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Mapping of a gene responsible for the difference in amylopectin structure between *japonica*-type and *indica*-type rice varieties

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Abstract The present investigation revealed that the *alk* and *gel(t)* genes, which cause the differences between a *japonica* rice variety Nipponbare and an *indica* rice variety Kasalath in terms of the disintegration of endosperm starch granules in alkali solution and their gelatinisation in a 4 M urea solution, respectively, cosegregated in backcross inbred lines derived from a cross between the two varieties. The segregation pattern of the profile for amylopectin chain-length, which was distinguished by enrichment in short chains of DP≦11 and depletion in intermediate-size chains of 12≦DP≦24 in *japonica* as compared with *indica*, was exactly the same as those of the above physico-chemical properties of starch granules, and the gene was designated as *acl(t)*. Gene-mapping analysis showed that the *starch synthase IIa* (*SSIIa*) gene is located at the *alk* locus on chromosome 6 in the rice genome. These results lead us to the possibility that different alleles of the *SSIIa* gene are responsible for differences in amylopectin structure between the two varieties, in that SSIIa plays a distinct role in the elongation of short chains within clusters ($A+B_1$ chains) of amylopectin. It is proposed that the activity of SSIIa in *japonica* rice is reduced in amount or functional capacity relative to the activity of this enzyme in *indica* rice. This, in turn, would explain why starch from *japonica* rice has a lower gelatinisation temperature than starch from *indica* rice and is more susceptible to disintegration in alkali or urea. The evidence for this hypothesis is that the *alk(t)*, *gel(t)*, *acl(t)* and *SSIIa* genes all map to the same locus.

Keywords *Indica* rice · *Japonica* rice · Amylopectin · Starch · Starch synthase

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Introduction

It is known that the structure and crystalline organization of starch granules, and the molecular fine structure of amylopectin and amylose, are distinctly different among plant species and tissues. Rice (*Oryza sativa* L.) varieties have been classified into either *japonica*-type or *indica*-type (Oka and Morishima 1997) based on their morphological and physiological differences in, for example, the length of hull-hairs, seed dormancy, cold tolerance, and the disintegration of endosperm starch granules in alkali solution. Varietal differences in the disintegration of endosperm starch granules in alkali (KOH) solution were reported first by Warth and Darabsett (1914). Later, an association between the alkali disintegration and cooking property of rice was found (Little et al. 1958). A significant correlation was also found between disintegration in alkali and the gelatinisation temperature of milled rice (Juliano et al. 1964). Genetic studies with rice of *japonica*-type and *indica*-type revealed that a gene controlling the varietal difference in the alkali disintegration of starch granules, designated the *alk* gene, mapped on chromosome 6 (Kudo 1968). However, identification of the gene remained to be discovered.

Starch is composed of linear amylose and branched amylopectin. Amylose is synthesised by starch granule-bound starch synthase I (GBSSI) encoded by the *waxy* gene. The fact that amylose content is usually higher in endosperm starch of *indica* rice than that of *japonica* rice

has been explained by the presence of two types of *waxy* alleles, Wx^a and Wx^b (Sano 1984). Rice plants having the Wx^a allele produce several times more GBSSI protein in their endosperm than plants with the Wx^b allele. The fact that many *indica* varieties possess the Wx^a allele, while *japonica* varieties usually have the Wx^b allele, accounts for the higher amylose content in endosperm of *indica* rice than in *japonica* rice (Sano et al. 1985).

The rice GBSSI gene was mapped on chromosome 6 (Saito et al. 1991). However, the location of this gene (position 7.9 cM) is clearly different from that of the *alk* locus (position 36.7 cM) on the high-density genetic linkage map (Harushima et al. 1998) (see Fig. 3). These results suggest the possibility that variation in a gene encoding an amylopectin-synthesising enzyme between *japonica* and *indica* varieties is responsible for the difference in physico-chemical properties of starch granules through structural alteration of their amylopectin molecules.

