Effects of Various Allelic Combinations of Starch Biosynthetic Genes on the Properties of Endosperm Starch in Rice

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Abstract
Rice endosperm accumulates large amounts of photosynthetic products as insoluble starch within amyloplasts by properly arranging structured, highly branched, large amylopectin molecules, thus avoiding osmotic imbalance. The amount and characteristics of starch directly influence the yield and quality of rice grains, which in turn influence their application and market value. Therefore, understanding how various allelic combinations of starch biosynthetic genes, with different expression levels, affect starch properties is important for the identification of targets for breeding new rice cultivars. Research over the past few decades has revealed the spatiotemporal expression patterns and allelic variants of starch biosynthetic genes, and enhanced our understanding of the specific roles and compensatory functions of individual isozymes of starch biosynthetic enzymes through biochemical analyses of purified enzymes and characterization of japonica rice mutants lacking these enzymes. Furthermore, it has been shown that starch biosynthetic enzymes can mutually and synergistically increase their activities by forming protein complexes. This review focuses on the more recent discoveries made in the last several years. Generation of single and double mutants and/or high-level expression of specific starch synthases (SSs) allowed us to better understand how the starch granule morphology is determined; how the complete absence of SSIIa affects starch structure; why the rice endosperm stores insoluble starch rather than soluble phytoglycogen; how to elevate amylose and resistant starch (RS) content to improve health benefits; and how SS isozymes mutually complement their activities. The introduction of active-type SSIIa and/or high-expression type GBSSI into ss3a ss4b, isa1, be2b, and ss3a be2b japonica rice mutants, with unique starch properties, and analyses of their starch properties are summarized in this review. High-level accumulation of RS is often accompanied by a reduction in grain yield as a trade-off. Backcrossing rice mutants with a high-yielding elite rice cultivar enabled the improvement of agricultural traits, while maintaining high RS levels. Designing starch structures for additional values, breeding and cultivating to increase yield will enable the development of a new type of rice starch that can be used in a wide variety of applications, and that can contribute to food and agricultural industries in the near future.

Keywords: Amylopectin, Amylose, Backcrossing, Endosperm, Indica rice, Japonica rice, Starch biosynthesis, Starch branching enzyme, Starch debranching enzyme, Starch synthase

Background
Starch (α-glucan), which is synthesized mainly from photosynthetic products, is deposited in the storage tissues of plants and represents the most important source of carbohydrates in the human diet. Starch is a gigantic polymer of glucose molecules connected by α-1,4- and α-1,6-glucosidic bonds. The major component of starch
is amylopectin, a highly branched structure, while that remaining is composed of amyllose, which is essentially a linear polymer. The structure of starch, as described above, has been extensively studied at the molecular level; however, the higher order structure of starch has yet to be fully understood. To date, the higher order structure of starch has been postulated by two models and is currently under debate: the Cluster Model (Nikuni 1969; French 1972; Hizukuri 1986) has been proposed by many researchers, and the Backbone Model (Bertot et al. 2012) is currently being challenged (Crini et al. 2021; Nakamura and Kainuma 2021).

Starch biosynthesis involves at least four classes of enzymes (Smith et al. 1997): ADP-glucose pyrophosphorylase (AGPase), which supplies glucose moiety as a substrate; starch synthases (SSs), which use the substrate to elongate α-1,4-linked linear glucose chains; starch branching enzymes (BEs), which create α-1,6-linked branches; and starch debranching enzymes (DBEs), which trim off improper branches (Nakamura 2002). Additional enzymes are also involved in starch biosynthesis: starch phosphorylase, which functions in the initiation step of starch biosynthesis (Satoh et al. 2008; Nakamura et al. 2012); disproportionating enzyme, which transfers α-1,4-linked glucans (Colleoni et al. 1999a, b); and Protein Targeting to Starch (PTST), which guides starch biosynthetic enzymes to the starch granules (Seung et al. 2018). The function of each starch biosynthetic enzyme was elucidated in the 1960s to 1980s using biochemical approaches, and the DNA sequence of the corresponding genes was determined in major crops in the 1990s. Our understanding of the mechanisms of starch biosynthesis rapidly improved after the 2000s because of the isolation, identification, and characterization of mutants lacking starch biosynthetic enzymes in major crops such as maize, rice, wheat, barley, potato, and beans, in addition to model organisms such as Arabidopsis and Chlamydomonas. Our research group revealed the functions of each starch biosynthetic enzyme by exhaustively isolating japonica rice mutants lacking these enzymes and by comparing the characteristics of these mutants with those of the wild-type in detail. The results of our studies served as the basis for the model of starch biosynthesis (Nakamura 2002; Fujita 2014).

