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Cloning and characterization of a nuclear gene encoding a starch-branching enzyme from the marine red alga *Gracilaria gracilis*

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Abstract The biosynthesis of starch in red algae occurs in the cytosol, in contrast to green plants where it takes place in the plastid. We have cloned a nuclear gene from the red alga *Gracilaria gracilis* that encodes a homolog of starch-branching enzymes (SBEs); this gene, which is apparently intron-free, was designated as *GgSBE1*. A potential TATA box, CAAT boxes, and other potential regulatory elements were observed in its 5′ flanking region. The encoded 766-aa peptide shares significant sequence similarity with SBEs from green plants (at least 40%), and with glycogen-branching enzymes (GBEs) from human (46%) and *Saccharomyces cerevisiae* (45%). Southern-hybridization analysis indicates that the gene is single-copy, although weaker signals suggest that related genes exist in the genome of *G. gracilis*. Phylogenetic analyses indicate that GgSBE1 groups within the eukaryote branching enzymes (BEs) and not with eubacterial GBEs, suggesting that its gene has not been derived directly from an endosymbiotic cyanobacterium, but instead is ancestrally eukaryotic.

Key words Branching enzyme · Floridean starch · *Gracilaria* · Rhodophyta

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Introduction

Red algae (Rhodophyta) utilize α-1,4-glucans, known as floridean starch, as reserve polysaccharides (Raven et al. 1990). A number of characteristics distinguish the biosynthesis and structure of floridean starch from those of starches and glycogen in other organisms. Floridean starch is synthesized not in plastids, as in green plants, but in the cytosol (Pueschel 1990). It differs from green-plant amylopectin, and even more from animal glycogen, in its structure and properties, particularly in average unit chain length, average internal chain length, affinity for iodine, and limiting viscosity number (Percival and McDowell 1967; Aspinall 1970). The floridean starch granule, in contrast with that of plant starch, shows greater variability in shape; in addition to the more common spherical shape, ovoid, obovoid, cylindrical, and pyriform shapes have been observed (Sheath et al. 1981). Moreover, whereas plant starch consists of both amylose and amylopectin, floridean starch in most red algae consists solely of amylopectin-like material (Craigie 1974; Percival and McDowell 1967). Amylose fractions have, however, been observed in floridean starch in some species (McCracken and Cain 1981). These differences indicate that the physiology, molecular biology, and perhaps the evolutionary history, of floridean starch biosynthesis may differ from those of starch or glycogen biosynthesis in other organisms.

One of the key enzymes in starch biosynthesis is the starch-branching enzyme (SBE) [1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase, EC 2.4.1.18], responsible for the formation of amylopectin from α-1,4-D-glucan chains. Early enzymological studies of plant SBEs led to the recognition of two types of SBEs based on substrate preference. The Q-enzymes use only amylose as a substrate and produce only moderately branched amylopectins, whereas the amylopectin-branching enzymes act on both amylose and amylopectin; this distinction may not, however, always be clear-cut (Manners 1985). Recent cloning and characterization of SBE-encoding genes from green plants has led to the recogni-

tion of at least two families of plant SBEs (Smith-White and Preiss 1994; Burton et al. 1995), the *Sbe1* family (or family B), and the *Sbe2* family (or family A). These families apparently became established before the divergence of the monocots and dicots, as isoforms of both families occur in the two plant groups. Members of these two families differ in enzymological properties (Martin and Smith 1995; Guan et al. 1997).

Biochemical studies in the late 1960s by Fredrick (1968, 1971) showed that three SBE isozymes occur in the red alga *Rhodymenia pertusa*; two of these were found to be Q-type enzymes, while the third is a "dual action" enzyme, capable of branching both amylose and amylopectin. Biochemical research on red-algal SBEs has been in the doldrums since the 1970s. The biochemistry and enzymology of this enzyme in red algae have remained poorly known, and much work remains to be done to understand its role in floridean starch biosynthesis, especially at the molecular level. The evolutionary relationship of red-algal SBEs with those of green plants also remains to be clarified. Herein we report the cloning and characterization of an SBE gene from the agar-producing marine red alga *Gracilaria gracilis* as a first step toward elucidating the molecular biology of floridean starch biosynthesis, and to confirm the presence of other potential SBE-like genes in the genome of *G. gracilis*.