Our previous study (Umemoto et al. 1999) showed that the amylopectin fine-structure of the *japonica* rice variety Kinmaze differs distinctly from that of the *indica* variety IR36 in that the former is enriched in short chains of DP≦10, but has fewer intermediate-size chains of 13≦DP≦22 as compared with the latter. The distribution of long chains of DP≧25 is the same in both types of amylopectin.

In *amylose-extender* mutants of rice endosperm, amylopectin has few short chains of DP≦17. However, it should be noted that the *ae* amylopectin has more long chains of DP≧37 as compared with wild-type amylopectin (Nakamura et al. 1998; Nishi et al. 2001). Therefore, the pattern of alteration in amylopectin chain length in the *ae* mutant apparently differs from that observed between *japonica* and *indica* rice varieties.

Recent investigations established that amylopectin is synthesised by the concerted actions of ADPglucose pyrophosphorylase (AGPase), soluble forms of starch synthase (SSS), the starch branching enzyme (SBE), and the starch debranching enzyme (DBE) (Martin and Smith 1995; Smith et al. 1997). Multiple forms of all amylopectin-synthesising enzymes have been reported. For example, maize endosperm contains large and small subunits for AGPase (Preiss and Levi 1980); BEI, BEIIa and BEIIb forms for SBE (Preiss and Levi 1980; Gao et al. 1997); SSI and SSIII forms (Cao et al. 1999), or in addition an SSIIa form for SSS (Harn et al. 1998); and isoamylase and pullulanase for DBE (Beatty et al. 1999).

The present investigation was conducted to reveal the gene responsible for the structure of either *indica*- or *japonica*-type amylopectin. To achieve this, backcross inbred lines between *japonica* and *indica* rice varieties, Nipponbare and Kasalath, respectively, were used for mapping the genes which cause differences in the physico-chemical properties of starch granules and in the amylopectin fine structure between the two rice varieties, and a candidate gene(s) encoding an amylopectin synthesising enzyme(s).

Materials and methods

Plant materials

The backcross inbred lines (BILs) derived from Nipponbare (a *japonica* variety)/Kasalath (an *indica* variety)//Nipponbare were used (Lin et al. 1998). Namely, Nipponbare was crossed with Kasalath and the resultant F_1 plant was crossed with Nipponbare to produce BC_1F_1 seeds. Ninety eight BC_1F_5 lines were developed from the resulting BC_1F_1 progeny by the single-seed descent method. The BILs used for amylopectin chain-length analysis were advanced to generation BC_1F_8 seeds which matured under field condition in Tsukuba, Japan. The BILs used in other experiments were BC_1F_9 seeds harvested from BC_1F_8 plants. These BILs and parental lines were grown in plastic pots in a greenhouse. Each pot contained 3 to 5 plants from the same line. After flowering, plants were transferred into growth chambers in which the day/night temperatures were controlled at 25°C/20°C (changed at 6:00 and 18:00 h) under natural day length condition s, and ears were harvested at maturity.

Analysis of chain-length profiles for amylopectin

One grain was used for each analysis of amylopectin chain distribution from BILs and their parental lines, Nipponbare and Kasalath. The grain was hulled, the embryo removed with tweezers, and ground in a mortar and pestle. Debranched amylopectin was prepared as described by Umemoto et al. (1999). The chain-length distribution was determined by HPAEC-PAD as described by Nakamura et al. (1997).

Disintegration of starch granules in alkali solution and gelatinisation in urea solution

Three grains each of Nipponbare, Kasalath and the BILs were used. The seeds were hulled and halved vertically by a razor. Apical halves were used to examine the disintegration in alkali solution, while dorsal halves, including the embryo, were used for gelatinisation in urea solution. Three halved-grains were put into a well of a 24-well plate (Asahi Techno Glass Co., Tokyo, Japan). The disintegration and gelatinisation of starch granules were detected by standing the grains in 1 ml/well of 1.3% (w/v) KOH solution for 20 h at room temperature, or in 4 M urea solution (pH adjusted to 6.4 with 10% acetic acid) for 18 h at 30°C, respectively. The degree of disintegration and the gelatinisation of BIL grains were determined using Nipponbare and Kasalath as the positive and negative standards, respectively.

