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Linkage mapping of starch branching enzyme III in rice (*Oryza sativa* L.) and prediction of location of orthologous genes in other grasses

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Abstract The chromosomal position of Starch Branching Enzyme III (SBEIII) was determined via linkage to RFLP markers on an existing molecular map of rice (*Oryza sativa* L.). A cDNA of 890 bp was generated using specific PCR primers designed from available SBEIII sequence data and used as a probe in Southern analysis. The SBEIII cDNA hybridized to multiple restriction fragments, but these fragments mapped to a single locus on rice chromosome 2, flanked by *CDO*718 and *RG*157. The detection of a multiple-copy hybridization pattern suggested the possibility of a tandemly duplicated gene at this locus. The map location of orthologous SBE genes in maize, wheat, and oat were predicted based on previously published genetic studies and comparative maps of the grass family.

Key words Rice · Molecular mapping · Grain quality · Starch branching enzyme (SBE) · Amylose extender (*ae*)

Introduction

Consumer preferences for cooking properties of rice (*Oryza sativa* L.) vary across the world and are an important consideration in the breeding of new rice varieties. Consumers from Japan, Korea, and parts of China prefer a soft, viscous rice containing low levels of amylose (Juliano et al. 1965), while consumers in most other countries prefer firmer, long-grain varieties. The texture of cooked rice is largely determined by the combination of the two types of storage starch found in rice seeds, amylose and amylopectin (Reddy et al. 1993). These same starch molecules are found in the storage organs of many other plant species, including other cereal crops as well as dicots such as potato, yam, and pea (Wang et al. 1995). As a result, the genetics of starch synthesis is of interest.

Both amylose and amylopectin are glucan polymers. Amylose is a predominantly linear chain of $\alpha(1–4)$ linked glucose residues, while amylopectin is a highly branched glucan chain.

Amylopectin is formed when starch branching enzymes (SBE) form an $\alpha(1–6)$ linkage between the reducing end of one glucan chain and the C6 of a second glucose residue (Martin and Smith 1995). Multiple forms of starch branching enzymes are known, and these have been grouped into two distinct families based on primary structure and functional analysis (Burton et al. 1995; Martin and Smith 1995; Preiss 1991). Family A consists of rice SBEIII, maize SBEII, pea SBEI, and *Arabidopsis* SBE2.1 and 2.2. Family B consists of rice SBEI, maize SBEI, potato SBE, cassava SBE, and pea SBEII (Fisher et al. 1996a; Martin and Smith 1995). Each isoform may be responsible for a unique aspect of amylopectin biosynthesis and structure in developing plants.

Two isoforms of SBE (also known as Rice Branching Enzyme, RBE) have been characterized in rice, SBEI and SBEIII. Rice mutants deficient in SBEIII exhibit the amylose extender (*ae*) phenotype (Mizuno et al. 1993), which is characterized by exceptionally high levels (29–35%) of apparent amylose (Yano et al. 1985).

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The amylose extender phenotype has been mapped to chromosome 2 using trisomic analysis (Kaushik and Khush 1991).

The objective of the study presented here was to determine the map location of SBEIII, one of the key enzymes in starch biosynthesis, on the molecular linkage map of rice.

Materials and methods

Generation of SBEIII probe

The sequence of SBEIII (Mizuno et al. 1993) (GenBank Acc. # D16201) was used as the basis for primer design (primers 423 and 436) and polymerase chain reaction (PCR) amplification of the SBEIII probe used for mapping in this study. RNA was extracted from immature rice seeds (mid-milky stage) by grinding approximately 20 seeds under liquid nitrogen and extracting the total RNA using RNAzol B (Biotecx, Houston, Tex.) according to the manufacturer's instructions. Messenger RNA was then purified from this using the PolyATtract System III (Promega, Madison, Wis.) according to the manufacturer's instructions. Reverse transcription was performed by mixing 26.5 µl mRNA with 2.5 µl primer 423 (75 pmoles) sequence TTCCGCTGGAGCATAGACAA and incubating the mixture at 70°C for 10 min. After cooling on ice, dNTP(ATP, CTP, GTP, and TTP)(final concentration = 0.15 m*M*), DTT(final concentration = 0.01 *M*), reverse transcriptase buffer (1 × concentration) (Gibco BRL Products, Grand Island, N.Y.), 200 U SuperScript II (Gibco BRL) and 20 U RNasin (Promega) were added (total reaction volume of 50 µl). The reaction was incubated at 25°C for 10 min, 37°C for 1 h, 90°C for 5 min and finally on ice for 10 min. This mix was used directly as the PCR template.