The abovementioned rice mutants not only contributed to the discovery of starch biosynthesis mechanisms but also displayed great potential for expanding the use of rice starch in various applications. The starch accumulated in some of these rice mutants exhibited unique physicochemical properties. For example, the texture of high amylose mutant rice ss3a, after cooking, is utterly different from that of wild-type japonica rice. The be2b mutants, which contain high levels of resistant starch (RS), are likely to be utilized as functional rice because the intake of RS is thought to promote human health. Greater variation in the properties of rice starch will likely expand its utilization in various food applications. In this review, the process of generating new rice lines, with unique and novel starch properties, by designing the starch structure, is explained based on accumulating evidence obtained through the analysis of single and double mutants, and by the introduction of high-expressing alleles of starch biosynthetic genes. Please refer to the book “Starch” on rice lines generated before 2015 (Fujita 2015). The present review focuses on rice lines generated after 2015, and describes the breeding of new promising rice cultivars and their potential application in the food industry.

Methods
Mutant rice lines described in this article were isolated from the N-Methyl-N-nitosourea (MNU)-treated populations of japonica rice cultivars (Kinmaze and Taichung 65) or from the Tos17 insertion mutant panels of japonica rice cultivar, Nipponbare. Mutant lines were identified by western blot analysis of mature seed extracts to confirm the absence of specific proteins. The presence of single nucleotide polymorphisms (SNPs) was confirmed by PCR amplification of genomic DNA isolated from seedlings, followed by genotyping using cleaved amplified polymorphic sequence (CAPS) or derived cleaved amplified polymorphic sequence (dCAPS) markers. SSIIa and/or GBSSI genes derived from indica rice were selected by PCR using SNP-specific primers. Mutant lines lacking starch biosynthetic enzyme(s) were backcrossed with elite rice cultivars, ‘Akita 63’ or ‘Akita Komachi’, and homozygous BC2F2 plants were identified as described above. The selected homozygous plants were grown to maturity, and the seeds were used for extracting starch using the cold-alkaline method. The purified starch was gelatinized, debranched using Pseudomonas isoamylase, and separated by gel filtration chromatography using ToyoPearl HW55s connected in series to three ToyoPearl 50S columns to analyze the apparent amyllose content. The amylopectin branch structure was analyzed by capillary electrophoresis, and starch granule morphology was observed by scanning electron microscopy.

SSIIa Alleles
Most of the indica rice cultivars possess active-type SSIIa (wild-type), whereas typical japonica rice cultivars possess mutant SSIIa harboring three amino acid substitutions, thus exhibiting only 10% of SSIIa activity relative to indica rice (Nakamura et al. 2005). Low SSIIa activity in japonica rice leads to a reduction in amylopectin branches with degree of polymerization (DP) 13–24, and
an increase in short amylopectin chains with DP \leq 12 (Umemoto et al. 1999, 2002; Nakamura et al. 2005). These changes in amylopectin branch structure drastically affect the gelatinization temperature of starch (Noda et al. 1998), which is 5–10 °C lower in japonica rice than in indica rice (Nakamura et al. 2002). The effects of SSIIa absence on amylopectin branch structure have also been reported in other plant species such as maize (Zhang et al. 2004; Liu et al. 2012), wheat (Yamamori et al. 2000), barley (Morell et al. 2003), sweet potato (Katayama et al. 2004; Liu et al. 2012), wheat (Yamamori et al. 2000), barley (Morell et al. 2003), and Arabidopsis (Zhang et al. 2008). Loss of SSIIa in maize (Takeda and Preiss 1993), wheat (Yamamori et al. 2000), barley (Morell et al. 2003), and Arabidopsis (Zhang et al. 2008) was accompanied by an increase in amylose content. However, unlike other plant species, accurate evaluation of the association between the increase in amylose content and the loss of SSIIa is not straightforward in rice. This is because the amylose biosynthesis gene granule bound-starch synthase I (GBSSI), which is located close to the SSIIa gene on chromosome 6, carries a SNP at the exon1–intron1 boundary in japonica rice, which reduces the amount of GBSSI protein, thus decreasing the amylose content in japonica rice compared with indica rice (Sano 1984).

We isolated a null mutant of the SSIIa gene, EM204, from the MNJ mutant panel of the indica rice cultivar Kinmaze (Miura et al. 2018). EM204 possesses a SNP at the end of the 5th intron, resulting in the skipping of the 6th exon during translation, eventually producing a negligible amount of the truncated SSIIa protein lacking 15 amino acids (Miura et al. 2018). EM204 showed no SSIIa activity, higher amounts of short amylopectin chains (DP \leq 12), and lower amounts of amylopectin branches with DP 13–24 compared with wild-type japonica rice, expressing SSIIa with low activity (Fig. 1; Miura et al. 2018). Comparison of amylose content between lines containing or lacking SSIIa under the same japonica-type gbss1 allele revealed that loss of SSIIa led to a higher amylose content, as in other plant species (Miura et al. 2018).