Materials and methods

DNA extraction and construction of a G. gracilis genomic library. *G. gracilis* ("grass" strain) was obtained from cultures maintained in large tanks at the Aquaculture Research Station (Sandy Cove, Halifax County) of the NRC Institute for Marine Biosciences. Genomic DNA was extracted from clean tips of *G. gracilis* using a protocol described previously (Zhou and Ragan 1993), partially digested with *Sau*3AI, and ligated to the Lambda-DASH II vector (Stratagene, La Jolla, Calif.). The recombinant phage was packaged using the Gigapack III Gold Packaging Extract (Stratagene, La Jolla, Calif.). The library was constructed with *Escherichia coli* XL1-Blue MRA (P2) strain (Stratagene, La Jolla, Calif.) as host.

Isolation of genomic clones. The strategy we employed to clone an SBE gene from *G. gracilis* is based on the use of PCR to generate a homologous probe. Degenerate PCR primers were designed to anneal to conserved regions of the SBE gene revealed by multiple alignments of amino-acid sequences of SBEs obtained from the NCBI protein database. Although several primers were tested, only one pair yielded a desired product. PCR reactions using the "forward" primer Fb [5′-TA(TC)GCNGA(GA)(AT)(GC)(GCT)CA(TC)GA(TC)-CA-3′], which corresponds to the conserved protein region YAESHDQ, and the "reverse" primer Rb [5′-(TC)TCNGG(GA)-TGNCC(GA)AA(TC)TC(GA)TT-3′], which corresponds to the conserved region NEFGHPE, with *G. gracilis* genomic DNA as a template, yielded a product of about 220 bp; subsequent sequencing confirmed that the product arises from a probable SBE gene. This PCR product was labelled and used as a probe to screen the genomic library of *G. gracilis* using standard protocols (Sambrook et al. 1989). Two clones were isolated, and one of these was sequenced.

Sequencing. Lambda DNA was isolated and purified using the protocol described by Sambrook et al. (1989) and employed directly for sequencing; both strands were fully sequenced. For the the initial sequencing, primers were designed based on the known sequence of the previously sequenced fragment; sequencing in both directions thereafter proceeded by primer walking. When necessary, fragments of the gene were PCR-amplified using *Pfu* polymerase (Stratagene, La Jolla, Calif.) to generate more template for sequencing. Sequencing was carried out on an Applied Biosystems 373A sequencer (Applied Biosystems, Foster City, Calif.) following the manufacturer's Dye Deoxy terminator cycle sequencing protocol. The sequence data were assembled and edited using the Pregap and Gap4 programs within the Staden package (Staden 1996).

Southern hybridization. *G. gracilis* DNA (5 μg per reaction) was digested with restriction enzymes, and blotted onto Zeta-Probe GT membranes (Bio-Rad Laboratories, Hercules, Calif.) following the manufacturer's protocols. A fragment of the *SBE* clone (from position 759 to position 1504 in Fig. 1) was PCR-amplified and employed as a template for the synthesis and α-^{32}P-dCTP labelling of the probe using the Random Primed DNA labelling kit (Boehringer Mannheim, Mannheim, Germany). Hybridization was performed essentially following standard protocols (Sambrook et al. 1989).

Mapping of the 5′ and 3′ ends of the GgSBE1 mRNA. The mRNA was extracted, using the Invitrogen FastTrack 2.0 kit (Invitrogen Corp., San Diego, Calif.), from 2 g tissue of *G. gracilis* that had been cultured in the laboratory under approximately 30 $\mu Em^{-2} s^{-1}$ of fluorescent white light with a 12 h:12 h light:dark cycle. The 5′ end was reverse-transcribed and PCR-amplified by 5′ RACE using the Boehringer Manheimm 5′/3′ RACE kit (Boehringer Mannheim, Mannheim, Germany); the PCR product was de-salted by centrifugation through Centricon-100 concentrators (Amicon, Beverly, Mass.) with 2 ml of distilled water, and sequenced using the primer r3c (see Fig. 1). To obtain the sequence at the 3′ end, the mRNA was reverse-transcribed using the Pharmacia T-Primed First-Strand Ready-To-Go kit (Pharmacia Biotech, Uppsala, Sweden). Aliquots of the reaction were then used in PCR, with the primer f2 (see Fig. 1) as the gene-specific primer, and the anchor primer 5′-ATTCGC-GGCCGCAGGAATT-3′. The PCR product was de-salted similarly, and sequenced using primer f2.

