBSSla encoded an active GBSSI. However, the specific activity of GBSSlb in the amf background was much higher than that of GBSSla. Ia.12 produced GBSSla protein at a level equivalent to the level of potato GBSSI in wild-type potato (0.25 μg GBSSI protein/mg starch; Figure 5A), whereas Ib.12 synthesized 250 times less GBSSlb protein (0.001 μg GBSSI protein/mg starch; Figure 5B). The results are summarized in Table 4 and show that although GBSSla is active in vivo, GBSSlb encodes a distinct protein with a significantly higher specific activity than GBSSla (at least 10-fold higher) in vivo.

Interestingly, comparison of enzyme activities in solution (Table 1) and on the starch granule (Table 4) implies that both proteins are activated significantly by association with the starch granule. In fact, activation probably requires the incorporation of GBSSla into the starch granule, because in vitro assays of soluble GBSSla (expressed in E. coli) with added starch granules did not show starch synthase activity. The precise degree of activation was impossible to calculate for GBSSla, because no activity could be detected for the soluble form. For GBSSlb, the specific activity of the soluble form expressed in E. coli was calculated to be 0.3 μmol-min⁻¹·mg⁻¹ GBSSI protein (with maltooligosaccharides as substrate; Table 2), and on the amf starch, it was measured as 3.4 μmol-min⁻¹·mg⁻¹ GBSSI protein (a 10-fold activation).

High-Performance Anion-Exchange Chromatography Analysis of the Soluble Products of GBSSla and GBSSlb

To determine whether GBSSla and GBSSlb expressed in the transgenic amf lines had a processive or a distributive reaction mechanism, isolated starch granules from these lines were incubated with ADP-¹⁴C-Glc, maltotriose, and amylopectin, and the sizes of the reaction products were analyzed by high-performance anion-exchange chromatography. Control granules from the amf line and the amf line

Table 2. Activity of Pea GBSSlb and Potato GBSSI in the Presence of Different Concentrations of Amylopectin Substrate and Other Effectors

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Pea Leaf GBSSlb Activity (μmol·min⁻¹·mg⁻¹ GBSSI protein)</th>
<th>Potato GBSSI Activity (μmol·min⁻¹·mg⁻¹ GBSSI protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg/mL amylopectin</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>50 mg/mL amylopectin</td>
<td>2.9 ± 1.5</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Fold activation by amylopectin</td>
<td>7.3</td>
<td>5.6</td>
</tr>
<tr>
<td>0.5 M citrate + 5 mg/mL amylopectin</td>
<td>6.4 ± 3.2</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>Fold activation by citrate</td>
<td>10.7</td>
<td>9.3</td>
</tr>
<tr>
<td>0.5 M citrate + 50 mg/mL amylopectin</td>
<td>17.6 ± 8.8</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>Fold activation by amylopectin at 0.5 M citrate</td>
<td>6.0</td>
<td>2.5</td>
</tr>
<tr>
<td>50 mM MOS</td>
<td>0.3 ± 0.1</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Fold activity relative to 5 mg/mL amylopectin</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>100 mM MOS + 5 mg/mL amylopectin</td>
<td>2.4 ± 0.1</td>
<td>0.6 ± 0.0</td>
</tr>
<tr>
<td>Fold activation by amylopectin on MOS</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

MOS, maltooligosaccharides.
expressing potato GBSSI (BW101) were used. The amf granules made only maltotetrose (as a result of the distributive reaction of endogenous potato SSII and SSIII). BW101 granules (expressing potato GBSSI) displayed a highly processive reaction. Granules expressing either pea GBSSIa or GBSSIb showed processive elongation of glucan chains (data not shown).

Analysis of Transgenic Starch

Iodine Staining

Transgenic starch was stained with Lugol’s iodine and compared with amf starch. Granules from the amf line stained red-brown because of the absence of amylose (Figure 6B). Wild-type starch granules stained blue with clearly defined darker staining rings caused by the presence of amylose (Figure 6A). Blue-staining material was seen in both la and lb lines expressing the pea GBSSI proteins (Figures 6C and 6D). In the la lines, the blue-staining material was confined essentially to a series of rings, although sometimes there were granules with solid blue-staining cores. The rings tended to fade toward the periphery, but the staining pattern was quite uneven.

In some granules, the most intensely staining ring was closer to the periphery than to the center. In the lb lines, there were many fewer blue cores and fewer but more evenly stained rings. These differences in amylose localization were reproduced in independent transgenic lines. The significance of these differences in staining pattern between GBSSIa and GBSSIb starch is under investigation, but they suggest that the two isoforms may synthesize amylose preferentially in different regions of the starch granule.