Sequencing of a cDNA fragment coding for rice SSIIa

A partial cDNA clone (EST) for *OsSSIIa* (clone E11025, accession no. C73554) which was identified by homology searching using maize starch synthase IIa (*ZmSSIIa*) (Harn et al. 1998) was sequenced. The sequence was determined on both strands by the dideoxy chain-termination method using the Dye Primer Sequencing Kit (PE Applied Biosystems, USA). Results were analyzed using Genetyx Mac (Tokyo, Japan).

Southern-blot analysis and mapping of the rice SSIIa gene

Southern analysis was performed according to the method of Maniatis et al. (1982) using a full-length DNA insert of a cDNA clone (E11025) as the probe, as previously described (Nakamura et al. 1996). The chromosomal location of the *OsSSIIa* gene was determined using the above-described 98 BILs and the genotype data on RFLP markers for each BIL together with the genetic linkage map, provided by the Rice Research Genome Program (http://rgp.dna.affrc.go.jp/).

Results

The chain-length profiles of amylopectin in the endosperm of the parental rice varieties used for the backcross inbred lines

To compare the structural differences in endosperm amylopectin between the *japonica* rice variety Nipponbare and the *indica* rice variety Kasalath, parental lines of backcross inbred lines (BILs), or between another *japonica* rice Kinmaze and *indica* rice IR36, the distribution of the chain-length of amylopectin was analysed by means of high-performance anion-exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD) after debranching the starch fraction with *Pseudomonas amyloderamosa* isoamylase (Fig. 1A). The amylopectin chain-length profiles of Nipponbare and Kasalath were clearly different in that Nipponbare had more short chains, particularly with DP 7 to 10. A similar difference was observed between Kinmaze and IR36 (Fig. 1A). Figure 1B compares the chain-length distribution of amylopectin from IR36 with those from other varieties in the range of DP 6 to 24. There was no significant difference in amylopectin structure between the two *indica* varieties, Kasalath and IR36. In contrast, amylopectin in the two *japonica* varieties, Nipponbare and Kinmaze, was markedly enriched in short chains with DP 7 to 10, and depleted in intermediate-sized chains with DP 12 to around 21 compared to IR36. The pattern of differences in chain lengths of amylopectin was similar in the two *japonica* varieties. These results suggest that the amylopectin fine structure in terms of its chain-length profile is determined by the genetic background of the rice varieties. This observation predicts the possibility that the gene(s) which determine(s) either type of amylopectin chain-profile in rice endosperm can be mapped by using BILs developed by crossing Nipponbare and Kasalath, since RFLP markers, together with data on the genotype of 98 BILs (BC_1F_5), are available from the database provided by the Rice Genome Research Program (http://rgp.dna.affrc.go.jp/).

Genetic analysis and chromosomal location of the gene which causes the difference in amylopectin chain-length between two rice varieties

The chain-length distribution of debranched amylopectin from the 98 BILs endosperm was measured by HPAEC-PAD. The ratio of the sum of peak areas for DP 6–11 to that for DP 12–24 was used to characterize the chain-length distribution of amylopectin of the BILs. Figure 2 illustrates that the BILs were clearly divided into two groups, one with ratios ranging from 0.24 to 0.33 and another from 0.36 to 0.48. The former group including Kasalath with the ratio of 0.28, and the latter group including Nipponbare with the ratio of 0.40, consisted of 28 and 70 BILs, respectively. Therefore, the proportion