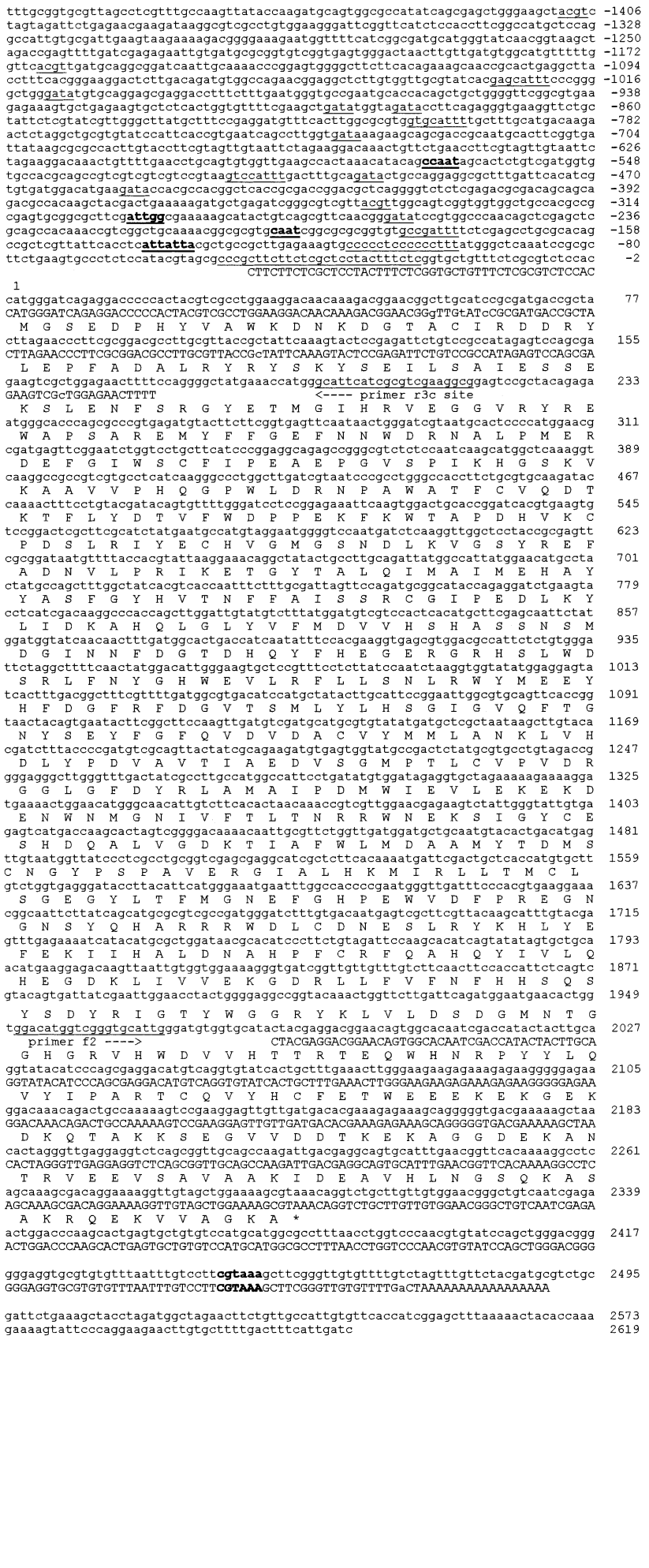
Sequence analysis. Amino-acid sequences of branching enzymes (BEs, which include SBEs and glycogen-branching enzymes, or GBEs) were obtained from the NCBI protein database (http://www.ncbi.nlm.nih.gov) and aligned using CLUSTAL W (Thompson et al. 1994) under its default parameters: pairwise alignments = slow (accurate), gap opening penalty = 10, gap extension penalty = 0.10 or 0.05; scoring utilized the BLOSUM series (Henikoff and Henikoff 1992). Phylogenetic trees were constructed by neighbor-joining (Saitou and Nei 1987) as implemented in Treecon (version 1.15) (Van de Peer and De Wachter 1994), and by protein parsimony as implemented in the PHYLIP package (Felsenstein 1989). The N- and C-terminal portions of the alignment were excluded from our analyses, as the quality of the alignment and placement of gaps in these regions appear unreliable (detailed alignment available from the authors). Trees were inferred either including or excluding insertion-deletion (indel) regions.