Table 3. Starch Synthase Activity Measured on Starch Granules from Potato Lines

<table>
<thead>
<tr>
<th>Line</th>
<th>GBSS Activity (nmol·min⁻¹·mg⁻¹ starch)</th>
</tr>
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<tbody>
<tr>
<td>Amf</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>Diploid</td>
<td>0.287 ± 0.061</td>
</tr>
<tr>
<td>BW101</td>
<td>0.463 ± 0.061</td>
</tr>
<tr>
<td>PeaIa.3</td>
<td>0.018 ± 0.004</td>
</tr>
<tr>
<td>PeaIa.5</td>
<td>0.048 ± 0.011</td>
</tr>
<tr>
<td>PeaIa.7</td>
<td>0.067 ± 0.009</td>
</tr>
<tr>
<td>PeaIa.12</td>
<td>0.074 ± 0.001</td>
</tr>
<tr>
<td>PeaIb.8</td>
<td>0.009 ± 0.003</td>
</tr>
<tr>
<td>PeaIb.4</td>
<td>0.032 ± 0.010</td>
</tr>
<tr>
<td>PeaIb.12</td>
<td>0.034 ± 0.006</td>
</tr>
</tbody>
</table>
Quantitative and Qualitative Analysis of Amylose

Amylose, as judged by iodine staining, was synthesized by both GBSSla and GBSSlb in the amf background. The amylose contents of the starch from the highest expressing lines for la.12 and lb.12 were calculated to be 1 and 0.8%, respectively, compared with 20% for the wild-type diploid and the potato GBSSI-complemented amf line, BW101. This represents a 5% restoration of amylose synthesis by pea GBSSla and a 4% restoration by pea GBSSlb.

In the case of pea GBSSlb, the failure of the pea protein to fully complement amylose production in the amf line probably was attributable to the low levels of GBSSlb production achieved (Figure 5B). However, in the case of GBSSla, high levels of protein (equivalent to those of wild-type GBSSI) were produced in the transgenic lines (Figure 5A). This finding implies that GBSSla encodes an isoform of GBSSI distinct from that in potato that cannot fully complement potato GBSSI activity.

When amyloses from lines la.12 and lb.12 were concentrated by butanol precipitation and analyzed by gel permeation chromatography, they had different molecular masses (Figures 7A and 7B). In line la.12, the mass of amylose (peak fractions 42 to 43; peak measured as 367,320 ± 10 D) was significantly smaller than that of lb.12 (peak fraction 39; peak measured as 655,390 ± 10 D). Amylose from line lb.12 appeared to have a molecular mass that was very similar to that of pea leaf amylose (peak at 655,390 ± 10 D; Figure 1A). The amylose from pea embryos appeared to be intermediate in size (peak at 470,210 ± 10 D; Figure 1B) between that in la.12 potato starch (peak at 367,320 ± 10 D) and that in lb.12 potato starch (peak at 655,390 ± 10 D).

These findings suggested that GBSSla was not the only enzyme that contributed to amylose synthesis in pea embryo. To investigate this further, amylose was concentrated from the lam mutant of pea (SIM 503; Denyer et al., 1995), which lacks GBSSla protein and activity. Gel permeation chromatography revealed that the molecular mass of this amylose (peak at 614,240 ± 0 D) was similar to that of pea leaf, potato lb.12, and wild-type potato starch (Figures 7C and 7D) and implied that GBSSlb contributes to amylose synthesis in pea embryos, as does GBSSla. This conclusion is supported by the presence of a small amount of a 58-kD protein on starch from lam pea embryos (Denyer et al., 1997).

By contrast, GBSSlb synthesized most of the amylose in pea leaves. Although GBSSla was expressed at a low level in pea leaves (Figure 3), the low specific activity of this protein suggests that it makes a very minor contribution to the synthesis of amylose in pea leaves compared with GBSSlb. To confirm that the size of the amylose synthesized was principally a function of the activity of the specific isoform of GBSSI in a particular tissue, amylose from amf lines expressing GBSSla was extracted. The amount of amylose in this leaf starch was extremely low, but it clearly had a low peak molecular mass (370 kD), typical of the product of GBSSlb, as seen in amf potato tubers (Figure 7E).

The sizes of amyloses from wild-type and GBSSlb potato leaves are shown, for comparison, in the supplementary material (see supplementary data online). No amylose was detected in amf leaf starch. Therefore, GBSSlb makes low molecular mass amylose in both tubers and leaves, establishing GBSSI isoform specificity as a major determinant of amylose functionality in higher plants.

Our kinetic data indicated that GBSSlb was very similar to potato GBSSI in its activity and specificity (Tables 1 and 2). Interestingly, the potato amylose from the diploid control line had a relatively high molecular mass, comparable to that synthesized by GBSSlb in the amf potato line (Figures 7C and 7D).

Effects of GBSSI Isoforms on Short Chains of Amylopectin

It has been suggested that GBSSI also contributes to amylopectin biosynthesis, either through the synthesis of intermediates in amylose production (van de Wal et al., 1998) or through a direct contribution to the long chains in amylopectin formation, as suggested for Chlamydomonas (Colleoni et al., 1999) and higher plants (McPherson and Jane, 1999).