PCR was performed using 5 µl of the reverse transcription reaction as template in a 50-µl reaction volume containing 0.2 m*M* dNTP, 20 pmoles of primers 423 and 436 (sequence 5′ GATGTCAGTGGAATGCCTAC 3′), and 1 U *Taq* polymerase (Boehringer Mannheim, Indianapolis, Ind.). The reaction was cycled 40 times at 94°C for 1 min, 50°C for 2 min, and 72°C for 1.5 min. A band of approximately 890 bp (as expected from the published sequence) was amplified and then isolated from an agarose gel using Geneclean II (Bio 101, LaJolla, Calif.) according to the manufacturer's instructions. The DNA fragment was ligated into pTZ19R (Pharmacia Biotech, Piscataway, N.J.) at the *Sma*I site to create pBC185. The 5′ end of the plasmid insert was sequenced and compared with the published sequence to confirm that the fragment was from the SBEIII gene.

Southern blotting and hybridization

A backcross population of 113 plants, derived from the cross *Oryza sativa*/*O. longistaminata*/*O. sativa*, was used for linkage analysis (Causse et al. 1994). Ten micrograms of DNA from the indica recurrent parent (BS125) and the interspecific F_1 (BS125/WLO2) was digested with each of six restriction enzymes (*Eco*RI, *Hin*dIII, *Xba*I, *Sca*I, *Dra*I, and *Eco*RV), run overnight on 0.9% agarose gels and blotted onto Hybond N + membranes according to the manufacturer's instructions (Amersham, Arlington Heights, Ill.) for use in parental polymorphism surveys. For mapping filters, DNA from 113 backcross progeny was digested and blotted using the same procedures.

The SBEIII cDNA insert was PCR-amplified from the pTZ19R vector using M13 forward and reverse primers (Gibco BRL) to give a product of approximately 890 bp. The amplified insert was labeled with [^{32}P]-dCTP (DuPont, Boston, Mass.) using the random hexamer method (Feinberg and Vogelstein 1983) and used as probe in Southern analysis. Filters were hybridized overnight at 65°C and washed three times for 20 min each at 65°C at successive stringencies of 2 ×, 1 ×, and 0.5 × SSC (each wash contained 0.1% SDS). Filters hybridized with the labeled probes were exposed to X-ray film with two intensifying screens for 5 days at −80°C.

Germplasm survey filters were made by digesting DNA from several cultivated rice germplasm accessions with *Eco*RV, *Hin*dIII, *Sca*I, *Dra*I, and *Eco*RI and blotting it onto Hybond N + membranes. The surveys were hybridized with the 890-bp SBEIII insert, washed, and exposed as described above.

Segregation in the backcross progeny was scored by the presence or absence of the polymorphic band. Linkage analysis was performed using MAPMAKER version 2.0 (Lander et al. 1987) on a Macintosh Performa 475. Genetic distance is expressed in Kosambi centiMorgans (cM) (Kosambi 1944), and the map was constructed using a LOD 2.0 significance threshold.

Results and discussion

When the 890-bp SBEIII probe was hybridized to *Eco*RI-digested DNA a single band was visible in the inbred parent, while multiple bands were observed for the other five enzymes tested (Fig. 1). All of the enzymes detected polymorphism in one fragment, but only *Eco*RV detected more than one polymorphism. Using *Sca*I-digested DNA we mapped the SBEIII gene to a single locus on chromosome 2 (flanked by markers *RG*157 and *CDO*718) (Fig. 2). Segregation analysis of the two polymorphic bands produced by digestion with *Eco*RV was also done to check for the possibility of more than one locus. The 5.5-kb and the 15-kb bands cosegregated and mapped to the same locus on chromosome 2.

Although each of the SBEIII hybridizing fragments observed in parental surveys mapped to a single locus, the multiple banding pattern seen in all enzymes except *Eco*RI-digested DNA suggested the possibility that

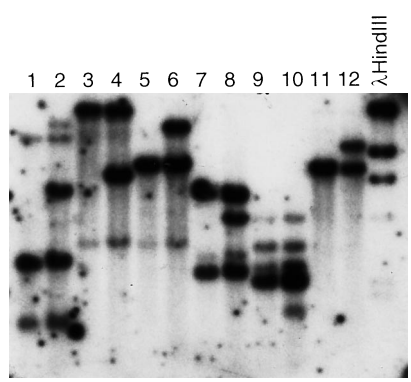

Fig. 1 Autoradiogram of parental survey probed with 890-bp SBEIII PCR product. *Odd-numbered lanes* BS125 (indica) parent; *even-numbered lanes* F1 (BS125/WLO2) parent. *Pairs of lanes* contain DNA digested with the same enzyme as follows: *lanes 1, 2 Eco*RV, *lanes 3, 4 Hin*dIII, *lanes 5, 6 Xba*I, *lanes 7, 8 Sca*I, *lanes 9, 10 Dra*I, *lanes 11, 12 Eco*RI