Knocking down SSIIa from wild-type japonica rice, Nipponbare, by RNAi also did not show any increase in amylose content in endosperm starch. Its amylopectin structure, physicochemical properties of starch, and seed phenotype resembled those of EM204 (Butardo et al. 2020). SSIIa locus is known to be the same as ALK (Umemoto et al. 1999, 2002), and there are four known alleles, ALKa, ALKb, ALKc, ALKd, depending on the combinations of amino acid substitutions (Zhang et al. 2020; Chen et al. 2020). ALKc encodes indica-type active SSIIa, whereas ALKa encodes japonica-type SSIIa with Glu88Asp, Gly604Ser, Val737Met substitutions resulting in low activity and an increase in amylopectin short chains with DP < 12 (Nakamura et al. 2005; Zhang et al. 2020). ALKb encodes japonica-type SSIIa with Glu88Asp, Gly604Ser, Leu781Phe substitutions, and analyses of near isogenic lines showed a further increase in amylopectin short chains with DP < 12 and decrease in gelatinization temperature compared to ALKc. In contrast, ALKd with Glu to Asp substitution in Exon 1 of ALKc showed a decrease in short amylopectin chains with DP < 12 and an increase in gelatinization temperature (Zhang et al. 2020). Given abovementioned starch structure characteristics of SSIIa alleles, it is no doubt that the strength of SSIIa activity determines the ratio of short amylopectin chains with DP < 12 and intermediate amylopectin chains with DP 13–24. These greatly influence physicochemical properties such as gelatinization temperature and retrogradation of starch. Particularly, the ss2a mutant or SSIIa knockdown rice lines could be utilized practically as a genetic material for preventing the retrogradation of starch, and for breeding rice that tastes excellent after cooking and that maintains its stickiness and softness after cooking.

Ultra-High RS Content Rice

Rice and maize genomes encode three BE isozymes, BEI, BEIIa, and BEIIb, and single mutants of each BE gene have been isolated in japonica rice. BEI is expressed in both endosperm and vegetative tissues (Yamanouchi...
and Nakamura 1992). The phenotype of the be1 single mutant generally resembles that of the wild-type, with minor differences in endosperm amyllopectin structure; the amount of amyllopectin branches with DP 12–21 and DP > 37 is slightly higher in be1, whereas that of DP < 10 is slightly increased, compared with the wild-type (Sato et al. 2003). BEI is highly expressed in vegetative organs, and its absence does not affect the structure of endosperm starch (Nakamura 2002). By contrast, BEIIb is expressed exclusively in the endosperm, and be2b mutants exhibit a drastically different endosperm starch structure compared with the wild-type japonica rice: the amount of amyllopectin chains with DP < 14 is much lower, while the amylose content is considerably higher in be2b mutants than in the wild-type (Nishi et al. 2001). Owing to its unique structure, starch in be2b mutants resists degradation by digestive enzymes, resulting in high RS content (Tsuki et al. 2016; Miura et al. 2021).

Knocking out BEIIb in indica rice by CRISPR/Cas9 approach gave similar effects on starch properties, and increased amyllopectin branches with DP 6, 7 and > 25, decreased amyllopectin chains with DP 9–24, increased gelatinization temperature by 6 °C, and increased amylose content by 7% (Tappibani et al. 2021).

RS is defined as the starch that is not easily digested by human digestive enzymes and therefore reaches the large intestine, where it prevents a sudden increase in post-prandial glucose levels and improves the intestinal environment, thus promoting human health (Englyst et al. 1992; Nugent 2005; Matsuji 2010). RS contents were analyzed in various rice cultivars and mutants, including indica rice cultivars with high amylose content, single and double mutants of ss3a with high amylose content, and single and double mutants of be2b with high levels of long amyllopectin chains. The results of the survey revealed that the RS contents of be2b mutants were an order of magnitude higher than those of other lines. The RS content of high amylose rice, such as indica rice and the ss3a single mutant, was only several times higher than that of wild-type japonica rice. Previously, amyllose was considered to be one of the factors contributing to the RS content; however, our study clearly demonstrated that high levels of long amyllopectin branches is the primary factor responsible for the increase in RS content (Tsuki et al. 2016).

Compared with the be2b single mutant, the be1 be2b double mutant contained fewer amyllopectin chains with DP 10–20 and more amyllopectin chains with DP ≥ 21, and showed an amylose content of 52%, which was the highest in the entire rice lines (Fig. 2; Miura et al. 2021). Given its extreme starch composition, the be1 be2b double mutant showed an increase in the gelatinization temperature of starch by 7.5 °C, and in the RS content of its cooked rice (76%) by threefold, compared with the be2b single mutant. Further analysis demonstrated that RS extracted from the cooked rice of be1 be2b consisted of partially degraded amylose and long amyllopectin branches. Although the be1 be2b double mutant lacked two major BE isozymes, its plant growth and fertility rate were not affected, and it showed rather higher seed weight than the be2b single mutant (Miura et al. 2021).