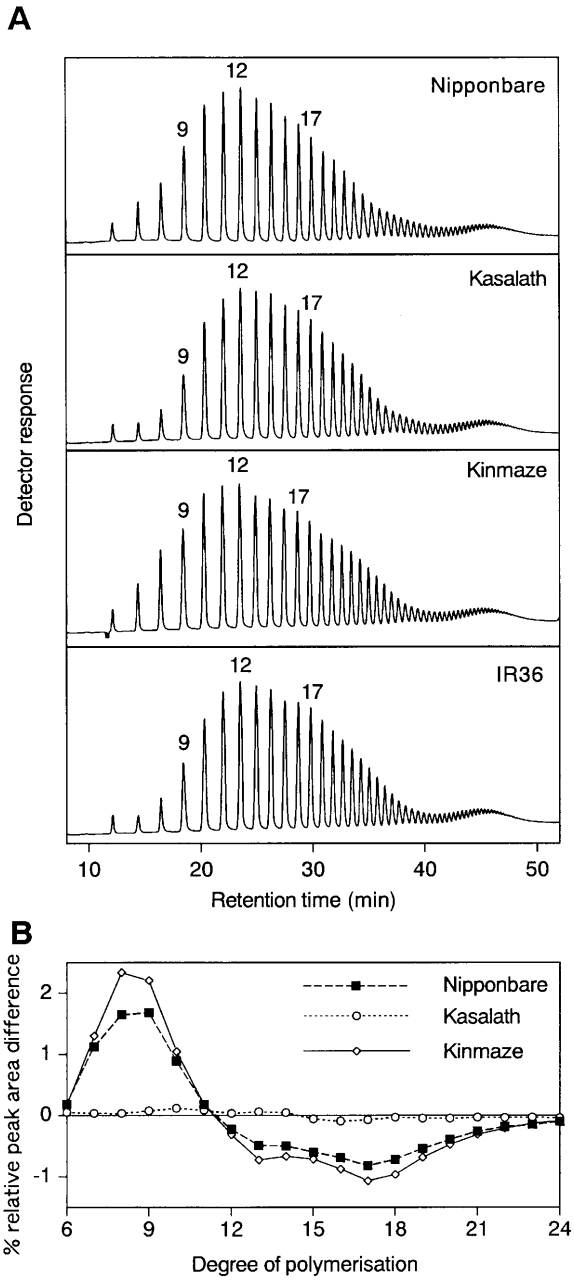

Fig. 1A, B Comparison of the chain-length profile of amylopectin in rice endosperm from various *japonica* (Nipponbare and Kinmaze) and *indica* (Kasalath and IR36) rice cultivars. **A** HPAEC-PAD analysis of debranched amylopectin from rice endosperm. From top to bottom, rice cultivars: Nipponbare; Kasalath; Kinmaze; IR36. Nipponbare and Kasalath are the parental varieties of BILs used in this study. The *number* above the peak means the degree of polymerization (*DP*). Note that there are no significant differences in the distribution of chains with DP≧25 among the four rice cultivars. **B** Difference in the chain-length distribution of amylopectin. The difference in the relative peak area between Nipponbare, Kinmaze or Kasalath and IR36 is shown in the DP range of 6 to 24

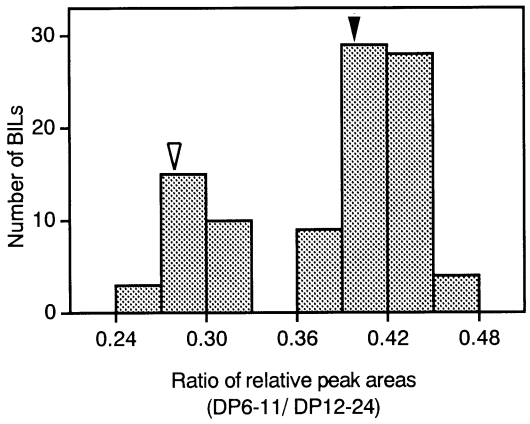

Fig. 2 Segregation of the amylopectin chain-length distribution from BILs and their parent cultivars Nipponbare and Kasalath. The distribution pattern of amylopectin chain length in each BIL was characterized by the ratio of the total peak areas of DP 6–11 to those of DP 12–24. The ratios in Nipponbare (0.40) and Kasalath (0.28) are shown by *closed and open arrowheads*, respectively