Results and discussion

Cloning and sequencing of an SBE gene from *G. gracilis*

Degenerate oligonucleotide primers were used in the polymerase chain reaction (PCR) to amplify a portion of an SBE-encoding gene from genomic DNA of *G. gracilis*. A PCR product was confirmed by sequencing to encode a portion of a probable SBE gene, and was used as a probe to screen a *G. gracilis* genomic library, resulting in the recovery of two lambda clones. The sequence obtained from one clone (λ25) is shown in Fig. 1. It contains an ORF of 767 codons. The inferred amino-acid sequence aligns well

Fig. 1 The nucleotide sequence of *GgSBE1*. The numbering scheme assigns +1 to the first position of the start codon (marked with *1*). Potential *cis*-regulatory elements, including ACGT and GATA motifs, the GCCGATTT and G(AT)(GC)CATTT motifs (see text), and two pyrimidine-rich regions, are *underlined*. The potential TATA and CAAT boxes are in *bold and underlined*. The sequences of the 5′ and 3′ ends of cDNAs (shown in *uppercase letters*) are aligned with the genomic sequence, and the positions of the sequencing primers r3c (5′ RACE) and f2 (3′ RACE) are *underlined*. The conceptual translation is shown below the coding region; * marks the stop codon. The putative polyadenylation signal (CGTAAA) downstream from the protein-coding region is *in bold*. The last four residues (GATC, positions 2616–2619) comprise the restriction site where the vector and the genomic insert were ligated



with other SBE sequences, and is highly similar with those of green plants (40–45% sequence identity), human (46%) and *Saccharomyces cerevisiae* (45%), indicating that the ORF encodes an SBE. Since there are apparently two or more SBE-like genes in *G. gracilis*, based on the results of the Southern analysis (see below), we designate this gene as *GgSBE1*.

More than 1.4 kb of the 5′ flanking region was sequenced. ORFs found in this region contain fewer than 100 codons except for one which is at least 240 codons in length. A search through the NCBI database using BLASTX yielded no sequences with significant similarity (i.e., BLASTX scores >80) to these ORFs.

Features of the gene

We mapped the 5′ end of the *GgSBE1* transcript by 5′ RACE. The alignment of the 5′ end-fragment of this cDNA with the genomic sequence is shown in Fig. 1. Our results indicate that the 5′ end is at, or close to, position –47. A potential TATA box occurs 85 bp further upstream of this site. Upstream of this putative TATA box are three CAAT elements, one in an inverted orientation. Potential GATA boxes and ACGT elements, and three motifs with the consensus sequence G(AT)(CG)CATTT, which may function as regulatory elements, were likewise identified. A GCCGATTT motif, closely resembling the sequence GCAGATTT that occurs in the promoter region of the *G. gracilis* nuclear genes *GgGALT1* (three closely-spaced repeats: Lluisma and Ragan 1998) and *GapA* (Zhou and Ragan 1994), was found slightly upstream of the TATA box. Pyrimidine-rich regions were found downstream from the TATA box. The first AUG codon downstream from the putative 5′ end of the transcript appears to be in the right context for translation initiation; the sequence UCCACC**AUG** (as TCCACCATG, with the translation initiation codon in bold) conforms to the canonical sequence found at the translation initiation site in red-algal nuclear genes (YYCRCY**AUG**: Zhou and Ragan 1996), and in fact is identical to the sequence in the corresponding site in the polyubiquitin-encoding gene from *G. gracilis* (Zhou and Ragan 1995b).

