Starch Biosynthesis in Rice Endosperm

Naoko Fujita

Department of Biological Production, Faculty of Bioresource Sciences
Akita Prefectural University
241-438 Kaidobata-nishi, Shimoshinjo-nakano, Akita 010-0195, Japan
e-mail: naokof@akita-pu.ac.jp

Abstract
Starches are the most important form of carbohydrates for most organisms on earth. However, the starch structure and biosynthesis mechanisms have not been completely resolved. At least four classes of enzymes catalyze the reactions of starch biosynthesis in plants: starch synthase (SS) elongates α-glucan chains of starch, ADP-glucose pyrophosphorylase (AGPase) supplies the substrate for SS, branching enzyme (BE) forms the α-1,6-glycosidic bonds of amylopectin, and debranching enzyme (DBE) trims improper branches generated by BE. Many isozymes of these enzymes encoding different genes exist in green plants. To understand the starch biosynthesis mechanisms, the author tried to isolate rice mutant lines and transgenic rice lines of the genes that account for starch biosynthetic enzymes. Through the biochemical and physiological analyses of these materials during the last 15 years, the function of the isozymes expressed in the endosperm of rice has been better understood. We built the model of amylopectin biosynthesis based on the function of each isozyme. The unique starches that accumulate in the endosperm of mutant lines are quite different from those of the wild type. In the near future, the author hopes that unique starches that accumulate in the mutant lines will be useful for industrial applications.

Received on March 31, 2013
Accepted on August 30, 2013
Online published on February 26, 2014

Keywords
- amylopectin
- mutant lines
- rice
- starch biosynthesis
- transgenic rice lines

1. Prologue

Starches are large biopolymers consisting of glucose molecules joined by glycosidic bonds. Starches are the most important form of carbohydrate for every organism on earth. Starches also represent a storage form of polysaccharides, because excess glucose produced by plants during photosynthesis can be stored in starch molecules. It is thought that the molecular structure of starch molecules evolved during plant evolution. The large pool of stored starch molecules in seeds, embryos, and tubers of plants provide for smooth growth of new generations. The research effort devoted to understanding the chemistry and biology of starches is considerably smaller than that focusing on DNA and protein, although starch is one of the most important biopolymers. Starch has traditionally been considered a stable and bored material. However, research during the last 15 years shows that the structural and physicochemical properties of starches can be regulated by genetic manipulation of genes involved in starch biosynthesis.

Starch shows specific traits named gelatinization and retrogradation. Starch is gelatinized by heating it with water (**Fig. 1**) or treating it with alkaline, urea, or dimethyl sulfoxide through the cutting of hydrogen bonds between the starch molecules. It is thought that digestion of hydrogen bonds results in the unwinding of the double helices of parallel α-1,4 glucan chains in the crystal lamellae of amylopectin molecules. The reactions involved in the formation of gelatinized starch are not reversible (**Fig. 1**). Retrogradation of starch is thought to be the incomplete winding of neighboring chains of gelatinized starch. Retrograded starch can be gelatinized again by heating, indicating that the reactions between starch retrogradation and gelatinization are reversible (**Fig. 1**). Both gelatinization and retrogradation of starch play essential roles for food chemistry and industrial applications. Starches from plants with different genetic backgrounds have different physico-chemical properties and can be used for different purposes. Corn starches have widespread use for many applications because of their low cost and high performance. The advent of genetic engineering expanded the possibilities for production of tailor-made starches with specific properties that can be synthesized in the storage tissues of plants. People must use recyclable carbohydrate starches whose properties are

doi:10.5047/agbm.2014.00401.0001

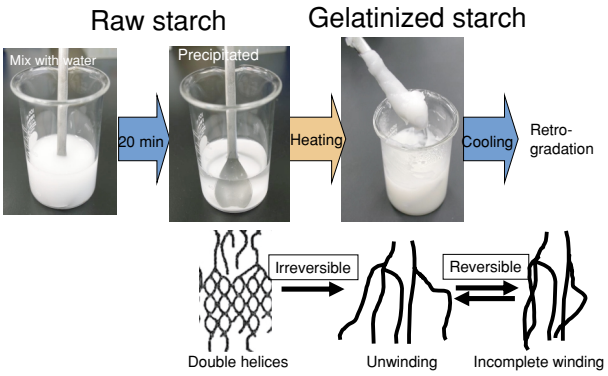

Fig. 1. "Insolubility", "gelatinization" and "retrogradation" of starch and its molecular model. Reprinted with permission from *Kagaku to Seibutsu*, **51**(6), Fujita, Denpunhenitaimai no kaiseki to riyou, 400–407, Fig. 1, © 2013, Japan Society for Bioscience, Biotechnology, and Agrochemistry.

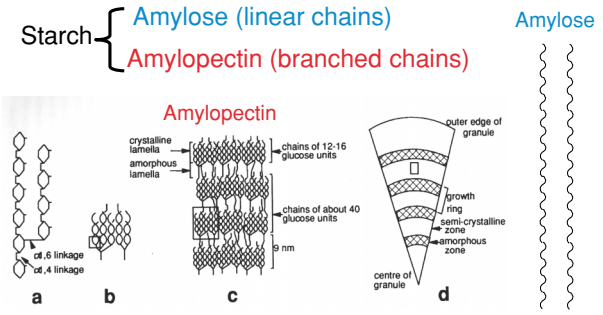

Fig. 2. Schematic representation of starch structure and the levels of organization within the starch granule. The boxes within the diagrams in panels b, c and d represent the area occupied by the structure in the preceding panel. (a) Structure of two branches of an amylopectin molecules, showing individual glucose units. (b) A single cluster within an amylopectin molecule, showing association of adjacent branches to form double helices. (c) Arrangement of clusters to form alternating crystalline and amorphous lamellae. The crystalline lamellae are produced by the packing of double helices in ordered arrays. Chains of 12–16 glucose units span one cluster, chains of about 40 glucose units span two clusters. (d) Slice through a granules, showing alternating zones of semicrystalline material, consisting of crystalline and amorphous lamellae, and amorphous material (Smith *et al.* 1997). Reprinted with permission from *Annu. Rev. Plant Physiol. Mol. Biol.*, **48**, Smith *et al.*, The synthesis of the starch granule, 67–87, Fig. 2, © 1997, Annual Reviews.

adaptable and efficient for the production of food and energy in the future.