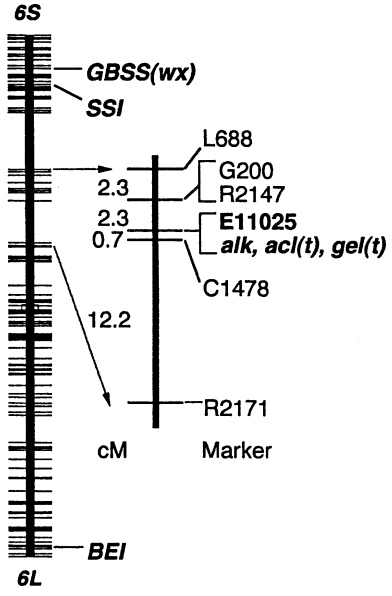

Fig. 3 Location of the *alk* gene and the gene corresponding to E11025 on chromosome 6 in the rice genome. The *left vertical bar* indicates an RFLP linkage map constructed from the F_2 population of Nipponbare and Kasalath (Harushima et al. 1998). The *right vertical bar* is the linkage map constructed in this study. The EST clone E11025, the *alk* gene, the *acl(t)* gene and the *gel(t)* gene were located between RFLP markers G200, R2147 and C1478 with a map distance of 2.3 cM and 0.7 cM, respectively

of the Nipponbare-group lines to the Kasalath-group lines is 2.5:1. This segregation ratio is not statistically significantly different from the 3:1 segregation ratio expected for a single gene (χ^2=0.67, p=0.50–0.30). It is noteworthy that the ratio of the Nipponbare group to the Kasalath group is expected to be 3:1 since Nipponbare was used for backcrossing (BC_1F_1). Thus, it is highly probable that the difference in chain-length distribution between Nipponbare and Kasalath is controlled by a single gene. We have tentatively designated this allele *acl(t)* since it markedly affected *a*mylopectin *c*hain *l*ength. The chromosomal location of *acl(t)* was determined by comparing the genotype of each BIL with respect to RFLP markers and the amylopectin type. As shown in Fig. 3, the *acl(t)* gene was mapped at 36.7 cM from the top of the short arm of chromosome 6, based on the high-density genetic linkage map (Harushima et al. 1998). It mapped between the RFLP markers G200, R2147 (at 33.5 cM) and C1478 (at 37.7 cM), in a position that is the same as the *alk* locus of rice (Harushima et al. 1998).

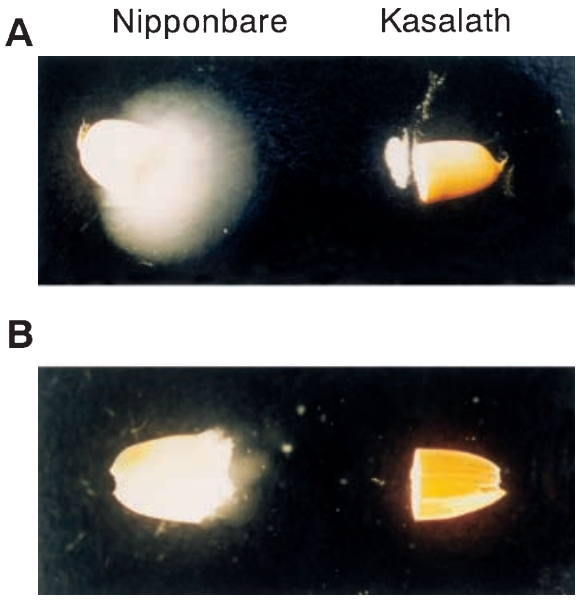

Fig. 4 Disintegration of starch granules in rice kernels of *japonica* variety Nipponbare and *indica* variety Kasalath in 1.3% KOH (**A**) and 4 M urea (**B**) solutions

Mapping of the genes determining the physico-chemical properties of starch granules in terms of degradation in alkaline solution and of gelatinisation in urea solution

Figure 4 shows that the difference between Nipponbare and Kasalath in the physico-chemical properties of their starch granules could be assessed by incubating the halved kernels in 1.3% KOH solution at room temperature or in 4 M urea solution at 30°C. The endosperm starch granules from Nipponbare were apparently disintegrated in both solutions while those from Kasalath were scarcely degraded. The phenotype was also examined in every BIL line, and the *alk* gene was mapped at 36.7 cM on chromosome 6, consistent with the report by Harushima et al. (1998).