Fluorophore-assisted gel electrophoresis was used to

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<th>Table 4. Starch Synthase Activity on Washed Granules from Wild Type, amf plus GBSSla, and amf plus GBSSlb Potato Lines</th>
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<tr>
<td>Wild Type</td>
</tr>
<tr>
<td>GBSS activity (nmol·min⁻¹·mg⁻¹ starch)</td>
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<tr>
<td>GBSS activity (% of wild type)</td>
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<td>GBSS protein (µg/mg starch)</td>
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<td>GBSS protein (% of wild type)</td>
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<tr>
<td>Specific activity (µmol·min⁻¹·mg⁻¹ GBSS)</td>
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<td>Specific activity (% of potato GBSSI activity in wild type)</td>
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Assays were performed on freshly purified, acetone-washed starch from individual tubers of plants late in tuber development. Values are means ± SE from measurements on batches of starch from five separate tubers. For each line, tubers were from at least two separate plants.
examine the chain length distribution of the amylopectin shorter chains (6 to 35 Glc units) after debranching with isoamylase. No significant differences were observed between any of the transgenic lines, the amf mutant, the wild type, or the fully complemented mutant BW101 (data not shown). We conclude that GBSSla and GBSSlb make no contribution to the synthesis of short glucan chains in amylopectin. However, in our hands, fluorophore-assisted gel electrophoresis did not resolve chain lengths with a degree of polymerization >35 with accuracy, so it is possible that these proteins could contribute to the synthesis of long chains in amylopectin.

Physicochemical Analysis of Potato Starches

Starch is used for many applications in food processing and for nonfood industrial uses. These applications of starch depend on its physicochemical properties, which depend on many factors, including the ratio of amylose to amylopectin. The cooking properties of many flours also are determined by the physicochemical characteristics of their starches (Shibanuma et al., 1996; Zeng et al., 1997; Noda et al., 2001), which are correlated closely with the staling properties of bread flours and the cooking quality of noodles (Collado and Corke, 1997; Boggini et al., 2001; Noda et al., 2001). We examined the physicochemical properties of the starches from the potato lines expressing GBSSla and GBSSlb to determine whether the molecular mass of amylose has a significant impact on the physical properties of starch.

We measured two features, the Tm of the starch granules and the solution properties of the starches, because these parameters are particularly dependent on amylose content. The starches of the wild type, amf, amf expressing GBSSla (lines Ia.5 and Ia.12), and amf expressing GBSSlb (line Ib.12) were compared. Line Ia.5 was included in this comparison because its amylose content was exactly the same as that of line Ib.12 (0.8%), whereas line Ia.12 had slightly higher amylose composition (1.0%).

Differences in the Tm of the starches were measured by differential scanning calorimetry (DSC). The onset temperature (T onset) for melting of wild-type potato starch was 63.9°C and that of amf was 70.3°C, confirming that amylose reduces Tm significantly. The T onset of Ia.12 starch was 70.7°C, that of Ia.5 was 70.5°C, and that of Ib.12 was 67.5°C. These data show that the small amount of amylose present in the Ib.12 starch significantly decreased the T onset for starch melting. However, the equivalent amount of amylose in Ia.5 or slightly more in Ia.12 starch did not shift the T onset significantly from that observed for amf starch. These data indicate that the molecular mass of amylose does influence the T onset for melting of starch granules.

The solution properties of the starches were examined using a rheometer, which involves measuring viscosity changes while the starch suspension is heated and then cooled with constant stirring. This type of analysis provides

Figure 6. Iodine Staining of Starch Granules.
Starch granules were viewed in a hydrated state in a 10-fold dilution of Lugol’s iodine.
(A) Wild type.
(B) amf.
(C) Transgenic line Ia.12.
(D) Transgenic line Ib.12.
Discrete Forms of Amylose Made by GBSSI Isoforms

Insight into how the starch may behave as a paste or a sauce. Upon heating, the granules swell and the viscosity of the suspension increases. A point of maximum swelling is reached, known as the peak paste viscosity. Upon further heating, the viscosity decreases (Flipse et al., 1996; Visser et al., 1997). After heating and cooling, the starch suspensions are kept at 20°C for 15 min.

During this period, the viscosity increases again, a process known as retrogradation, which is a negative attribute of starches for food uses and for nonfood uses as coatings and paints, because it involves the formation of “skins.” Although retrogradation can be strongly influenced by the amylpectin component of starch, it also is correlated positively to increasing amylose content (Visser et al., 1997).

The details of the rheological analysis of wild-type, amf, la.5, and la.12 starches are shown in Figure 8A. All of the starches started to increase in viscosity at a similar temperature, and the differences in T_onset observed in the DSC analysis were not observed using this method. The peak pasting viscosities differed considerably between wild-type and amf starch, but the inclusion of low levels of amylose in lines la.5 and la.12 reduced the peak pasting viscosity compared with that in amf starch, indicating that this feature is not related linearly to amylose content.

Upon cooling, the setback (the final viscosity of the starch gel) was much higher for wild-type starch than for amf starch, demonstrating the importance of amylose content to retrogradation. The increasing amounts of amylose in la.5 and la.12 starches increased the setback.

Figure 7. Gel Permeation Chromatography of Partially Purified Amylose from Transgenic amf TUBERS, Pea Embryos, Wild-Type Potato, and Potato Leaves.

Amylose was partially purified by butanol precipitation, solubilized, and subjected to chromatography on Sepharose CL-2B columns. Fractions eluted from the columns were mixed with an iodine solution, and absorbance was measured at 595 nm (lines).

(A) Amylose from transgenic line GBSSIa.
(B) Amylose from transgenic line GBSSIb.
(C) Amylose from laam pea embryos (the 503 allele, which produces no GBSSIa RNA or protein, was used). For comparison, amylose from wild-type pea embryos is shown as a dotted line.
(D) Amylose from a wild-type (WT) diploid line of potato. The molecular mass of wild-type potato amylose is very similar to that synthesized by GBSSIb in amf potato (B). The regression line for the dexTRAN size markers used to calibrate the column for all samples is shown as a dotted line.
(E) Amylose from leaves of an amf line expressing GBSSIa.