2. Starch structure has not been resolved

Research on starch was delayed compared with that of other biopolymers due to the complexities of the starch structure. Starches are composed of multiple molecules with different molecular weights; nevertheless, the basic structure is simple and consists of glucose homopolymers joined through α-1,4- and α-1,6-glycosidic bonds. Starch consists of branched amylopectin and linear amylose (**Fig. 2**). The molecular weight of the major component of starch, amylopectin, is thought to be 10^{8-10} Da (Yoo and Jane 2002), which is as large as DNA (the largest molecules on earth). Starch is water-insoluble (**Fig. 1**). The Chinese characters for starch mean "the precipitated powder." The insolubility of starch is due to the semi-crystallinity of amylopectin. Amylopectin has a tandem-cluster structure, and the cluster is composed of amorphous lamellae and crystalline lamellae (**Fig. 2**). Amylopectin chains having branches and no branch are called B chains and A chains, respectively. The B chains within one cluster are called B_1 chains, by contrast, the B chains connecting 2 and 3 clusters are called B_2 and B_3 chains. The chains having a reduced end are called C chains (Peat *et al.* 1952). Most branch points in amylopectin molecules are located in amorphous lamellae (Jane *et al.* 1997), and a single cluster has a fixed length in a range of 9–10 nm (Jenkins *et al.* 1993). The fact that the crystalline lamellae, which are double helices of parallel α-glucan chains (DP $\geq$ 10), exclude water is what causes the insolubility of starch. Amylose is much smaller (MW = 10^{5-6}) than amylopectin and is composed of mainly linear chains (**Fig. 2**). The distribution and location of amylopectin and amylose in starch molecules remains unclear. It is thought that one starch granule contains both amylopectin and amylose and turns to purple stained with iodine (there are no blue starch granules containing only amylose and red starch granules containing only amylopectin stained with iodine in the endosperm cell at the same time). Many unanswered questions about starch structure make starch research a challenging field. Technical innovation including chromatography, solubility of starch molecules, observation of the inside of starch granules and so on are necessary to resolve the structure of starches. At the same time, we need to prepare various sorts of starch having a different structure with different genetic background and to know the effects of genes on the starch structure. I focus on the latter in this monograph.

3. Starch is produced by many kinds of enzymes (isozymes)

At least four enzymes classes catalyze the reactions of starch biosynthesis in plants: starch synthase (SS, EC2.4.1.21) elongates α-glucan chains of starch, ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27) supplies the substrate for SS, branching enzyme (BE, EC 2.4.1.18) forms the α-1,6-glycosidic bonds of amy-

doi:10.5047/agbm.2014.00401.0001

lopectin, and debranching enzyme (DBE) trims improper branches generated by BE (Smith *et al*. 1997; Myers *et al*. 2000; Nakamura 2002; Ball and Morell 2003). Starch biosynthesis functions to expand glucose polymers. There is no doubt that SS, AGPase, and BE are involved in starch biosynthesis. However, the role of DBEs (isoamylase, ISA, EC 3.2.1.68, and pullulanase, PUL, EC3.2.1.41) in starch biosynthesis was only confirmed when the corresponding gene of maize and rice *sugary-1* mutants, which accumulate glycogen-like α-glucan (phytoglycogen) instead of starch, were determined to be identical to the *Isoamylase1* (*ISA1*) of maize and rice in the late 1990s. The other enzymes, phosphorylase (PHO, EC 2.4.1.1) and disproportionating enzyme (DE, 2.4.1.25), are also thought to be involved in starch biosynthesis (Satoh *et al*. 2008; Colleoni *et al*. 1999).

Many isozymes of these enzymes encoding different genes exist in green plants. Most cyanobacteria, which are thought to be ancestors of chloroplasts in green plants, produce glycogen instead of starch, whereas a few cyanobacteria (Nakamura *et al*. 2005a) and red algae (Shimonaga *et al*. 2008) accumulate starch-like α-glucan. Green algae and higher plants accumulate starch. The structures of starches evolved during plant evolution, and these structures reflect the distinct roles of multiple isozymes. For example, SS in green plants can be divided into six types: SSI, SSII, SSIII, SSIV, SSV, and GBSSI (Hirose and Terao 2004). Each SS often has multiple isozymes. Rice has one SSI, three SSII, two SSIII, two SSIV, one SSV, and two GBSS isozymes. *Arabidopsis* has only a single isozyme of each type of SS. Elucidation of the distinct roles and specificities of each isozyme in different plant species will expand the understanding of starch biosynthesis.

4. Advantage of using rice for starch biosynthesis

To understand the function of each isozyme involved in starch biosynthesis, we are performing *in vivo* analysis using rice mutants and transgenic lines. Rice (*Oryza sativa* L.) is a monocot and important crop species, whereas *Arabidopsis* is a dicot and important as a research model plant. The entire DNA sequence of the rice cultivar "Nipponbare" was determined in 2005 (International Rice Genome Sequencing Project 2005). It is easy to isolate mutant lines in rice compared with the isolation of mutants in polyploid crops, such as wheat or potato, which have duplicated genes on homoeologous chromosomes. The methods for producing transgenic rice have been established for more than 20 years. There are two further advantages for starch scientists to use rice. The first is the extremely high concentration of starch in the rice endosperm compared with other crops. This is a great advantage for the purification of starch and starch biosynthetic enzymes. The second is that the parent cultivars of our mutant lines are japonica cultivars (Nipponbare, Taichung 65, and Kinmaze), which have an inactive SSIIa isozyme due to two point mutations in the *SSIIa* gene. By contrast, indica rice cultivars have an active SSIIa (Nakamura *et al*. 2005b). The phenotype of the SSI-deficient mutant does not appear unless the *ss2a* mutant is used as a parent mutant, because the functions in chain elongation by SSI and SSIIa are partially overlapping, as subsequently discussed (Fujita *et al*. 2006).

Most japonica rice cultivars are also GBSSI-leaky mutants whereas indica cultivars are the wild-type (Sano 1984). A point mutation in the boundary between exon 1 and intron 1 in the *GBSSI* gene leads to incomplete mRNA. This long mRNA reduces the normal amount of GBSSI in most japonica cultivars (Cai *et al*. 1998; Isshiki *et al*. 1998). GBSSI is involved in the synthesis of amylose in the endosperm. The average amylose content of japonica cultivars is lower (*ca*. 20%) than that of indica cultivars (*ca*. 25%). The differences of amylose content affect the physicochemical properties of starch; cooked rice of japonica cultivars is sticky, which is the Japanese preference.