The gene which distinguishes the gelatinisation-behavior of *japonica*-type starch granules from that of *indica*-type granules in the urea solution was also mapped at the same position to the *alk* gene and the *acl(t)* gene in all BIL lines. We tentatively designated it as the *gel(t)* gene.

Fig. 5 Alignment of amino-acid sequences of the EST clone (E11025) coding for rice SSIIa (OsSSIIa), maize SSIIa (ZmSSIIa) (Harn et al. 1998) and maize SSIIb (ZmSSIIb) (Harn et al. 1998). The consensus regions for SSII are *shaded*, as described in Li et al. (1999). *Asterisks* show the identical amino acids among the three sequences

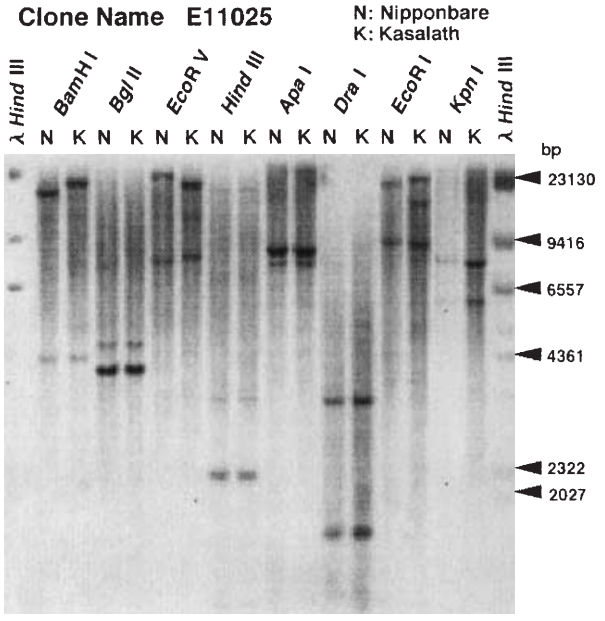

Fig. 6 Genomic Southern-blot analysis of the EST clone (E11025). Rice genomic DNA from Nipponbare (*N*) and Kasalath (*K*) was digested separately with various restriction enzymes as shown in the figure

Characterization of a rice cDNA coding for SSIIa and genetic-linkage mapping of the *SSIIa* gene

Partial cDNA clones coding for SSIIa were identified using maize endosperm SSIIa (Harn et al. 1998) by Blast analysis of the rice endosperm-expressed sequence tag (EST) data (Yamamoto and Sasaki 1997). The nucleotide sequence of the longest EST clone (E11025) designated *OsSSIIa* is 1,724 bp in length. The deduced amino-acid sequence of OsSSIIa encodes a polypeptide of 466 residues, and contains eight out of possible nine distinct SS motifs (Li et al. 1999) (Fig. 5). OsSSIIa is most-closely similar to maize SSIIa (87.6% identity), and to a lesser-extent to maize SSIIb (77.9% identity) (Fig. 5). OsSSIIa showed significant similarities with other SS proteins; the identities to rice endosperm SSI (Baba et al. 1993), maize endosperm SSIII (DU1) (Gao et al. 1998) and potato SSIII (Abel et al. 1996; Marshall et al. 1996) being 48.0% (when 435 amino acids were compared), 33.9% (254 amino acids) and 33.0% (300 amino acids), respectively.