Closed circles indicate the elution profile of glucan determined by iodine staining. Closed squares indicate the wavelength of maximum absorbance of the starch-iodine complex (λ_max), and the peak λ_max values indicate clearly the size of amylose in this leaf starch. For a comparative analysis of the amylose in total starch preparations from GBSSIa, GBSSIb, wild type, and amf potato leaves, see the supplementary data online. In each case, results from typical experiments are shown.
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Figure 8. Changes in the Storage Moduli of 2.5% (w/v) Potato Starch Suspensions during Heating and Cooling (rapid viscometric analysis).

(A) Profiles of starches with increasing amylose contents. Solid line, amf starch; open circles, starch from line la.5 (0.8% amylose); closed circles, starch from line la.12 (1.0% amylose); open squares, wild-type starch; dotted line, temperature.

(B) Profiles from la.5 starch with 0.8% amylose of lower molecular mass (open circles) and from lb.12 starch with 0.8% amylose of higher molecular mass (open triangles); dotted line, temperature.

We compared the starches from lines la.5 and lb.12, which had identical amylose contents (0.8%), to determine the influence of amylose molecular mass on the rheological profiles. These data are shown in Figure 8B. The T\textsubscript{onset} and the peak pasting viscosities of the two starches were identical, but the setback of la.5 starch was lower than that of lb.12 starch, indicating that the longer amylose produced by GBSSIb is more susceptible to retrogradation than the shorter amylose produced by GBSSIa.

DISCUSSION

Isoforms of GBSSI in Pea

Our analysis of genes encoding GBSSI in pea has demonstrated that at least two genes that encode isoforms can catalyze the incorporation of Gic from ADP-Glc into glucan. Both isoforms are tightly bound to starch granules and therefore are granule-bound starch synthases. Their deduced primary amino acid sequences support this interpretation. Despite their structural similarity, these isoforms of GBSSI are not identical. First, they are expressed differentially in plant organs, GBSSIa being expressed predominately in pea embryos, where storage starch is synthesized, and GBSSIb being expressed predominantly in leaves, where transitory starch is synthesized, although it is expressed in embryos as well (Denyer et al., 1997). This is similar to the situation reported for wheat, in which GBSSI is expressed in endosperm and a second isoform, GBSSII, is expressed in leaves, pericarp, and aleurone (Vrinten and Nakamura, 2000).

The two isoforms of GBSSI from pea also have distinct catalytic properties. GBSSIa activity cannot be measured in vitro using standard assay procedures for starch synthases, whereas GBSSIb activity is detected readily in vitro. Comparison of the kinetic properties of GBSSIb with those of other GBSSI proteins suggests that they are very similar to those of potato GBSSI in terms of its specific catalytic properties and the size of the amylose it synthesizes in vivo. Interestingly, both GBSSIa and GBSSIb are activated upon incorporation into starch granules. The degree of activation is impossible to estimate for GBSSIa because no activity can be detected in vitro (implying a very significant activation upon association with starch granules). For GBSSIb, we estimate activation upon association with the starch granule to be at least 10-fold.

Differences in the Products of GBSSI Isoforms

The two isoforms of GBSSI from pea synthesize distinct forms of amylose. GBSSIa forms a shorter type of amylose than GBSSIb. This is shown quite clearly by comparison of the amylose made in potato amf lines transformed with either GBSSIa or GBSSIb. The features of the enzymes that determine product specificity are not clear. It is possible that differences in the affinity of the isoforms for glucans favor the production of shorter amylose by GBSSIa and longer amylose by GBSSIb. Alternatively, the location of
each GBSSI isoform within the starch granule might influence the product it synthesizes. Differences in the location of the amylose synthesized by each isoform across starch granules were apparent from analysis of amf transgenic lines.

Differences in the location of the enzymes (or active enzymes) within the granule might influence product specificity through differences in the supply of ADP-Glc substrate (concentration and rate of diffusion) to different places and in the space available for the synthesis of new glucan. The importance of granule association to GBSS1a activity is particularly clear, because no activity of this isoform was detected in vitro. In addition, microheterogeneity within the granule might influence both where this enzyme is active and the specific nature of its product.

Our analysis showed that pea leaves contain a longer form of amylose in their starch granules than do pea embryos. There have been reports of many factors that influence the molecular mass of amylose, including the developmental stage of the tissue (Yun and Matheson, 1992), whether the starch is transitory or storage starch, the relative activity of GBSSI (Fulton et al., 2002), and the particular location of activity within the starch granule (Jane and Shen, 1992). Our data show that the specific isoform of GBSSI also is a major determinant of the molecular mass of amylose.

This specificity in the product of different GBSSI isoforms represents an additional explanation for the differences in the molecular mass of amylose that has not been considered previously. Although GBSSIa is expressed weakly in leaves, its relatively low expression, coupled with its relatively low specific activity (compared with that of GBSSIb), indicates that GBSSIb is the major GBSSI isoform activity in leaves. Embryo starch consists of amylose that is intermediate in size between the product of GBSSIa and that of GBSSIb. This finding indicates that both GBSSIa and GBSSIb operate to synthesize amylose in pea embryos.