5. Research of starch in other plants

Biochemical research on starch biosynthesis by measuring enzyme activities and determining the substrate specificities of the partially purified enzymes started in the 1960s. This research was accelerated with the development of column chromatography for enzyme purification in the 1990s. After 2000, many investigations using mutant and transgenic lines and genome information were performed. The main research groups investigating starch biosynthesis are the following: the groups of J. Preiss, A. Myers, and P. Keeling researching maize in USA; the groups of M. Emes and I. Tetlow researching maize in Canada; R. Visser's group in the Netherlands researching potato; A. Smith's group researching pea and potato in the UK; M. Morell's group researching wheat and barley in Australia; R. Chibbar's group researching wheat in Canada; J. Kossmann's group researching wheat in Germany; K. Denyer' group in the UK researching barley; research on *Arabidopsis* by S. Zeeman's group in Switzerland and C. D'Hulst's group in France; and S. Ball's group in France researching algae. Research on starch biosynthesis in rice is performed by H. Satoh's group and our group (Y. Nakamura and N. Fujita's group) using rice mutant lines. P. Wu's group and Q. Liu's group in China and J-S Jeon's group in Korea also study starch biosynthesis using rice.

A comparison of the reports on starch sciences by these researchers suggested that these plant species have common enzyme sets involved in starch biosynthesis, and the functions of these enzymes also appeared to be common. However, the distinct starch

doi:10.5047/agbm.2014.00401.0001

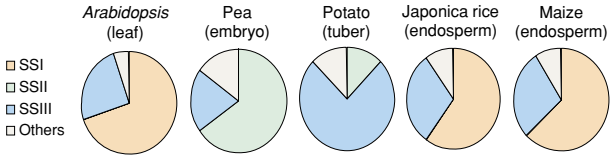

Fig. 3. Relative activities of SS isozymes as expressed by percentages of total SS activities in *Arabidopsis* leaves, pea embryo, potato tuber, rice endosperm and maize endosperm. Each SS isoform activity in *Arabidopsis* (Zhang *et al*. 2005; Szydlowski *et al*. 2011) and japonica rice (Fujita *et al*. 2006) was estimated by native-PAGE/SS activity staining. That in pea (Craig *et al*. 1998), potato (Marshall *et al*. 1996) and maize (Cao *et al*. 1999) was estimated by immunoprecipitation method (Fujita and Nakamura 2012). Reprinted with permission from Tetlow, I. (ed), *Essential Reviews in Experimental Biology, Volume 5, Starch: Origins, Structure and Metabolism*, Fujita and Nakamura, Distinct and overlapping functions of starch synthase isoforms, 115–140, Fig. 2, © 2012, Society for Experimental Biology, London.

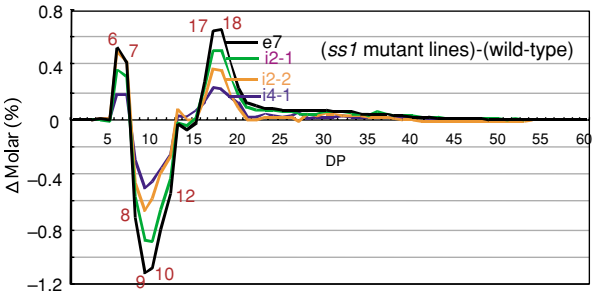

Fig. 4. Differences in chain-length distribution patterns of endosperm amylopectin between the mature endosperm of *ss1* mutant lines and the wild type. *e7*, *i2-1*, *i2-2*, *i4*-1 are the name of *ss1* mutant lines that *Tos17* is inserted into exon7, intron2, intron2 and intron4 of rice *SSI* gene, respectively. The SSI activity in these lines are 0, 1/6, 1/5, and 1/4 of the wild type, respectively (Fujita *et al*. 2006). Reprinted with permission from *Plant Physiol*., **140**, Fujita *et al*., Function and characterization of starch synthase I using mutants in rice, 1070–1084, Fig. 5B, © 2006, American Society of Plant Biologists (www.plantphysiol.org).

structures and physico-chemical properties in different plant species indicate that the presence and relative activities of isozymes greatly differ among plant species and tissues (**Fig. 3**; Fujita and Nakamura 2012). Rice has a large number of mutant lines, as many as maize and *Arabidopsis*, which were utilized to resolve the function of the corresponding isozymes. Starch research in rice and maize led to the studies on biosynthesis of the storage starches, which are utilized in industrial applications. The starch of *Arabidopsis*, which is the transient starch in the leaf, is not to be used for industrial applications. The studies of starch biosynthetic isozymes in mutant and transgenic rice mainly performed by our group will be introduced in the following section.

6. The function of isozymes related to the starch biosynthesis using rice mutant lines

Many starch mutant lines were isolated by Prof. Satoh in Kyushu University, Japan. Fertilized embryos of rice flowers were treated with the chemical mutagen *N*-methyl-*N*-nitrosourea (MNU) (Satoh and Omura 1979). The biochemical traits and starch properties of specific seed morphology lines were analyzed, and the corresponding genes were identified. This strategy is called "forward genetics". A "reverse genetics" strategy can also be used for mutant analysis. In this strategy, a single nucleotide polymorphism (SNP) of specific genes from the mutagenized rice population can be detected by the TILLING (targeting induced local lesions in genomes) method (Colbert *et al*. 2001); subsequently, the phenotypes are determined by the mutations.

Retrotransposon *Tos17* rice mutant stocks of greater than 40,000 populations have been prepared by Dr. Hirochika's group at NIAS in Japan (Hirochika 2001). The japonica rice cultivar Nipponbare has two copies of *Tos17* in the genome, and these duplicate and transport to the other position on the chromosome during culture as plant callus. The gene that *Tos17* inserts into is not able to be expressed and results in a mutant. To isolate a specific mutant of the gene *A*, PCR is performed using primer pairs between specific regions of gene *A* and *Tos17*. If the sequences of gene *A* and *Tos17* are detected from the amplified PCR fragment, *Tos17* must be inserted into gene *A*. Our mutant lines for starch biosynthesis were isolated from these mutants and analyzed by reverse genetics. By 2000, only three mutant lines, including *waxy* (*gbss1*), *sugary-1* (*isa1*), and *amylose-extender* (*be2b*), had been isolated by Prof. Satoh's group. Therefore, our group isolated additional mutant lines, especially for SS isozymes, which are the largest family of the starch biosynthetic enzymes. Single-mutant rice lines of SS, BE, and DBE isozymes account for starch biosynthesis, whereas PHO must be important for initiation of starch biosynthesis in the early stage of developing endosperm have been isolated. These are described in the following sections.