Introduction of SS2a from Indica Rice into ss1 and ss3a
Among all starch biosynthetic enzymes, SSs possess the largest numbers of isozymes. Among these isozymes, SSI, SSIIa, and SSIIIa play major roles in amyllopectin biosynthesis in the endosperm. As described above, SSIa found in indica rice is the highly active wild-type enzyme, whereas that found in japonica rice exhibits only 10% of the activity of indica SSIa (Nakamura et al. 2005). Because of the low-level activity of SSIa in japonica rice, the genotype of wild-type japonica rice is shown as SS1 ss2a L SS3a. Japonica rice mutants lacking SSI (Fujita et al. 2006) or SSIIIa (Fujita et al. 2007) have been isolated previously, both of which show no activity of the corresponding enzymes and different amyllopectin structures relative to wild-type japonica rice. However, the ss1 and ss3a mutants accumulate starch to levels similar to the wild-type, which implies that other SS isozymes complement the functions of missing isozymes. In fact, the ss3a mutant showed increased levels of SSI and GBSSI proteins, and these effects were clearly reflected in the starch structure (Fujita et al. 2007). Homozygous ss1 ss3a double mutant is sterile; however, genotypes heterozygous for one of mutant alleles exhibit starch accumulation (Fujita et al. 2011).

Active-type SSIa derived from indica rice was introduced into the ss1 and ss3a single mutant lines of japonica rice to generate ss1 SS2a SS3a and SS1 SS2a ss3a mutants, and their amyllopectin structures were investigated. The results showed that rice lines with the SS2a allele, such as ss1 SS2a SS3a and SS1 SS2a ss3a, showed a reduction in amyllopectin chains with DP ≤ 11 and an increase in amyllopectin chains with 12 ≤ DP ≤ 24 compared with lines harboring the ss2aL allele, such as ss1 ss2aL SS3a and SS1 ss2aL ss3a. These results indicate that
SSIIa functions in the absence of SSI or SSIIIa (Crofts et al. 2017a).

Analysis of japonica ss1 or ss3a mutant rice revealed that each SS isozyme plays a distinct role in amylopectin biosynthesis. SSI elongates the non-reducing ends of amylopectin chains with DP 6 or 7 to DP 8–12 (Fujita et al. 2006); SSIIa functions to elongate amylopectin chains with DP 6–12 to DP 13–24 (Nakamura et al. 2005); and SSIIIa synthesizes long amylopectin chains with DP > 30 (Fujita et al. 2006, 2007). Generation of new rice lines by introducing indica rice-derived active-type SS2a allele into japonica ss1 or ss3a mutant rice enabled the demonstration of compensatory roles among SSI, SSIIa, and SSIIIa (Crofts et al. 2017a). The chain-length distribution pattern of indica rice (SSI SS2a SS3a) was similar to that of the ss1 SS2a SS3a line, containing active-type SSIIa in the absence of SSI. The absolute value of the subtraction curve of “ss1 SS2a SS3a” (rice containing active-type SSIIa in the absence of SSI)—“SSI SS2a SS3a” (wild-type indica rice)” was considerably lower than that of “ss1 ss2aL SS3a” (japonica ss1 mutant)—“SSI ss2aL SS3a” (wild-type japonica rice). These results indicated that the active-type SSIIa enzyme derived from indica rice compensated most of the functions of SSI. The increase in the amount of amylopectin chains with DP 6–7 and the reduction in that of chains with DP 8–12 in the absolute value of the subtraction curve “ss1 SS2a SS3a—SSI SS2a SS3a” were minimized by 80%; however, the increase of DP 16–19 was minimized by only 50%. This implies that SSIIa derived from indica rice compensates the function of SSI in elongating the non-reducing ends of A-chains with DP 6–7 into those with DP 8–12, but does not perfectly compensate its role in the elongation of the non-reducing ends of B1-chains into those with DP 16–19. By
contrast, the SSI SS2a ss3a line generated by introducing indica rice-derived active-type SS2a allele into the ss3a japonica mutant did not increase the amount of long amylopectin chains with DP > 30. This clearly indicates that SSIIa derived from indica rice does not compensate for the function of SSIIIa. Similarly, SSI could not compensate for the function of SSIIa or SSIIIa, and SSIIa could not compensate for the function of SSI or SSIIa (Fig. 3; Crofts et al. 2017a).