Both proteins can be detected in embryos bound tightly to starch (Denyer et al., 1997), and the analysis of starch from lam embryos confirms GBSSIb to be present and active, because lam (line 503) embryos lack GBSSIa activity yet still make some amylose (estimated at between 4 and 10% total glucan compared with 30% for wild-type embryos) (Wang et al., 1998). This amylose is of higher average molecular mass than that in wild-type embryos and is similar to the product of GBSSIb in potato.

GBSSIa and GBSSIb may be expressed in the same cells of the embryo, and the amylose produced would be the result of their combined specificities. Alternatively, GBSSIa and GBSSIb may be expressed in different cells of the embryo, but upon extraction, the starch granules they synthesize would become mixed. The fact that GBSSIa and GBSSIb synthesize discrete forms of amylose in pea embryos suggests that isoform specificity is a major determinant of amylose molecular mass, at least in the starch of pea embryos.

Our data provide evidence for specificity in starch composition being provided by the differential use of GBSSI isoforms in different tissues of the plant. A recent phylogenetic analysis of the Rosaceae has shown two loci encoding GBSSI in all subfamilies (Evans et al., 2000), suggesting that more than one isoform of GBSSI may be active in some other dicot species, although only one GBSSI gene has been found in the genome sequence of Arabidopsis and only one active form has been identified in potato. In wheat, two structurally distinct isoforms are expressed in different tissues (Fujita and Taira, 1998; Nakamura et al., 1998; Vrinten and Nakamura, 2000), and there is evidence for at least two genes that encode GBSSI in rice, barley, and maize (Vrinten and Nakamura, 2000).

Based on structural analysis, the GBSSI proteins of cereal storage organs appear quite distinct from those active in dicot storage organs (Figure 2B). These structural differences may imply differences in catalytic activity/specificity, which also could affect product specificity. It has been reported that cereal endosperm starch contains amylose of higher molecular mass than that in pea embryos but lower than that in potato tubers (Gerard et al., 2001). If the cereal endosperm GBSSIs constitute a catalytically distinct subclass of GBSSI proteins, it might be possible to introduce new specificity to amylose production into dicot species such as potato using a cereal GBSSI gene, as we have done with GBSSIa from pea, or, more significantly, to introduce new specificity to amylose production in cereal endosperm using the potato GBSSI or the pea GBSSIb gene.

Interestingly, a GBSSI gene from cassava failed to complement the amf mutant in full production of amylose when transformed into this line of potato. These data suggest that cassava GBSSI and potato GBSSI also may be distinct in their activities in plants (Salehuzzaman et al., 1999).

Our experimental analysis of GBSSI isoforms from pea in potato was complicated by the very low levels of expression of GBSSIb we were able to obtain in the amf line. This meant that none of the transgenic lines showed full complementation of the amf phenotype, despite GBSSIb being a protein with very similar activity and specificity to the potato GBSSI encoded by the AMF locus and expressed in potato tubers. We do not know the reason why such low levels of GBSSIb protein were obtained in our transgenic experiments, but we suspect problems as a result of inefficient translation caused by the short leader sequence in the gene construct or differences in codon use between pea and potato, which might have limited GBSSIb protein production, because mRNA levels for the two genes in the different potato lines were equivalent.

However, one advantage of comparing lines with low GBSSI activity with those expressing GBSSIa is that lines with similar production of amylose were compared despite the very different specific activities of the two isoforms in vivo. We conclude that differences between the amyloses synthesized by the two isoforms resulted from different product
Given the facts that GBSSIb is a significantly more active enzyme than GBSSIa, both in vitro and in vivo (i.e., it has a much higher specific activity), and that recent data suggest that the molecular mass of amylose is correlated positively with the level of GBSSI activity in potato (Fulton et al., 2002), comparison of starch products from lines with similar GBSSI activities has been central to establishing the product specificity of the different isoforms. Comparison of starches from lines with equivalent amounts of amylose also has been important in establishing whether or not the distinct forms of amylose synthesized by GBSSIa and GBSSIb confer distinct physical properties on the starches.

**Physical Properties of Starches Synthesized by Different GBSSI Isoforms**

The amylose composition of the starches affected their melting behavior, as shown by DSC, and their pasting behavior, as shown by rheometer measurements. The $T_{\text{onset}}$ for wild-type starch was significantly lower than that of amf starch, which lacks amylose. The starch containing a small amount of amylose synthesized by GBSSIb had an intermediate $T_{\text{onset}}$, whereas the starch containing amylose synthesized by GBSSIa had the same $T_{\text{onset}}$ as amf starch. These data suggest that the molecular mass of the amylose does influence this parameter, because Ia.5 and Ib.12 starches had equivalent amylose contents. However, these differences in $T_{\text{onset}}$ were not evident by rheological analysis.

Retrogradation also is a very important feature of starches; it involves the tendency of starches to form skins after heating. Retrogradation can be measured from the viscosity changes that occur upon cooling of starches. Amylose composition is associated strongly with retrogradation, so the wild-type starch increased in viscosity quickly after cooling, whereas the amf starch showed a very slow, low-level increase in viscosity. Significantly, the starch synthesized by GBSSIb (Ib.12) showed a more rapid increase to a higher viscosity than the starch synthesized by GBSSIa (Ia.5) after cooling. These results suggest that the shorter amylose synthesized by GBSSIa may provide beneficial properties to starch pastes in terms of retrogradation.