Comparison of the cDNA and genomic sequences (Fig. 1), and comparison of the inferred GgSBE1 amino-acid sequence with those of other SBEs, indicate that *GgSBE1* is devoid of introns; the sequence similarity between the deduced GgSBE1 protein and other SBEs extends throughout the entire length of GgSBE1. The yeast glycogen-branching enzyme is also intronless (Thon et al. 1992), although the *sbe1* gene from rice contains 13 introns (Kawasaki et al. 1993). The absence of introns from *GgSBE1* is, however, not entirely surprising; known red-algal nuclear genes are either intronless [e.g., genes encoding triose phosphate isomerase in *G. gracilis* (as *G. verrucosa*: Zhou and Ragan 1995c) and GapC in *Chondrus crispus* (Liaud et al. 1993)] or, more commonly, are interrupted by a single intron [e.g., genes encoding GapA, GapC, and mitochondrial aconitase in *G. gracilis* (Zhou and Ragan 1994, 1995a, d), and GapA and β-tubulin in *C. crispus* (Liaud et al. 1993, 1995)].

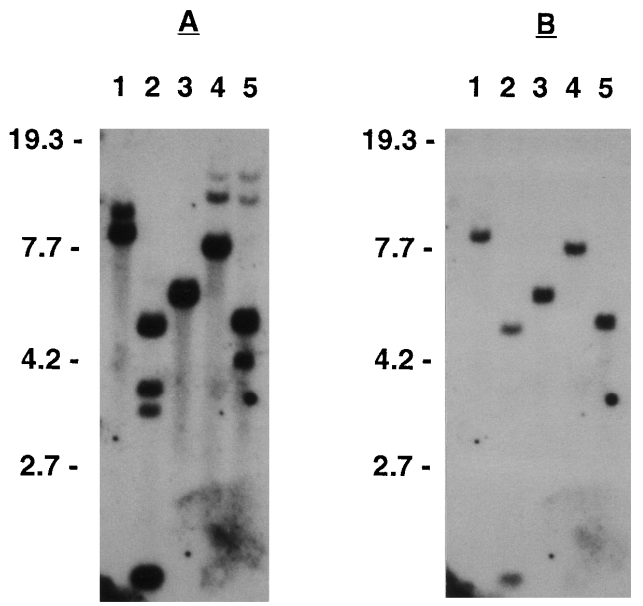


Fig. 2A, B Determination of *GgSBE1* copy number by Southern-hybridization analysis. Genomic DNA (5 µg per reaction) from *G. gracilis* was digested with *Eco*RI (*lane 1*), *Hin*dIII (*lane 2*), *Sal*I (*lane 3*), *Xba*I (*lane 4*), and *Xho*I (*lane 5*). The probe was prepared by PCR from a *GgSBE1* fragment (position 759 to position 1504, Fig. 1), which contains no sites for *Eco*RI, *Sal*I, *Xba*I, or *Xho*I, and one site for *Hin*dIII (positions 1159–1164). Final washing was with 0.5×SSC/0.1% SDS at 65 °C for 30 min, performed twice (**A**). The same blot was re-washed twice with 0.1×SSC/0.1% SDS at 65 °C for 30 min (**B**). The *numbers* on the left of each panel indicate the size of the markers, in kb

The 3′ end of the transcript was sequenced using the 3′ RACE technique. Comparison of its sequence with the corresponding region of the genomic clone (Fig. 1) reveals that the polyA (cleavage) site occurs 171 bp downstream from the termination codon; a potential polyA signal, CGUAAA, is seen 21 bp upstream of the polyA site. All *G. gracilis* cDNAs that have been characterized so-far contain the UAAA motif, occurring close to, and upstream of, the polyA site. In green plants, AAUAAA-like elements, analogous to the highly conserved AAUAAA motif in animals, are degenerate or may even be absent (Hunt 1994; Wu et al. 1995). The short region between the polyA site and the putative polyA signal is rich (86%) in G and U residues; GU-rich regions have been shown to be important for efficient polyadenylation in animals and green plants, and are located upstream of (in plants) or downstream from (in animals) the AAUAAA-like motifs (Wu et al. 1995).