Southern-blot analysis was performed to examine the genomic organization of the *OsSSIIa* gene in the rice genome and the RFLP pattern between Nipponbare and Kasalath (Fig. 6). Signals on the blot of the genomic DNAs digested with a variety of restriction enzymes indicated that *OsSSIIa* is a single-copy gene. DNA polymorphism was detected with *BamH*I, *EcoR*V and *EcoR*I digests. The segregation of BILs was assessed using the 20-kb fragment (Nipponbare genotype) or the 23-kb fragment (Kasalath genotype) with *BamH*I digestion. The location of the *OsSSIIa* gene was determined to be at the same position in the rice genome as the *alk* gene.

Discussion

The present studies are the first to show that the genes *OsSSIIa*, *alk*, *gel(t)* and *acl(t)* very probably are identical, all being mapped at the same location on chromosome 6 in the rice genome (Fig. 3). If this is the case, the *OsSSIIa* gene must be responsible for differences between the *japonica* variety, Nipponbare, and the *indica* variety, Kasalath, in terms of amylopectin chain-length distribution and the physico-chemical properties of their starch granules.

The OsSSIIa protein deduced from the nucleotide sequence of the EST clone (E11025, accession no. C73554) is quite similar in amino-acid sequence to that of maize SSIIa (ZmSSIIa) (Harn et al. 1998), and to a

lesser extent to that of ZmSSIIb (Harn et al. 1998). Blast analysis indicated the existence of three additional genes coding for OsSSI, OsSSIIb and OsSSIII in the rice genome (unpublished data). It was shown that the *OsSSI* gene is localized on chromosome 6 (Tanaka et al. 1995), but its location is clearly apart from that of the *alk* gene (Fig. 3). The *OsSSIIb* and *OsSSIII* genes are mapped on chromosomes 10 and 8, respectively (unpublished data).

It is widely accepted that SBE and DBE also play essential roles in amylopectin biosynthesis (Smith et al. 1997). Our previous studies showed that there are three SBE isoforms (Yamanouchi and Nakamura 1992) and two DBEs (pullulanase and isoamylase) (Nakamura et al. 1996; Fujita et al. 1999) in developing rice endosperm, and each of these enzymes presumably plays a distinct role in amylopectin biosynthesis (Nakamura et al. 1998; Kubo et al. 1999). The *BEI* gene was mapped on chromosome 6 (Nakamura et al. 1994), but its location is clearly far from the *alk* gene (Fig. 3). The rice *BEIIb* gene encoding an *Amylose-Extender*-type protein from maize (Stinard et al. 1993), previously designated QEIIa by Yamanouchi and Nakamura (1992) and SBE3 by Mizuno et al. (1992), was mapped on chromosome 2 (Harrington et al. 1997). Our preliminary result indicates that the rice gene coding for BEIIa (previously designated as QEIIb by Yamanouchi and Nakamura 1992, and SBE4 by Mizuno et al. 1992) is on chromosome 4. These observations seem to exclude the possibility that some form of SBE or DBE plays a primary part in producing the distribution difference in amylopectin chain-lengths between the *indica* and *japonica* rice varieties.

SSII is a major constituent of SSS in the pea embryo and potato tuber, accounting for 60–70% (Craig et al. 1998) and 10–15% (Edwards et al. 1995; Abel et al. 1996; Marshall et al. 1996), respectively, while SSII contributes a minor part, if any, of the total SSS activity in cereal endosperm, such as maize (Cao et al. 1999) and wheat (Li et al. 1999). Craig et al. (1998) proposed that SSII plays a distinct role in the synthesis of the B_2 and B_3 chains of amylopectin on the basis of the observation that the amylopectin of *rugosus5* pea embryo is markedly deficient in chains with intermediate length. Edwards et al. (1999) found that in transgenic potato tubers where SSII activity is reduced (antisense SSII lines), amylopectin is enriched in short chains of DP 6–12, whereas it has fewer chains in the range of DP 13–25. Since SSII and SSIII account for about 10–15% and 80%, respectively, of the total SSS activity in potato tubers (Edwards et al. 1995; Marshall et al. 1996), the result strongly suggests that SSIII is unable to substitute for the function of SSII in the elongation of short chains of amylopectin. In agreement with these results, Fontaine et al. (1993) found that in *Chlamydomonas* SSII plays an essential role for the synthesis or maintenance of the intermediate-size glucans of the amylopectin chains.