**Introduction of SS2a and/or GBSS1 from Indica Rice into ss3a ss4b**

SS enzymes possess a total of 11 isozymes: one SSI isozyme, three SSII isozymes, two SSIII isozymes, two SSIV isozymes, one SSV isozyme, and two GBSS isozymes. The function of each SS isozyme, including SSI, SSIIa, SSIIa, and GBSSI, strongly expressed in the endosperm, became apparent through the characterization of the corresponding mutants and the biochemical analysis of purified enzymes. However, the functions of other SS isozymes have not yet been elucidated because they are expressed in tissues other than the endosperm. Mutants lacking SSIVb, one of the SSIV isozymes, have been isolated; however, their amylopectin structure and seed phenotype were the same as those of the wild-type. In contrast to the ss4b single mutant, double mutants lacking both SSIIa and SSIVb (ss2a ss3a ss4b) showed a remarkably different starch granule morphology (spherical) compared with the wild-type (polygonal) (Toyosawa et al. 2016). The amounts of B2 and B3 long amylopectin chains, which connect amylopectin clusters, were further reduced in ss3a ss4b mutant compared with ss3a. This suggests that SSIVb is involved in the synthesis of B2 and B3 chains of amylopectin, and therefore is functionally redundant to SSIIa. Each amyloplast contains multiple starch granules, which form a compound structure in rice (Matsushima et al. 2013), within which individual starch granules are separated from each other by a septum-like structure (Kawagoe 2013). Immuno-electron microscopy observation using anti-SSIVb antibody revealed that SSIVb localizes to septum-like structures in the amyloplast in the developing endosperm of rice (Toyosawa et al. 2016), while SSIIa localizes to the starch granules as well as the outer envelope membrane surrounding the amyloplast.

Loss of SSIIa alone results in slightly rounded starch granule morphology compared with the wild-type (Fujita et al. 2007). Confocal laser scanning microscopy analysis of the ss3a ss4b double mutant revealed the presence of compound-type starch granules, although they resembled the spherical simple-type starch granules in shape. Thus, the loss of both SSIIa and SSIVb made the septum-like structure fragile, reduced starch biosynthesis, and fully expanded the starch granules, resulting in spherical starch granules (Toyosawa et al. 2016).

The ss3a ss4b mutants were derived from japonica rice, and therefore showed low SSIIa activity and GBSSI protein levels compared with indica rice (Nakamura et al. 2005; Sano 1984). To determine whether the alteration of starch granule morphology in japonica ss3a ss4b mutants could be complemented by SSIIa and/or GBSSI derived from indica rice, the corresponding genes were introduced into ss2a ss3a ss4b gbss1 by crossing with indica rice (SS2a SS3a SS4b GBSSI1), and SS2a ss3a ss4b gbss1, ss2a ss3a ss4b GBSSI1, and SS2a ss3a ss4b GBSSI were generated (Crofts et al. 2021a). However, the expression of SS2a and/or GBSSI in the japonica ss3a ss4b mutant did not recover the starch granule morphology from spherical to polygonal (Fig. 4). This implies that the loss of SSIIa and SSIVb cannot be complemented by SSIIa or GBSSI (Crofts et al. 2021a). Lines containing active-type SSIIa, such as SS2a ss3a ss4b gbss1c and SS2a ss3a ss4b GBSSI1, showed lower levels of amylopectin chains with DP 7–12 and higher levels of those with DP ≥ 13 compared to the lines containing less active SSIIa. Amylose contents of ss2a ss3a ss4b GBSSI1, SS2a ss3a ss4b GBSSI1, and SS2a ss3a ss4b gbss1c were 40%, 36%, and 31%, respectively. The ADP-glucose content of the crude extract prepared from the developing endosperm of lines carrying the gbss1c allele but lacking SSIIa and SSIVb (such as ss2a ss3a ss4b gbss1c and SS2a ss3a ss4b gbss1c) was high, whereas that of lines with the GBSSI1 allele was low, perhaps because ADP-glucose was used for amylose synthesis (Crofts et al. 2021a).
Introduction of SS2a and/or GBSS1 from Indica Rice into High RS Mutant Rice

As a potential target locus for increasing RS content in indica rice, a loss of function mutation in SSIIIa in the presence of GBSS1 (Wxa) allele was identified by map-based cloning (Zhou et al. 2016). The rice grains of ss3a GBSS1 had high amylose content (approximately 35%) and more amylose–lipid complex, which increased the RS content to 6% compared to that of the control line (approximately 1%) (Zhou et al. 2016). In comparison to the loss of SSIIIa, the loss of BEIIb drastically increases the RS content of endosperm starch (Tsuiki et al. 2016), as described above. This is because a considerable decline in the amount of short amyllopectin branches proportionally increases the amount of long amyllopectin chains, and a reduction in amyllopectin biosynthesis elevates the amylose content; both of these factors contribute to the increase in RS content (Tsuiki et al. 2016; Zhou et al. 2016; Chen et al. 2017). To determine whether RS content can be further increased, SS2a and/or GBSS1 alleles from indica rice were introduced into japonica be2b single mutant or ss3a be2b double mutant (Itoh et al. 2017; Miura et al. submitted). To practically utilize high RS rice lines, it is important to increase their yield. However, elevation of the RS content of the endosperm and increase in seed weight and yield are often a trade-off. To solve this problem, RS-rich rice lines were backcrossed three times with the high-yielding and large grain bearing rice cultivar, ‘Akita 63’ (Makino et al. 2020).