Southern analysis

To determine the copy number of *GgSBE1*, we performed Southern analyses with a *GgSBE1* fragment as a probe. The results (Fig. 2A) indicate that there are at least two SBE-related sequences in the genome of *G. gracilis*, as indicated

by the multiple bands observed per lane after moderately stringent washing conditions (final wash with 0.5×SSC/0.1%SDS, 65 °C, 30 min, performed twice). However, a further, more stringent washing (0.1×SSC/0.1% SDS, 65 °C for 30 min, performed twice) of the same blot yielded only a single band per lane (Fig. 2B), suggesting that *GgSBE1* is single-copy in the *G. gracilis* genome. This finding is consistent with the observation that three distinct SBE isozymes occur in another red alga, *R. pertussa* (Fredrick 1968, 1971).

Multiple isoforms of SBEs also exist in green plants (Burton et al. 1995; Martin and Smith 1995). Interestingly, pea *SBE I* (a member of the *Sbe2* family) and pea *SBE II* (a member of the *Sbe1* family) genes do not cross-hybridize in Southern-blotting experiments, even at low stringency (2×SSC, 55 °C: Burton et al. 1995). Whether or not this indicates that SBEs in plants are more divergent than are SBEs in red algae remains to be investigated.

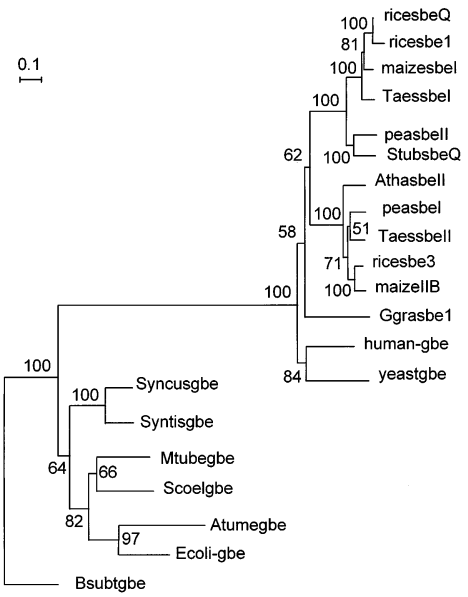


Fig. 3 A phylogenetic tree of selected starch- and glycogen-branching enzymes constructed using the neighbor-joining method. Distances were calculated using the formula of Kimura (1983); positions corresponding to insertion-deletion regions ("indels") were excluded from the analysis. *Numbers* represent bootstrap replicate values out of 100. The scale (top, left) indicates the branch length corresponding to 0.1 substitutions per site. The sequences are: Ggrasbe1, sequence deduced from *GgSBE1*, this paper; maize IIB, *Zea mays* sbeIIB (NCBI accession no. 1169911); ricesbe3, *Oryza sativa* sbe-3 (436052); TaessbeII, *Triticum aestivum* sbeII (1885344); peasbeI, *Pisum sativum* sbeI (1345570); AthasbeII, *Arabidopsis thaliana* sbe class II (726490); yeastgbe, *Saccharomyces cerevisiae* gbe (1076979); human-gbe, *Homo sapiens* gbe (1082408); ricesbeI, *O. sativa* sbe1 (421991); ricesbeQ, *O. sativa* sbeQ (399544; conflicts with another rice sbeQ sequence); maizesbeI, *Z. mays* sbeI (600872); TaessbeI, *T. aestivum* sbe (1935006); StubsbeQ, *Solanum tuberosum* sbe-Q (1169912); peasbeII, *P. sativum* sbeII (1345571); Syntisgbe, *Synechocystis* sp. strain PCC6803 gbe (1707936); Syncusgbe, *Synechococcus* sp. strain PCC7942 (121297); Scoelgbe, *Streptomyces coelicolor* gbe (2127516); Mtubegbe, *Mycobacterium tuberculosis* gbe (1707934); Ecoli-gbe, *Escherichia coli* gbe (66573); Atumegbe, *Agrobacterium tumefaciens* gbe (1707933); Bsubtgbe, *Bacillus subtilis* gbe (1084216)