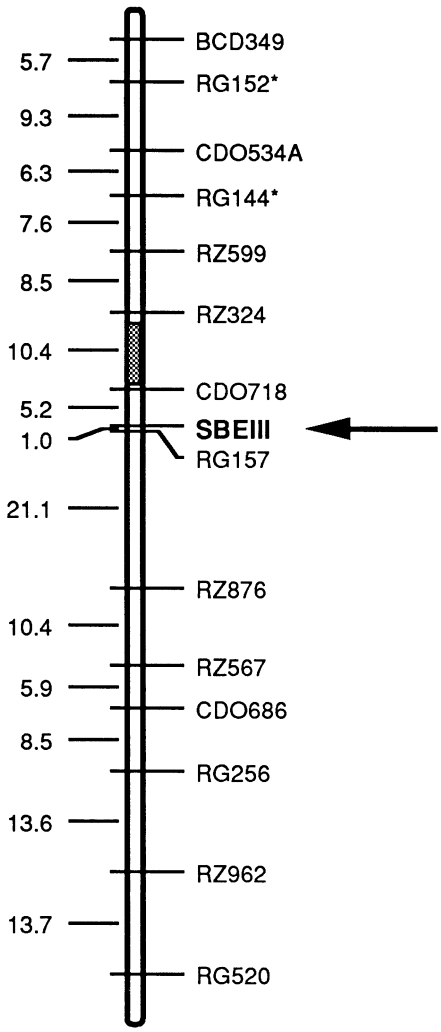

Fig. 2 Framework map of rice chromosome 2 showing position of SBEIII cDNA. NOTE: map has been inverted in relation to Causse et al. (1994) to reflect position of centromere (stippled region) (Singh et al. 1996)

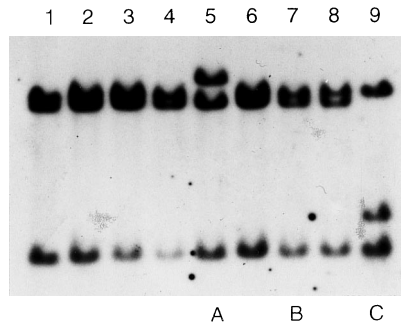

Fig. 3 Autoradiogram of *Sca*I-digested indica and japonica rice varieties hybridized with SBEIII cDNA clone showing three different banding patterns, labeled *A*, *B*, and *C*. *Lane 1* 'Lemont', *lane 2* 'Cypress', *lane 3* 'Jodan', *lane 4* 'L202', *lane 5* 'Panda', *lane 6* 'Pelde', *lane 7* 'Katy', *lane 8* 'Rexmont', *lane 9* 'Tong'.

more than one member of this gene family might be located in this region of chromosome 2. This would be consistent with the report of two closely related cDNAs encoding starch branching enzyme in *Arabidopsis* (Fisher et al. 1996a), and independent genes encoding SBE IIa and IIb in maize (Fisher et al. 1996b).

The unusually high frequency of polymorphism detected between *O. sativa* and *O. longistaminata* with this probe (all of the six enzymes) suggests that an insertion/deletion event(s) or small chromosomal rearrangement at or very near to the SBEIII gene is a likely explanation for the genetic differences between those two species, rather than the assumption of base-pair mutations at each restriction site. A high frequency of polymorphism in cultivated rice germplasm would support the possibility that this region was a hot spot for mutation. To investigate this hypothesis we conducted a survey of 37 indica and japonica varieties using six enzymes. The surveys revealed a maximum of three RFLP banding patterns at the SBEIII locus (Fig. 3). Banding patterns A, B and C were found in 51%, 43% and 5% of the varieties, respectively. This level of genetic diversity was not significantly different from that observed at other random genomic and cDNA marker loci (Wang and Tanksley 1989), and thus this study does not support the hypothesis that the region containing the SBEIII gene is a hotspot for mutation in cultivated rice.