**METHODS**

**Preparation of Starch**

Starch was prepared essentially according to the methods of Edwards et al. (1995) and Tomlinson et al. (1997). Starch was prepared from the amylose-free (amf) mutant of potato (*Solanum tuberosum*) (Hovenkamp-Hermelink et al., 1987) and from leaves and tubers of transgenic lines derived from it. Tubers were of comparable sizes when harvested. Leaves were harvested at the end of the day. Starch also was analyzed from a wild-type diploid line (which was formed by doubling up from the haploid progenitor of the amf line [Visser et al., 1989]; the amf line also is diploid after chromosome doubling of the haploid) and the amf mutant transformed with the genomic clone of potato GBSSI (BW101; van der Leij et al., 1991). Starch was prepared from pea (*Pisum sativum*) embryos and leaves of an inbred round-seeded line (BC1/9RR) and from the low-amylose (lam) line 503, which makes no GBSSIa protein (Denyer et al., 1997).

**Starch Analysis**

**Partial Purification of Amylose Using Butanol**

The method of amylose purification was based on that of Schoch (1942). Starch (1.33 g) was heated in 100 mL of distilled water at 100°C until gelatinized. Phosphate buffer (20% [w/v] phosphate solution: 16.4% anhydrous KH$_2$PO$_4$ and 3.6% anhydrous K$_2$HPO$_4$) was used to adjust the pH to 5.9 to 6.3, and the starch was autoclaved for 1 h at 121°C. The starch then was refluxed for 1 h at 100°C, after which 25 mL of butan-1-ol was added and refluxing was continued for 1 h. The starch was sealed in a 250-mL Duran bottle, placed in a Dewar flask containing boiling water, and left to cool for 36 to 48 h at room temperature. After centrifugation for 30 min at 1800g, crystalline amylose and butanol complexes were collected and dried in an air flow.

**Gel Filtration Chromatography**

Amylose was dissolved in 1 M NaOH at 13 mg/mL, diluted to 0.35 M NaOH with distilled water, and boiled for 5 min. After cooling, the solubilized amylose was diluted further to 0.2 M NaOH, and 2.4 mL was loaded onto the first of two Sepharose CL-2B columns (15 mm i.d. x 1 m) connected in series, equilibrated, and eluted with 10 mM NaOH. Fractions of 6 mL were collected at a rate of one fraction per 37.5 min. Samples (150 μL) of each fraction were mixed with 50 μL of Lugol’s iodine (Sigma) containing 10 mM acetic acid. $A_{595}$ was determined using a microtiter plate reader. The wavelength of maximal absorbance was measured. The columns were calibrated using dextrans of known molecular mass (Sigma).

**Chain Lengths of Amylopectin**

Short chains of amylopectin (between 6 and 35 Glc units) were analyzed by fluorophore-assisted gel electrophoresis exactly as described by Edwards et al. (1999b). Starch was debranched with isoamylase, labeled with 8-amino-1,3,6-pyrenetrisulfonic acid, and electrophoresed on an Applied Biosystems 373A DNA sequencer (Foster City, CA). Data were collected and analyzed using GeneScan 672 software (Applied Biosystems, Foster City, CA). The system was standardized using maltohexaose and maltoheptaose as standards.
Amylose Content

The amylose content of potato tubers was determined as described by Visser et al. (1991).

Iodine Staining

Samples of purified starch granules were suspended in a 10-fold dilution of Lugol’s iodine and viewed with a light microscope.

Isolation and Sequencing of cDNA Clones

The isolation and sequence of pea embryo GBSSIa has been described (Dry et al., 1992). To isolate pea leaf GBSSSb cDNA, mRNA was extracted from the leaves of a round-seeded line of pea (BC1/9 RR), and cDNA was synthesized as described by Dry et al. (1992). A λgt10 library was constructed according to the manufacturer’s instructions (Amersham International, Amersham, UK). A total of 2 × 10⁶ plaques were probed with a 2.2-kb BamHI fragment containing the full-length cDNA of potato GBSSI (Dry et al., 1992) at 55°C and washed with 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.5% (w/v) SDS.

Three positive clones were obtained and subcloned into the EcoRI site of pBluescript SK−, and the sequences were determined. All three clones were full length (2100 bp). The 5′ end of the GBSSb cDNA encodes a peptide sequence that conforms to the consensus sequence for chloroplast transit peptides (Gavel and von Heijne, 1990). The cleavage site for GBSSIb is GIIVC*G, compared with the consensus i/VXa/C*A (where * indicates the point of cleavage). V occupies position −2 (X) in a high proportion of chloroplast transit peptides. There also are characteristic basic residues (K and R) in the −6 to −10 region.