6-1. *ss1* mutant

SSI activity accounts for 60–70% of SS activity in the soluble fraction of developing endosperm in rice (Baba *et al*. 1993) and maize (Cao *et al*. 2000). As of 1999, SSI-deficient mutants were not isolated in any plant species. The reduction of potato SSI in antisense plants did not lead to any detectable changes in starch structure in the tuber. In potato, SSI is predominantly expressed in leaves and only to a lower extent in the

doi:10.5047/agbm.2014.00401.0001

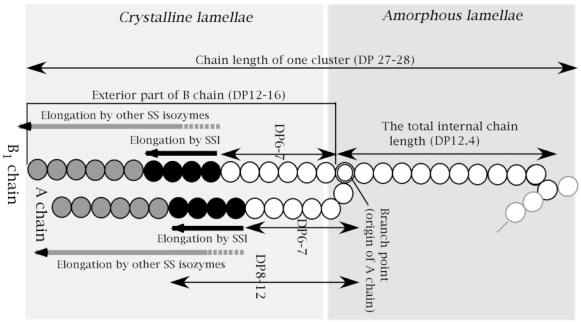

Fig. 5. Schematic representation of the proposed model for the elongation of rice amylopectin glucan chains by SSI and other SS isozymes. Circles represent glucose residues. In the wild-type, A and B_1 chains grow through the addition of 2–6 glucose residues (black circles) by SSI. Black and gray circles in A and B_1 chains are elongated by other SS isozymes when SSI is deficient. The double circle marks the point in the B_1 chain where a branch (A chain) emerges. The A chains and the exterior parts of B chains (from non-reduced end to branch point), both ranging from DP 12 to DP 16 in length (Hizukuri 1986), compose the crystalline domain of amylopectin clusters. The length of one cluster of amylopectin corresponds to DP 27-28 (Hizukuri 1986). In *waxy* maize, Bertoft (1991, 2004) estimated the total internal chain length of amylopectin to be DP 12.4. If this value holds true for rice amylopectin, the length of the B_1 chain would be the combined length of the exterior part plus DP 11. The partially broken arrows labeled "elongation by other SS isozymes" indicate compensatory function of other SS isozymes when SSI is deficient (Fujita *et al*. 2006). Reprinted with permission from *Plant Physiol*., **140**, Fujita *et al*., Function and characterization of starch synthase I using mutants in rice, 1070–1084, Fig. 8A, © 2006, American Society of Plant Biologists (www.plantphysiol.org).

tuber, in which SSII and SSIII are the major isozymes (Kossmann *et al*. 1999). Attempts were made to isolate rice *ss1* mutant lines from transposon *Tos17* populations using PCR screening. Four mutant lines carrying *Tos17* insertions in different positions of the *SSI* gene were isolated (Fujita *et al*. 2006). A strong SS activity band was detected in the middle of the gel on native-PAGE that was stained for SS activity in wild-type samples. However, this band was not detected in one *ss1* line, *e7*, in which *Tos17* is inserted in exon 7 of the *SSI* gene. This indicated that the middle migration band was SSI, and the *e7* line had no SSI activity band in the developing endosperm. The other lines, *i2-1*, *i2-2*, and *i4*, in which *Tos17* was inserted in intron 2(-1), intron 2(-2), and intron 4 of the *SSI* gene, had 1/6, 1/5, and 1/4 of the SSI activity of wild-type endosperm, respectively. *Tos17* inserted into an intron is usually spliced. However, the expression of normal mRNA is thought to be partially inhibited and results in a reduction of activity (leaky mutant). Although the chain-length distribution of the endosperm amylopectin in the *ss1* mutant lines showed specific patterns, the extent of the changes compared to those in wild-type were smaller than those of the *sugary-1* (ISA1-deficient) mutant lines and the *amylose-extender* (BEIIb-deficient) mutant lines. The degree of change in the pattern, a decrease in chains with a degree of polymerization (DP) 8–12 and an increase in chains with DP 6–7 and 16–19, was positively correlated with the extent of the decrease in SSI activity in the mutant lines (**Fig. 4**). These results strongly suggest that this pattern is caused by a deficiency of SSI activity (Fujita *et al*. 2006).

The interpretations of this pattern of amylopectin chain-length distribution were as follows. As shown in **Fig. 4**, the amount of DP 6–7 chains that were branched by BEIIb increased, because they were not elongated to DP 8–12 due to a lack of SSI. In other words, DP 8–12 chains decreased because the DP 6–7 chain precursors were not elongated and most of the available DP 8–12 chains were converted into longer chains by other SS isozymes. By contrast, the increase in DP 16–19 chains was primarily attributed to the increase in B_1 chains. Chains with DP 16–19 were predicted to have an exterior portion composed of DP 7. This was in agreement with the observed length of the A chains (DP 6–7), which increased in the mutant amylopectin. These results indicated that SSI distinctly generated DP 8–12 chains from short DP 6–7 chains that emerged from the branch point in the A or B_1 chains of amylopectin. These interpretations were supported by *in vitro* analysis of purified rice SSI expressed in *E. coli* (Fujita *et al*. 2008).

No *ss1* mutant has been isolated in plant species other than rice and *Arabidopsis*. Rice *ss1* mutant lines isolated by our group were the first *ss1* mutant lines that accumulate starch in storage tissues in higher plants. Surprisingly, the seed weight and accumulation of starch in the endosperm of the *ss1* null mutant line were similar to those of the wild-type, even though the SSI activity, which accounted for more than 60% of the SS activity in the crude extract of rice developing endosperm, was completely missing in this mutant. The seed morphology, crystallinity, and morphology of the starch granules in the lines were not significantly different from those of the wild-type. It was believed that an SSI-deficient maize mutant would be sterile; thus, an *ss1* mutant could not be identified (Commuri and Keeling 2001). These results indicated that the other SS isozymes must have complemented the function of SSI-deficiency in the rice endosperm.