Because of the structural and functional similarities of SBE enzymes in rice and maize (Martin and Smith 1995) it was of interest to compare the map location of SBEIII in rice with that of SBEII in maize based on comparative maps that have been developed for these species (Ahn and Tanksley 1993; Ahn et al. 1995; Paul et al. 1996). Mutants defective in SBEIII activity in rice and SBEII activity in maize are known as amylose extender (*ae*) mutants (Vineyard and Bear 1952). The gene *amylose extender* 1 (*ae*1) has been located on maize chromosome 5 and is possibly identical to maize SBEII (Maize database). On the basis of comparative maps of rice and maize (Ahn et al. 1995; Paul et al. 1996) the position of *ae*1 in maize and SBEIII in rice would suggest that these genes were orthologous. They are located in an array of markers whose order is conserved in rice, maize, oat, and wheat (Van Deynze et al. 1995a, b), suggesting that the region of rice chromosome 2 is homeologous to a large segment of maize chromosome 5 (Coe and Polacco 1996), oat linkage group G (Van Deyzne et al. 1995b) and wheat chromosome 6 (Ahn et al. 1993; Van Deynze et al. 1995a). Thus, we would predict the presence of an SBE (*ae*) gene at the positions indicated in Fig. 4 in these crops. The map position of homeologous genes in oat and wheat are unreported. This study demonstrates the potential of comparative genome analysis to predict the location of conserved gene sequences across genera, paving the way for functional comparisons of the different isoforms in a wide range of plant species.

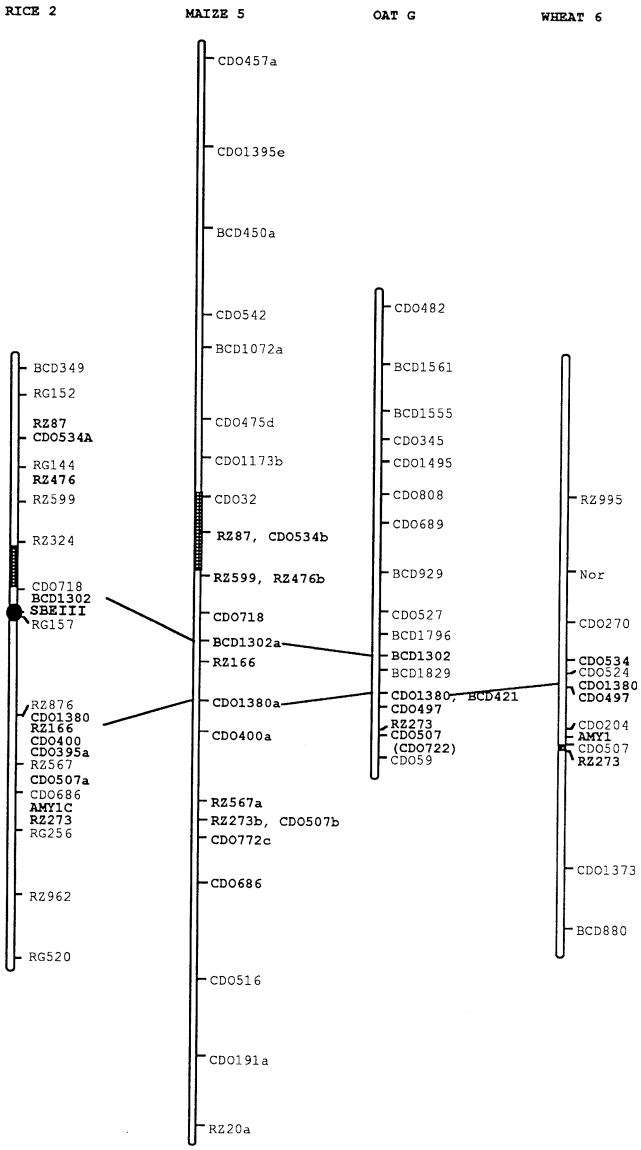

Fig. 4 Maps of rice chromosome 2, maize chromosome 5, oat linkage group G, and wheat chromosome 6 illustrating homoeologous relationships defined by conserved positions of *bold-faced* cDNA markers (Paul et al. 1996; Coe and Polacco 1996; Matthews 1996). Projected map positions of SBEIII orthologue indicated by *solid lines* connecting BCD1302 and CDO1380. *Stippled regions* indicate known centromere locations. NOTE: Rice chromosome 2 has been inverted in relation to Fig. 1 to correspond to order of markers on other genomes.

The molecular map position of SBEIII in rice helps unite the genetics of starch synthesis among members of the grass family. It contributes essential information that may be useful in producing consumer-preferred rice quality. This information also provides a link between biochemically well-characterized genes and economically valuable phenotypes.

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