Inferred features of the GgSBE1 protein: structural and phylogenetic analyses

Roughly 50% of the residues in GgSBE1 are identical with those at corresponding sites in plant SBEs and yeast and animal GBEs. Some residues that are highly conserved not only among SBEs and GBEs but also among members of the α-amylase family (to which the SBEs and GBEs belong: Romeo et al. 1988; Baba et al. 1991; Jespersen et al. 1993) are also conserved in GgSBE1. These include GsSBE1 positions H280, D346, E402, H470 and D471, residues involved in substrate binding or in catalysis (Svensson 1994; Kuriki et al. 1996).

It has been proposed that group-specific differences in the nature of residues on certain β-strand $\rightarrow$ α-helix connecting loops, and the lengths of these loops, help determine the substrate specificity of members of the α-amylase family (Jespersen et al. 1993; Svensson 1994). Burton et al. (1995) noted that members of the *Sbe2* family in green plants contain an 11-amino-acid insertion in the loop connecting β-strand 8 and α-helix 8 that is absent in members of the *Sbe1* family, and proposed that this difference might account for the difference in the average lengths of branches they transfer. This 11-amino-acid insertion is absent in GgSBE1, as it is in human and yeast GBEs.

The predicted GgSBE1 protein, like the GBEs of yeast and human, lacks an N-terminal region that is predicted to be present in SBEs from green plants. This N-terminal extension in green plant SBEs corresponds in part to a plastid-envelope transit peptide not present in mature BEs of animals or yeast. As starch synthesis is cytosolic in red algae (Pueschel 1990), red-algal SBEs would not be expected to contain a transit peptide. However, the mature peptides of Sbe2 isoforms in green plants, as well as the eubacterial GBEs, have an N-terminal extension absent in BEs from red algae, animals and fungi.

To examine relationships among BEs, we aligned 21 SBEs and GBEs, removed the ambiguously alignable regions, and inferred trees by the neighbor-joining and protein-parsimony methods. Both methods produced trees with essentially similar topologies. Essentially identical topologies were likewise recovered irrespective of whether the regions common to only a few of the sequences (e.g. shared insertions) were kept in the alignment, or removed. The neighbor-joining tree is shown in Fig. 3.

The BEs separate cleanly into two groups, each present in 100% of the bootstrap replicates: BEs from eukaryotes (*S. cerevisiae*, human, *G. gracilis* and green plants: Fig. 3, top 14 sequences), and GBEs from eubacteria including cyanobacteria (bottom seven sequences). GgSBE1 is resolved as one of the deeper branches within the eukaryote group, although bootstrap support within this region of the tree is modest. SBE isoforms in green plants divide solidly into two subgroups, corresponding to the *Sbe1* and *Sbe2* families, each supported in 100% of the bootstrap replicates. SBEs from both monocots and dicots are

present in each subgroup, strongly suggesting that these two isoforms became established prior to the divergence of monocot and dicot lineages. Complete interpretation of these results (e.g., whether GgSBE1 specifically resembles one or the other of the green plant SBE groups, and whether the common ancestor of red algae and green plants stored α-1,4 glucans in its cytoplasm) must await the identification and sequencing of other SBE-like sequences in a broader phyletic sample of green plants, green algae and red algae.

Conclusion

We have cloned an SBE gene from the red alga *G. gracilis*. The gene is apparently one of at least two SBE-like genes in this alga. Presumably, the products of these genes differ in function (as might be indicated by differences in expression or catalytic activity), as is the case in green plants. It would thus be important to isolate and sequence the other gene(s) so that comparative structural and functional analysis of the SBEs in *G. gracilis* can be conducted. It will be interesting to ascertain the function of each isoform in the biosynthesis of floridean starch.

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