6-2. *ss2a* mutant

Rice cultivars are divided into japonica and indica cultivar groups. The starch chain-length distribution pattern in japonica cultivars (Nipponbare and Kinmaze) is different from that of indica cultivars (Kasalath and

doi:10.5047/agbm.2014.00401.0001

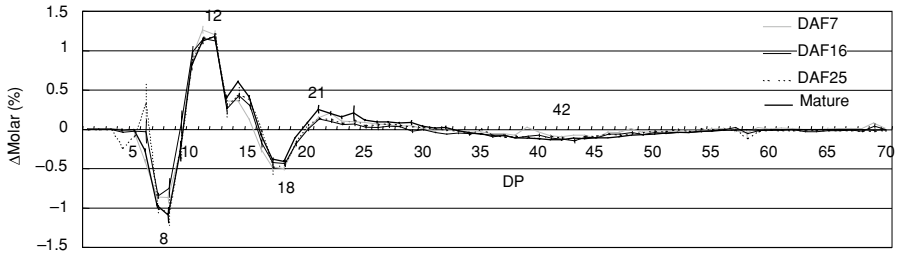

Fig. 6. Differences in the chain-length distribution patterns of amylopectin in developing endosperm at DAF 7, 16, and 25 and the mature endosperm of the *ss3a* mutant line and wild type Nipponbare. Vertical bars indicate standard errors (Fujita *et al*. 2007). Reprinted with permission from *Plant Physiol*., **144**, Fujita *et al*., Characterization of SSIIIa-deficient mutants of rice: the function of SSIIIa and pleiotropic effects by SSIIIa deficiency in the rice endosperm, 2009–2023, Fig. 7C, © 2007, American Society of Plant Biologists (www.plantphysiol.org).

IR36) (Umemoto *et al*. 1999, 2002). The short chains with DP 6–12 in the former are increased in the latter, and middle chains with DP 13–24 are decreased in the former compared with those in the latter. Analysis of the chain-length distribution of BILs (backcross inbred lines) between Nipponbare and Kasalath showed, that the gene regulating the chain-length distribution in indica and japonica rice, is located on the short arm of chromosome 6. This location is identical to the *alk* (starch disintegration by alkaline) locus and *SSIIa* gene (Kudo 1968; Umemoto *et al*. 2002). These results strongly suggest that the differences in chain-length distribution between japonica and indica cultivars are regulated by the *SSIIa* gene (Umemoto *et al*. 2002). The chain-length distribution analyses of endosperm starch in the global japonica and indica cultivars showed that chains with DP 6–12 and DP 13–24 in most japonica cultivars are increased and decreased (S-type amylopectin), respectively, compared with those in most indica rice cultivars (L-type amylopectin) (Nakamura *et al*. 2002). These changes in the amylopectin fine structure affect the physico-chemical properties; the gelatinization temperature of starch with enriched short chains in japonica cultivars is lower than that in indica cultivars (Nakamura *et al*. 2002). Two of four SNP of *SSIIa* gene between indica cultivars (Kasalath and IR36) and japonica cultivars (Nipponbare) are important for SSIIa activity. The activity of recombinant SSIIa of Nipponbare is less than 10% of that of wild-type SSIIa from indica cultivars (Nakamura *et al*. 2005b). Transgenic japonica rice (Kinmaze) expressing an introduced indica *SSIIa* gene change from japonica-type amylopectin (S-amylopectin) to L-amylopectin with alkaline-resistant starch (Nakamura *et al*. 2005b). SSIIa does not affect the amount of long chains with DP ≧ 25, indicating that SSIIa regulates the structure within one cluster of amylopectin and these changes affect the physico-chemical properties, such as gelatinization temperature and gelatinization-resistance to alkaline or 4 M urea. The function of SSIIa is common among maize (Zhang *et al*. 2004), wheat (Yamamori *et al*. 2000), barley (Morell *et al*. 2003), potato (Edwards *et al*. 1999), and pea (Craig *et al*. 1998).

6-3. *ss3a* mutant

SSIIIa accounted for the second major SS activity in the soluble fraction of developing rice and maize endosperm, followed by SSI (Fujita *et al*. 2006). Maize *dull-1* (*du1*) mutants were known as high-amylose mutants (Mangelsdorf 1947; Davis *et al*. 1955). It was reported that the *du1* gene was identical to the *SSIII* gene in maize using the gene tagging method (Gao *et al*. 1998). There was no *ss3* mutant in rice at that time; the *ss3a* rice mutant in which *Tos17* is inserted into exon 1 of the *SSIIIa* gene was isolated (Fujita *et al*. 2007). The seeds of the *ss3a* mutant lines had a chalky interior appearance and their hulled grain weight was similar to that of wild-type plants. The amylose content of the mutant lines was 1.3–1.5 times higher than that of the wild type. By contrast, the long chains connecting amylopectin clusters were only 60% of those of the wild type, indicating that the function of SSIIIa was the elongation of long chains connecting amylopectin clusters (**Fig. 6**, Fujita *et al*. 2007; Hanashiro *et al*. 2011). The deficiency of SSIIIa induced the expression of *SSI* and *GBSSI* genes. The AGPase activity increased in *ss3a* mutant compared with that in wild-type endosperm. A high-amylose content in this mutant line must be caused by the enhancement of GBSSI and AGPase activities (Fujita *et al*. 2007).

The function of SSIII(a) in the elongation of long B_{2-3} chains of amylopectin is common among maize (Cao *et al*. 1999), barley (Bertoft *et al*. 2011), potato (Edwards *et al*. 1999), and *Chlamydomonas* (Maddelein *et al*. 1994).

6-4. *gbss1* mutants

The *gbss1* mutant lines were the most well-known in many plant species; maize (Tsai 1974), rice (Sano

doi:10.5047/agbm.2014.00401.0001

1984), wheat (Nakamura *et al*. 1995; Fujita *et al*. 2001), barley (Eriksson 1962) *waxy* mutants, potato (Hovenkamp-Hermelink *et al*. 1987) *amf* (amylose free) mutant, and pea *lam* (*low amylose*) mutant. The trait of these mutants was that there was no amylose in storage starch, which was 100% amylopectin. This indicated that GBSSI was closely involved in the synthesis of amylose and extra-long chains of amylopectin, which are eluted in the similar retention time to the amylose chains by gel-filtration of debranched amylopectin (Takeda *et al*. 1987). The rice *gbss1* mutant lines were used for traditional rice cake foods, rice snacks, and sticky rice. The hardness of rice cake after incubation under low temperature depended on the cultivar background and affected the utilities of rice cakes; the low-hardness *gbss1* mutant was used for sticky rice, whereas the high-hardness *gbss1* mutant was used for rice snacks and rice cake. Unfortunately, the gene(s) regulating rice cake hardness are not isolated.