Compared with mutants lacking SS isozymes such as SSI, SSIIIa (Crofts et al. 2017a), or SSIIIa and SSIVb (Crofts et al. 2021a) described above, japonica be2b single mutant or ss3a be2b double mutant showed no significant change in starch structure upon the introduction of the indica SS2a allele (Itoh et al. 2017; Luo et al. 2020). This is because the number of short amyllopectin branches (DP < 12), which serve as primers for SSIIa, was greatly reduced in the absence of BEIIb. On the other hand, introduction of the GBSS1 allele into the japonica be2b single mutant increased the apparent amylose content to approximately 38% (equivalent to a 1.75-fold increase relative to the original japonica be2b mutant; Miura et al. submitted). Analyses of the recombinant inbred lines, which possess SS2a and GBSS1 but lack BEIIb, also showed high amylose content (31–34%) but had some short branches on amylose (Zhang et al. 2022). Loss of SSIIIa is known to increase the apparent amylose content of japonica rice (ss2aL ss3a be2b) compared with wild-type and be2b single mutant japonica rice, because of a concomitant increase in GBSSI and AGPase protein levels (Asai et al. 2014). However, introduction of the GBSS1 allele into the japonica ss3a be2b double mutant did not cause any further increase in the apparent amylose content (Miura et al. submitted), presumably because the ADP-glucose content reached its upper limit and was insufficient to meet the demand of increased GBSSI levels. The RS content of rice lines lacking BEIIb was higher than that of lines lacking SSIIIa and BEIIb, regardless of the presence or absence of SS2a and/or GBSS1 alleles. This is because the loss of SSIIIa increases the expression levels of SS1, thus increasing the number of short amyllopectin branches and decreasing the number of long amyllopectin chains connecting amyllopectin clusters (Fujita et al. 2007). Introduction of SS2a and/or GBSS1 elevated the RS content, and backcrossing the resultant RS-rich lines increased their seed weight by up to 1.9-fold. This is likely because flowering time was advanced, and temperature during the seed development period became more...
suitable for efficient starch biosynthesis. The ss2a\textsuperscript{c} be2b GBSSI\textsubscript{1} rice line showed high RS content and outstanding seed weight (29 mg per grain) compared with typical japonica rice (20 mg per grain), indicating its potential for practical applications.

**Introduction of SS2a and/or GBSSI from Indica Rice into isa1**

In addition to elongation of glucans by SS and generation of branches by BE, the trimming of inappropriate glucan branches by one of the DBEs, called isoamylase 1 (ISA1), is essential for formulating properly structured insoluble starch and for generating plump seeds. Loss of ISA1 leads to the accumulation of water-soluble phytoglycogen and the production of shriveled seeds in many plant species, including maize (James et al. 1995) and rice (Nakamura et al. 1996, 1997; Kubo et al. 1999), as shown by the analyses of isa1 (sugary-1, sug-1) mutants (Nakamura 2002). *Japonica* isa1 mutants can be roughly divided into two groups: severe-type, with phytoglycogen accumulation in the entire endosperm; and mild-type, showing phytoglycogen accumulation in the seed core but starch accumulation in the seed periphery, which can be stained by iodine (Nakamura et al. 1997; Kubo et al. 1999). These differences are dependent on the site of mutation within the ISA1 gene, but their exact cause remains unknown. Introduction of the SS2a allele from *indica* rice into severe-type *japonica* isa1 mutant (EM914) using the transgenic approach resulted in the accumulation of iodine-stainable insoluble α-glucans in the endosperm (Fujita et al. 2012). This insoluble glucan contained longer short amylopectin branches than phytoglycogen, but showed no increase in the amount of long amylopectin chains (DP > 30). X-ray diffraction analyses revealed that this glucan showed weak B-type crystallinity, unlike phytoglycogen (no crystallinity) and wild-type rice (clear A-type crystallinity).

When SS2a and/or GBSSI\textsubscript{1} alleles were introduced into the mild-type *japonica* isa1 mutant (EM653) by conventional crossing, the resultant genotypes (SS2a gbss1\textsuperscript{L} isa1 and SS2a GBSSI\textsubscript{1} isa1) accumulated starch instead of phytoglycogen and produced plump seeds (Crofts et al. 2021b). While introduction of the GBSSI\textsubscript{1} allele alone into the mild-type *japonica* isa1 mutant (Fujita et al. 2012). The *GBSSI* allele resulted in displaying clear A-type crystallinity and polygonal starch granules, similar to the wild-type; however, this result was not obtained by the introduction of SS2a into the severe-type *japonica* isa1 mutant (Fujita et al. 2012).

Taken together, these findings suggest that SSIIa partially complements the loss of ISA1 by avoiding generation of phytoglycogen and by accumulating amylopectin. SSIIa could also sufficiently extend the branches of phytoglycogen. On the other hand, while the introduction of the GBSSI\textsubscript{1} allele alone into the *japonica* isa1 mutant could elevate the amyllose content by 10%, it could not convert phytoglycogen into amylopectin, and the seeds remained shriveled.