Isolation of RNA, Poly(A)+ RNA, Reverse Transcriptase–Mediated PCR, and DNA Gel Blot Analysis

Total RNA was isolated from pea leaves and pea embryos according to the method of Martin et al. (1985), and poly(A)+ mRNA was purified using an oligo(dT)-cellulose spin column (Pharmacia). First-strand cDNA was made to 1 µg of poly(A)+ RNA primed with a dT(17) adaptor (Frohman et al., 1988) using Superscript reverse transcriptase (Gibco BRL) in accordance with the manufacturer’s instructions. The PCR amplification used oligonucleotides of between 26 and 33 bases in length to amplify fragments of 0.9 kb (GBSSIa) and 1.7 kb (GBSSb). The oligonucleotide pairs were 5′-AATGGACAACAT-AACGGGATCTTCAATGCCGAC-3′ and 5′-CTCAAGTAAACATGACTTTTCTCTCAGAC-3′ and 5′-CTACATTTCTCTCAGAC-3′ and 5′-GCAATTTTGTGACACCTTCTTCC-3′ for GBSSb. Control reactions contained plasmid DNA containing full-length GBSSIa and GBSSb cDNA (pGBSSIa and pGBSSb), respectively.

Construction of Expression Plasmids for Mature GBSSIa and GBSSb

PCR mutagenesis was used to introduce ATG initiation codons at the 5′ end of the mature cDNAs and to optimize codon use for the first nine amino acids. The sequences of the oligonucleotides were as follows: GBSSIa, 5′-GAATTCCCATGGGTCTGGTTTTCGTTGGTGCTGAAGTTCACCTTGGG-3′; GBSSIb, 5′-GTGGTCAATGGGTCTGGTTTTCGTTGGTGCTGAAGTTCACCTTGGG-3′.

The expression constructs were prepared as in-frame S-tag fusions between the Ncol-BamHI sites (GBSSIa) or Ncol-EcoRI sites (GBSSIb) of pStag (Edwards et al., 1999a) to give plasmids pStag19 and pStag46, respectively. Recombinant proteins were synthesized with a 15-amino acid N-terminal tag, the S-peptide, which was used for quantification and detection on protein gel blots (Kim and Raines, 1993). The presence of the S-tag has been shown not to influence potato GBSSI activity in solution (Edwards et al., 1999a).

Expression of GBSSIa and GBSSb in Escherichia coli

Recombinant proteins were expressed in E. coli strain RH98 (a glycogen synthase mutant) exactly as described by Edwards et al. (1999a). Host cells also contained plasmids pLysE and pSBET for improved expression (Studier, 1991; Schenk et al., 1995). Protein expression was induced by the addition of isopropylthio-β-galactoside to a final concentration of 1 mM. Cells were harvested, resuspended in extraction buffer (100 mM Mops, pH 7.2, 5 mM MgCl₂, 2 mM DTT), and lysed using a French pressure cell. Soluble and insoluble fractions were separated by centrifugation at 4°C for 10 min at 10,000 g, and the soluble fractions were used for further analysis.

SDS-PAGE and Immunoblot Analysis

Protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and visualized using either an S-tag protein gel blot kit (Novagen, Madison, WI) or rabbit anti-GBSSI antiserum (Smith, 1990) followed by alkaline phosphatase–conjugated goat anti-rabbit serum (Sigma). For quantification of GBSSI protein expression in transgenic potato, starch-granule bound proteins were separated by SDS-PAGE, visualized with Coomassie brilliant blue R 250, and quantified by reference to BSA standards run on the same gel.

Starch Synthase Assays

All reactions had a final volume of 100 µL, and the buffer consisted of 100 mM Bicine, pH 8.5, 25 mM potassium acetate, 5 mM EDTA, and...
10 mM DTT. Unless stated otherwise, 0.23 kBq of ADP-U-14C-Glc was used per 100-μL reaction. Assays were incubated at 25°C for 20 min, terminated by heating at 90°C for 2 min, and processed by either the methanol/KCl method or the resin method (Denyer et al., 1999). All assays and controls were duplicated.

**Bacterial Extracts**

Assays were performed as described by Edwards et al. (1999a). Kinetic constants were determined using potato amylopectin at concentrations up to 80 mg/mL and ADP-Glc concentrations up to 2 mM. Specific activity measurements were made with the following: 5 mg/mL amylopectin; 50 mg/mL amylopectin; 2.5 mg/mL amylopectin and 0.5 M citrate; 50 mg/mL amylopectin and 0.5 M citrate; 100 mM maltotriose; 100 mM maltotriose and 5 mg/mL amylopectin.

GBSSs (pStag19) also was assayed in the presence of the following: 200 mg/mL amylopectin; 10 mg/mL glycogen; 25% (v/v) glyceral; 5 and 25 mg/mL amylose-free potato starch; percloric acid-treated amylose-free starch granules; 1 mM UDPG; debranched (isomylase-treated) amylopectin; 10 mg/mL amylose (solubilized by sonication and boiling); 10 mg/mL amylose and 100 mM maltotriose; 100 mM maltoligosaccharides from G3 to G7; 60 mM ADP-Glc; 100 mM maltotriose and 5 mg/mL amylopectin.

Processivity assays were performed in the presence of 1 mM ADP-Glc, 100 mM maltotriose, 2.5 mg/mL amylopectin, and 1.84 kBq ADP-U-14C-Glc. The reactions were allowed to proceed for 1 h at 25°C, and the products were processed using the resin method and analyzed by high-performance anion-exchange chromatography as described previously (Edwards et al., 1999a).