6-5. Other *SS* mutants

The *SSI*, *SSIIa*, *SSIIIa*, and *GBSSI* genes are highly expressed in developing endosperm. The functions of these genes have been resolved by analyses of the mutant lines. The mutants of the other SS isozymes have not been isolated and the functions are unknown. The mutants of *SSIIIb* and *SSIVb* genes have been isolated from the populations of *Tos17* mutant lines (Fujita *et al*. unpublished). *SSIIIb* and *SSIVb* genes are expressed primarily in leaves; a slight expression of *SSIVb* occurs in the early stage of developing endosperm (Hirose and Terao 2004). The seed phenotype, starch size, and structure of endosperm starch in both mutants is very similar to that of the wild type (Fujita *et al*. unpublished data). The *ss4b*/*ss3a* double mutant is quite different than either of the parent mutants and the wild-type plant.

6-6. *be1* mutants

Rice *be1* mutant lines have been isolated as lines that are deficient in the 83 kDa band on SDS-PAGE of endosperm samples from rice mutant stocks that were induced by the treatment of fertilized egg cells with MNU (Satoh *et al*. 2003). The BEI activity band in rice developing endosperm showed a strong and broad band on native-PAGE followed by BE-activity staining. There were no significant differences in seed phenotype and size between the BEI-deficient mutant and the wild type. The mutant amylopectin was characterized by a slight decrease in chains with DP ≥ 37 and short chains with DP 12–21, and an increase in short chains with DP ≤ 10 and DP 24–34. These results suggested that BEI specifically branches long chains (Satoh *et al*. 2003).

6-7. *be2a* mutant

The chain-length distribution pattern of the rice *be2a* mutant is very similar to that of the wild type (Nakamura 2002). This is caused by the low expression of the *BEIIa* gene in the endosperm, although the BEIIa activity bands are detected in a position of faster migration than the BEIIb activity bands in a of very early stage of developing endosperm on native-PAGE followed by BE-activity staining (Nishi *et al*. 2001). It might be possible that BEIIa is involved in the initiation of starch biosynthesis.

6-8. *be2b* mutants

Cereals such as maize, rice, wheat, and barley have two BEII isozymes (BEIIa and BEIIb). BEIIb of rice (Nishi *et al*. 2001) and maize (Baba and Arai 1984), and BEIIa of wheat (Regina *et al*. 2006) and barley (Regina *et al*. 2010) are involved in the branching of the short chains in amylopectin. A BEIIb-deficient mutant of maize and rice is well-known as the *amylose-extender* (*ae*) mutant lines. The name *amylose-extender* is derived from the blue-staining with iodine. It was thought that the high-amylose starch accumulated in the endosperm. However, analyses of the *wx*/*ae* double mutant showed that the increasing blue staining with iodine was due primarily to the enrichment of the long chains of amylopectin with DP ≧ 14 in the *ae* mutant (Nishi *et al*. 2001). These results suggested that the function of BEIIb was branching short chains in the crystalline lamellae of amylopectin. The shortage of the amylopectin short chains in the *be2b* mutant resulted in high-resistance to gelatinization (Nishi *et al*. 2001). The gelatinization onset temperature of the mutant starch is 12°C higher than that of the wild type (Tanaka *et al*. 2004).

The amylose content of the *be2b* rice mutant is 1.5-times higher than that of the wild type (Abe *et al*. in preparation). By contrast, the amylose content in the maize *ae* mutant starch is significantly increased (50–70%, Wang *et al*. 1993a, b) compared with that in wild-type maize (25%). The different amylose contents in rice and maize *ae* mutant lines might be caused by the fact that japonica rice cultivars have inactive SSIIa and low GBSSI content.

6-9. *isa1* mutants

Isoamylase1 (ISA1)-deficient mutants (*isa1*) were called *sugary-1* mutants in rice (*sug-1*) and maize (*su1*). Many allelic *isa1* mutant lines were isolated and the α-glucans in the endosperm were characterized (Nakamura *et al*. 1997; Kubo *et al*. 1999). The cross-sections of the *isa1* seeds were not stained with iodine in the whole seeds (severe type of *sug-1*) or part of the seeds (mild type of *sug-1*). The wild-type seeds showed

doi:10.5047/agbm.2014.00401.0001

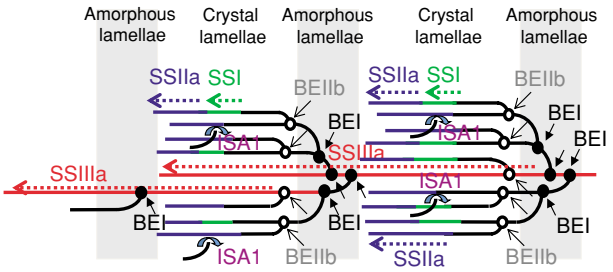

Fig. 7. The model of amylopectin biosynthesis of indica rice. SS: starch synthase, BE: branching enzyme, ISA1: isoamylase1, Black circles: branch points in the amorphous lamellae, open circle, branch points in the crystal lamellae. Dashed arrows: chain elongation by SS isozymes. Swing arrows: trimming of improper branch points by ISA1. Reprinted with permission from *Kagaku to Seibutsu*, **51**(6), Fujita, Denpunhenitaimai no kaiseki to riyou, 400–407, Fig. 2, © 2013, Japan Society for Bioscience, Biotechnology, and Agrochemistry.