**Practical Applications of Mutant Lines**

The absence of enzymes required for starch biosynthesis partially inhibits the production of starch in the developing endosperm, leading to smaller grains and poor yield; the loss of BEIIb is a classic example. In addition, loss of more than one enzyme, such as both SSI and SSIIa (Fujita et al. 2011) or both SSI and BEIIb (Abe et al. 2014), results in sterility. Unless the plant is completely sterile, the reduction in grain yield can be overcome by the introduction of loci responsible for high yield. As an attempt to improve agricultural traits, such as early flowering, large grain size, and high grain yield, we crossed rice mutants accumulating structurally unique starch with a high-yielding and elite rice cultivar ‘Akita 63’ (Makino et al. 2020).

The ss3a mutant accumulates a large amount of amyllose, with almost no reduction in seed weight, compared with the wild-type; however, its late-flowering phenotype, as observed in our research paddy filed at high latitude in Akita prefecture, suggests that ss3a is not suitable for commercial cultivation. The ss3a mutant exhibited delayed flowering because its genes controlling flowering time were derived from the late-flowering parental rice cultivar ‘Nipponbare’. We backcrossed the ss3a mutant three times either with a high-yielding elite rice cultivar ‘Akita 63’ or with an excellent tasting elite rice cultivar ‘Akita Komachi’, resulting in the production of ‘Akita Sarari’ or ‘Akita Parari’, respectively (Fujita 2020). Cultivars ‘Akita 63’ and ‘Akita Komachi’ were selected as recurrent parents for backcrossing, since both these cultivars are suitable for cultivation in high latitude regions such as the Akita prefecture. In theory, genes derived
from an elite parental rice cultivar account for 93.75% of the genetic background of the BC3 progeny. The flowering time of newly generated rice cultivars ‘Akita Sarari’ and ‘Akita Parari’ was similar to that of the elite rice cultivars (early August) and approximately 1 month earlier than that of the original ss3a mutant. The seed weight and yield of ‘Akita Sarari’ and ‘Akita Parari’ were also similar to those of ‘Akita 63’ and ‘Akita Komachi’, respectively. ‘Akita Sarari’ presented high yield and high amylose content, and its flour could be used for the production of noodles and bread. ‘Akita Parari’ exhibited a less sticky texture relative to the typical japonica rice cultivars and could be used for making pilaf, fried rice, and risotto. Both ‘Akita Sarari’ and ‘Akita Parari’ are now commercially available (https://starchtec.com/).

As described earlier, RS content and seed weight are often in a trade-off relationship in the absence of BEIIb. We tried to overcome this problem by crossing the high RS lines with a highly yielding rice cultivar. The high RS rice cultivar ‘Manpuku Surari’ was generated by backcrossing the ss3a be2b mutant (Asai et al. 2014) with ‘Akita 63’ three times. The resultant rice line ‘Manpuku Surari’ exhibited 1.5-fold higher seed weight compared with the original ss3a be2b mutant, and tenfold higher RS content in cooked rice and rice flour relative to the typical japonica rice, in addition to the ability to suppress

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**Fig. 5** Analysis of rice seed morphology. Images show the morphologies of dehulled mature rice seeds (top), stereo-micrographs of iodine-stained cross-section of mature seeds (middle), and scanning electron micrographs of the central region of mature seeds (bottom). Data were obtained from Crofts et al. (2021a)
postprandial increase in glucose levels (Saito et al. 2020). Thus, ‘Manpuku Surari’ is anticipated to be sold as “Foods with Function Claims”, as designated by the Consumer Affairs Agency.

Another effective way to overcome the low seed weight in high RS lines is to combine the absence of BEIIb with an absence of ISA1. ‘Chikushi-kona 85’ is a high RS japonica-rice mutant lacking BEIIb activity, but generated relatively plump seeds unlike shrunken seeds often seen in be2b mutants (Wada et al. 2018). The detailed genetic analyses of ‘Chikushi-kona 85’ and its parental mutant EM129 revealed that they were the double mutants lacking both BEIIb and ISA1 (Nagamatsu et al. 2022). BEIIb and ISA1 are the enzymes responsible for the generation of short amylopectin branches (Nishi et al. 2001; Tanaka et al. 2004) and removal of improper amylopectin branches (Nakamura 2002), respectively. The absence of these enzymes gives opposite effects on amylopectin structure. Loss of BEIIb drastically decreases short amylopectin chains with DP<14 and increases long amylopectin chains (Nishi et al. 2001), while loss of ISA1 greatly increases short amylopectin chains with DP<11 and decreases intermediate amylopectin chains (Nakamura et al. 1997). Both of these single mutant lines display shrunken or wrinkled seed phenotype with low seed weight (Nishi et al. 2001; Nakamura et al. 1997). Endosperm starch of ‘Chikushi-kona 85’ and EM129, both of which lack BEIIb and ISA1, showed milder starch phenotypes compared with the be2b single mutant, and accumulated a very small amount of phytoglycogen at the center of the seeds (Nagamatsu et al. 2022). In other words, the deficiency of BEIIb and ISA1 enzymes canceled each other’s effects, and their double mutant resulted in high RS content and high seed weight (Nagamatsu et al. 2022). ‘Chikushi-kona 85’, generated by crossing EM129 with a high yield elite rice cultivar, was registered as a rice variety that suppresses fluctuation of postprandial blood glucose levels (Wada et al. 2018).