**Potato Starch Granules**

Starch was resuspended in extraction buffer (100 mM Mops, pH 7.2, 1 mM EDTA, 10% [v/v] ethanediol, and 1 mM DTT) at a concentration of 20 to 40 mg/mL. Assays contained 50 μL (1 to 2 mg) of starch, 1 mM ADP-Glc, 5 mg/mL amylopectin, and 100 mM maltotriose and were processed by the methanol/KCl method (Denyer et al., 1999).

Processivity assays were performed as described above except that they contained 40 to 80 mg of starch granules and 1.84 kBq of ADP-U-14C-Glc, were incubated for 1 h at 25°C, and were not boiled to terminate. Reaction products were processed using the resin method and analyzed by high-performance anion-exchange chromatography as described previously (Edwards et al., 1999a).

**Construction of Sense Binary Vectors and Transformation of amf Potato**

A 2.0-kb fragment encoding full-length embryo GBSSs and a 1.8-kb fragment encoding full-length leaf GBSSs were subcloned in the sense orientation between the double 35S promoter of Cauliflower mosaic virus (CaMV) and the CaMV terminator in pJLT60 (Guertinova and Mullineaux, 1993), producing plasmids pGBSSs and pGBSSs, respectively. The KpnI-XhoI fragment from pGBSSs and the SalI-XhoI fragment from pGBSSs, both encompassing the double 35S promoter, cDNA, and CaMV terminator, were ligated into the plant transformation vector pBIN19 (Bevan, 1984), resulting in plasmids pBinGBSSs (embryo GBSSs) and pBinGBSSs (leaf GBSSs). In vitro–grown stem segments were used for the transformation of the potato amf mutant. Agrobacterium tumefaciens–inoculated stem explants were transferred to callus-induction and shoot-regeneration medium as described by Visser (1991).

**Measurement of Physicochemical Properties**

**Determination of the Tm of Starch Granules**

The temperature at which starch granules started to gelatinize was determined by differential scanning calorimetry using a Perkin-Elmer Pyris 1 calorimeter equipped with a ThermoeNeslab RTE-140 glyco-cooler (Portsmouth, NH). The instrument was calibrated with indium (Tm of 156.6°C) and zinc (Tm of 419.47°C). Before differential scanning calorimeter analysis, the moisture content of starch samples was determined by drying them overnight in an oven at 105°C and weighing them before and after drying.

Precisely 10 mg of starch was transferred to a stainless-steel pan, and the starch content of the pan was adjusted to 20% by adding an appropriate amount of water. The pan was sealed and allowed to equilibrate overnight. The samples were heated from 40 to 100°C at a scanning rate of 10°C/min. An empty sample pan was used as a reference. For each endotherm, the onset temperature of gelatinization and the difference in enthalpy were computed automatically.

**Determination of the Solution Properties of Transgenic Starches**

Dynamic rheological properties of 2.5% (w/v) starch suspensions at small deformations were determined by applying a small oscillating shear deformation using a Bohlin CVO rheometer (Mettler Toledo, Tiel, The Netherlands). The apparatus was equipped with a stainless-steel small sample cell with the following geometric parameters: cylinder i.d. of 25 mm, cylinder o.d. of 26 mm. The torque bar was adjusted automatically by the machine based on an initial stress of 0.019 Pa, a target strain of 0.100, and an oscillation frequency of 0.1 Hz. The suspensions were preheated to ~40°C under gentle stirring and loaded in the sample cell (preheated at 40°C). After this, the cell was subjected to the following temperature program: heating to 90°C, 15 min at 90°C, cooling to 20°C, and 15 min at 40°C. Heating and cooling were performed at a rate of 2°C/min. Data were collected automatically every 10 s.

**Accession Numbers**

The accession numbers for the plants described in Figure 2B are as follows: barley (Hordeum vulgare), X07932 (Rohde et al., 1988); wheat (Triticum aestivum), X57233 (Clark et al., 1991); maize (Zea mays), X60935 (Kloosgen et al., 1986); sorghum (Sorghum bicolor), Q43134 (Y.C. Haing, unpublished data); rice (Oryza sativa), X62134 (R.J. Okayaki, unpublished data); bean (Phaseolus vulgaris), AB029546 (N. Isono, K. Nozaki, H. Ito, H. Matsui, and M. Honma, un-
published data); peala (*Pisum sativum*), X88789 (Dry et al., 1992); *Antirrhinum* (*Antirrhinum majus*), AJ006293 (Mérida et al., 1999); potato (*Solanum tuberosum*), X58453 (van der Leij et al., 1991); sweet potato (*Ipomoea batatas*), U44126 (S.-J. Wang, K.-W. Yeh, and C.-Y. Tsai, unpublished data); cassava (*Manihot esculenta*), X74160 (Salehuzzaman et al., 1993); pea (**P. sativum**), U345045 (this article); *Arabidopsis* (*Arabidopsis thaliana*), O94727 (M. Bevan, G. Murphy, L. Drost, C. Hall, S. Hudson, P. Ridley, I. Bancroft, H.W. Mewes, K. Mayer, and C. Schueller, unpublished data); and wheat (*T. aestivum*), AF109395 (Vrinten and Nakamura, 2000). The accession number for the full-length cDNA of potato GBSSI (Dry et al., 1992) is X87988.

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