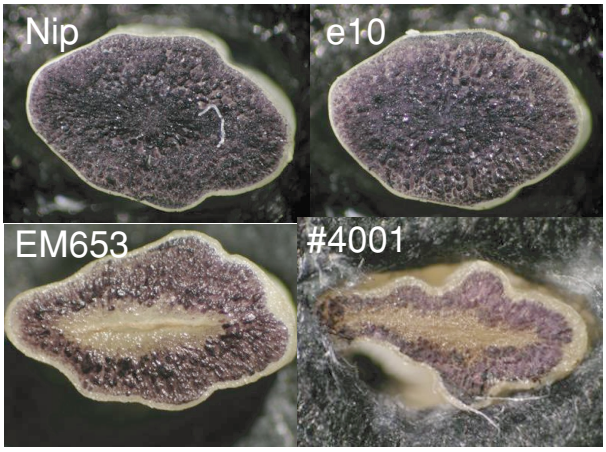

Fig. 8. Stereo micrographs of the cross sections of mature endosperm stained by iodine solution. Nip: Nipponbare (wild-type), e10: *pul* mutant, EM653: mild type *isa1* mutant, #4001: *isa1*/*pul* (*e10* × *EM653*). (Fujita *et al*. 2009). Reprinted with permission from *J. Exp. Bot*., **60**, Fujita *et al*., Characterization of pullulanase (PUL)-deficient mutants of rice (*Oryza sativa* L.) and the function of PUL on starch biosynthesis in the developing rice endosperm, 1009–1023, Fig. 6C, © 2009, Oxford University Press.

a purple color after staining with iodine. The part that was not stained with iodine is located in the center of the seeds in the mild-type of *sug-1* (Nakamura *et al*. 1997). The α-glucans in the part not stained with iodine in the endosperm were enriched with short-chain, highly branched starches that were water-soluble (phytoglycogen). Phytoglycogen had no crystallinity and showed no endothermal peak by differential scanning calorimetry (DSC), unlike that of amylopectin. The molecular weight of phytoglycogen (10^7) was lower than that of the wild-type amylopectin (10^{8-9}, Wong *et al*. 2003). The α-glucans in the part stained with iodine in the endosperm of *sug-1* were insoluble and similar to the amylopectin in the wild type, whereas the short chains with DP ≤ 12 were increased compared with the wild-type amylopectin (Kubo *et al*. 1999; Wong *et al*. 2003).

Numerous reports of *isa1* mutant lines suggested that ISA1 activity was essential for amylopectin crystallinity and the tandem cluster structure of amylopectin. This indicated that ISA1 was involved in the maintenance of the cluster structure of the amylopectin. The function of ISA1 was the trimming of improper branches produced by BEs in amylopectin molecules (see **Fig. 7**; Nakamura 2002).

6-10. *pul* mutants

Debranching enzymes (DBEs) were divided into two classes: the isoamylase (ISA) type debranched amylopectin and glycogen; the pullulanase (PUL) type debranched amylopectin and pullulan (Nakamura 1998). The *sugary-1* mutant lines in rice and maize were thought to be PUL-deficient mutants before 1995, because the PUL and ISA activities were reduced in the mutants (Nakamura *et al*. 1996a; Pan and Nelson 1984). James *et al*. (1995) isolated a *su-1* maize mutant using gene tagging and showed that the corresponding gene was similar to the isoamylase-type of DBE from *Pseudomonas*. The fact that *sug-1* alleles and *ISA1* genes were located on chromosome 8 in the rice genome (Yano *et al*. 1984; Fujita *et al*. 1999), whereas the *PUL* gene was located on chromosome 4 (Nakamura *et al*. 1996b), suggested that rice *sug-1* is not a PUL-deficient mutant but a ISA1-deficient mutant (*isa1*) (Nakamura *et al*. 1997; Fujita *et al*. 1999). We tried to isolate a PUL-deficient mutant, which had not been isolated at that time, from *Tos17* mutant stock (Fujita *et al*. 2009). The seed size and morphology of the *pul* null mutant line (complete lack of PUL) did not change, and the chain-length distribution of amylopectin was similar to that of the wild type, except for a slight increase of short chains. The area of cross section of the seeds stained with iodine was closely related with the PUL activity in the *isa1* allelic mutant (Nakamura *et al*. 1997). This suggests that when ISA1 was deficient, PUL complementation led to the prevention of phytoglycogen accumulation in the endosperm (Kubo *et al*. 1999). To clarify this speculation, double-mutant lines between mild-type *isa1*, which accumulated phytoglycogen in the endosperm and starch-like α-glucans (*sugary* amylopectin), and a null *pul* mutant were generated (Fujita *et al*. 2009). If the speculation was correct, the phenotype of mild-type *isa1* would transform to the severe-type *isa1*, which contained phytoglycogen in the whole endosperm cells. The actual results did not show such a phenotype, al-

doi:10.5047/agbm.2014.00401.0001

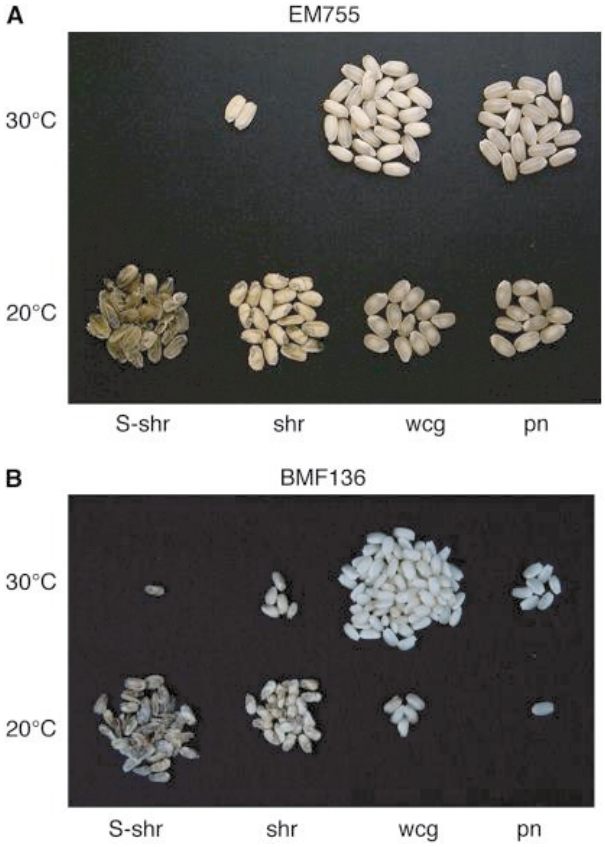

Fig. 9. Effects of temperature on kernel morphology during development of *pho1* mutant seeds. Mutant plants of EM755 (A) and BMF136 (B) were removed from the field plot at the maximum flowering stage and grown at temperatures of either 30°C (top rows) or 20°C (bottom rows) until they reached maturity. S-shr, severely shrunken grains; shr, shrunken grains; wcg, white-core endosperm grains; pn, pseudonormal grains (Satoh *et al*. 2008). Reprinted with permission from *Plant Cell*, **20**, Satoh *et al*., Mutation of the plastidial alpha-glucan phosphorylase gene in rice affects the synthesis and structure of starch in the endosperm, 1833–1849, Fig. 10, © 2008, American Society of Plant Biologists (www.plantcell.org).

though the phytoglycogen content was slightly increased in the double-mutant lines (**Fig. 8**). These results suggested that the compensation of PUL was limited even under a deficiency of ISA1, and the contribution of PUL for trimming of amylopectin was much smaller than that of ISA1 (Fujita *et al*. 2009).