**Conclusions**

Our results showed that the structure and properties of starch in the rice endosperm could be manipulated by abolishing and/or increasing the expression levels of various starch biosynthetic enzymes; however, the degree and type of change relative to wild-type rice varied widely. The longer the length between the non-reducing ends to the branch point of amylopectin branches within a cluster, the higher the temperature required for starch gelatinization. Moreover, as the gelatinization temperature increases, the retrogradation of starch becomes easier, while its degradation by digestive enzymes becomes more difficult. Conversely, the shorter the amylopectin branches, the easier the gelatinization and degradation, and the more difficult the retrogradation. Among the dozens of isozymes of starch biosynthetic enzymes, ISA1, SSIa, and BEIIb had the greatest influence on the structure and properties of amylopectin. The length of amylopectin branches decreased in the absence of ISA1 and SSIa, and increased in the absence of BEIIb, thus demonstrating that ISA1, SSIa, and BEIIb directly influence the physicochemical properties of starch, including gelatinization temperature, retrogradation, and enzymatic degradation.

Research has also elucidated the order of starch biosynthesis. A recent study showed that BEIIb generates amylopectin branches near the amorphous region of the crystalline lamella (Nakamura et al. 2020). Amylopectin branches generated by BEIIb are first elongated by SSI by the addition of two glucose residues, and then further elongated by active-type SSIa in indica rice (Abe et al. 2014; Crofts et al. 2017a). This is supported by the drastic reduction in SSI activity upon the loss of BEIIb (Nishi et al. 2001). Furthermore, SSI, SSIa, and BEIIb are thought to form a trimeric complex, which facilitates efficient starch biosynthesis during seed development (Liu et al. 2012; Crofts et al. 2017b).

High-level expression of GBSSI in japonica rice mutants, achieved by the introduction of the GBSSI allele from indica rice, generally increases their amylose content. However, introduction of the GBSSI allele into the ss3a be2b double mutant, in which the expression level of the endogenous GBSSI gene is also high, does not cause any further increase in amylose content, most likely because of the depletion of its substrate (ADP-glucose) (Miura et al. submitted).

The generation of new allelic combinations of starch biosynthetic genes exhibiting no and/or high expression levels in rice, and analysis of their starch properties are starting to allow us to freely design the starch structure according to the application-based requirements. We have isolated numerous japonica rice mutant lines lacking starch biosynthetic enzymes, analyzed their starch phenotypes, and revealed the function of individual enzymes (Fujita 2014). However, a considerable proportion of the world’s population consumes indica rice as their staples. Comparing with the rich source of japonica rice mutant panels, the number of mutants isolated from indica rice and their reports are currently limited. However, presence of unique allele(s) that affect starch properties in indica rice is expected, such as ALK described in the present review article. In fact, six starch biosynthetic enzymes, SSI, SSIa, GBSSI, BEI, BEIIa, and BEIIb, are known to have different alleles between japonica and indica rice cultivars. Their recombinant inbred lines showed that not only SSIa but also SSI, EBII, and BEIIa gave a minor effect on starch structure (Luo et al. 2015). These suggest that the presence of unique
alleles derived from indica rice that are different from the japonica rice. Accumulating the knowledge of different alleles in indica rice variety and combining with the alleles from japonica rice will enable to expand the variation of starch further. If these allelic combinations inhibit starch biosynthesis and deteriorate certain agricultural traits such as seed size, then the lines carrying these allelic combinations could be backcrossed with high-yielding elite varieties. Designing starch structures, breeding and cultivating to suit their growth environment and increase yield, and popularizing them will enable the development of a new type of rice starch, which can be used in a wide range of applications, and can contribute to the food industry and agricultural sector in the near future.

Abbreviations
AGPase: ADP-glucose pyrophosphorylase; BE: Starch branching enzymes; DBE: Starch debranching enzymes; DP: Degree of polymerization; GBSS: Granule-bound starch synthase; ISA: Isoamylase; RS: Resistant starch; SNP: Single nucleotide polymorphism; SS: Starch synthases.

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Author contributions
NF designed research, performed experiments and wrote the paper. SM isolated a double mutant and performed experiments, analyzed data. NC isolated a double mutant and wrote the paper. All authors read and approved the final manuscript.

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