It should be noted that the difference in the chain-length profile for amylopectin between an antisense SSII line and wild-type potato plants (Edwards et al. 1999) is similar to that between the *japonica* variety Nipponbare and *indica* variety Kasalath of rice (Fig. 1B). The result supports the view that the SSII isoform plays a specific role in the elongation of short chains of DP 6–11 to form longer chains of DP 12–24 (long A chains and B_1 chains) in the developing endosperm of rice plants.

Recently, Yamamori et al. (2000) reported that deficiency of starch granule protein-1 (SGP-1) in wheat affected the morphology of the starch granules in the endosperm, and that the amylopectin was enriched in chains of DP 6–10 and depleted in chains of DP 11–25. The cDNA sequences coding for the SGP-1 protein are highly similar to the maize SSIIa gene (Li et al. 1999). These results strongly suggest that SSIIa plays a distinct role in the elongation of short chains within a cluster ($A+B_1$ chains) of amylopectin in cereal endosperm, and this role can not be complemented by other SS isoforms.

Amylopectin side chains of DP$\geqq$10 form double helices, the length of which determines the gelatinisation temperature of the starch granules (Gidley and Bulpin 1987; Moates et al. 1997; Noda et al. 1998; Safford et al. 1998). Therefore, starch granules containing amylopectin with longer A and B_1 chains are predicted to be more-resistant to gelatinisation and are less-soluble in alkali solution. Thus, it is reasonable that *japonica* starch granules are more easily disintegrated in alkali solution and exhibit a lower gelatinisation temperature than *indica* starch granules (Little et al. 1958; Juliano et al. 1964), since *japonica* amylopectin is enriched in shorter A and B_1 chains relative to *indica* amylopectin.

What is the difference between the alleles of *SSIIa* in *japonica* and *indica* rice that causes the phenotypic difference between the two rice varieties in amylopectin structure? This could be a difference either in the amount of SSIIa or in the functional capacity of SSIIa in the two varietal types. It is possible that the amount of SSIIa protein is markedly higher in Kasalath (*indica*) than the in Nipponbare (*japonica*) due to reduced expression of the enzyme in the latter, which may be determined either at the transcriptional level or post-transcriptional level. Alternatively, if the intrinsic activity of SSIIa is hampered in the *japonica* rice variety, the catalytic capacity of the *japonica* SSIIa may be markedly lower than that of the *indica* SSIIa. It is also possible that some modification of the *japonica* SSIIa may occur, lowering its affinity for substrates or altering its binding properties for starch granules. Even if this is the case, the reduction in SSIIa activity in *japonica* rice would explain the higher proportion of short chains within clusters observed in its amylopectin. These ideas will be tested, for example, by inserting the *SSIIa* gene from the *indica* variety Kasalath into the *japonica* variety Nipponbare, and analysing the amylopectin chain-length distribution in the transformed plants.

It is stressed that our results can not necessarily exclude the possibility that other genes play additional roles in influencing the physico-chemical behavior of rice starch granules. Recently, two groups reported QTL analysis of rice grain qualities including alkali disintegration of endosperm starch granules. He et al. (1999)

detected two loci controlling alkali disintegration, and found that the one exhibiting the major effect was mapped at the *alk* locus. On the other hand, Tan et al. (1999) mapped a single gene controlling alkali disintegration at the *waxy* locus. The apparent discrepancy between the two results may be due to the differences in the plant materials used. He et al. (1999) used a double-haploid population via anther culture of an F_1 hybrid between *japonica* and *indica* varieties, while Tan et al. (1999) used the recombinant inbred lines between *indica* varieties as parental lines.

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