6-11. *ISA2*-deficient mutant

There are three *ISA* genes (*ISA1*, *ISA2*, and *ISA3*) in green plants. ISA2 has no activity in itself. The hetero-oligomers ISA1 and ISA2 display debranching activity in potato tubers and *Arabidopsis* (Hussain *et al*. 2003; Delatte *et al*. 2005; Wattebled *et al*. 2005). In rice and maize endosperm, homo-oligomers of ISA1 and the hetero-oligomers are present. The former is more important for the trimming of improper branches than the latter (Utsumi and Nakamura 2006; Kubo *et al*. 2010; Utsumi *et al*. 2011). The starches are not affected with ISA2 inhibition in the transgenic rice, whereas soluble polysaccharide in ISA2 overexpression of transgenic rice was significantly increased (Utsumi *et al*. 2011). This indicates that the increase of ISA2 leads to the increase of nonfunctional hetero-oligomers of ISA1–ISA2 and the decrease of the functional homo-oligomers of ISA1. Only hetero-oligomers are present in rice leaves. The function of ISA2 is still unclear, but it might maintain debranching under severe environmental conditions, such as high temperature (Utsumi *et al*. 2011).

6-12. *isa3* mutant

The *isa3* mutants have been isolated from *Tos17* mutant stocks by Dr. Kawagoe's group (Yun *et al*. 2011). *ISA3* is expressed mainly in rice leaves and to a lesser extent in endosperm (Kubo *et al*. 2005). The phenotype of *isa3* endosperm starch is similar to that of the wild type, although the starch content in the bran was increased. By contrast, starch degradation in this mutant was inhibited in the dark in leaves. The morphologies of amyloplasts in the *isa3* mutants and the transgenic lines overexpressing *ISA3* were abnormal. The fact that the *isa1* (*sug-1*) phenotype was not compensated by the introduction of *ISA3* gene indicates that the functions in ISA1 and ISA3 are different (Yun *et al*. 2011).

6-13. *pho1* mutants

Phosphorylase (Pho) adds a glucose residue to the non-reduced end of glucose primed with glucose-1-phosphate and releases Pi. This enzyme also catalyzes the reverse reaction. Approximately 96% of rice Pho was the plastid-type Pho1, and the remaining was the cytosol-type Pho2. The seed phenotypes of *pho1* mutant lines screened from the MNU-treated population showed a wide range of variability, from pseudonormal seeds to thin seeds containing few starch molecules (**Fig. 9**, Satoh *et al*. 2008). It appeared that these seed phenotypes were affected by the temperature during endosperm development. These results suggested that Pho1 was involved in the early stage of starch biosynthesis. It was assumed that the other factor(s) were related to the initiation of starch biosynthesis under high temperature condition during endosperm development (Satoh *et al*. 2008).

7. The model of amylopectin biosynthesis

The model of amylopectin biosynthesis was established by the *in vitro* analyses of recombinant isozymes

doi:10.5047/agbm.2014.00401.0001

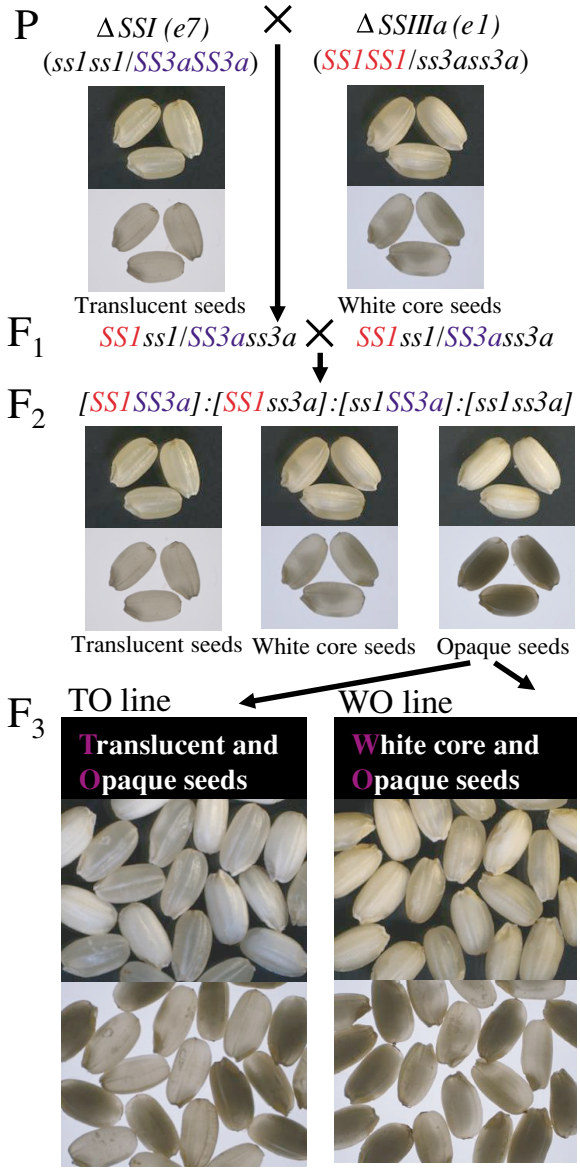

Fig. 10. Pedigree of opaque seeds from crossing between *ss1* and *ss3a* null mutant lines and seed morphology. The morphology of rice dehulled seeds was observed using a stereo-microscope with overhead light (upper panels) and on a light box (lower panel) (Fujita *et al*. 2011). Reprinted with permission from *J. Exp. Bot*., **62**, Fujita *et al*., Starch biosynthesis in rice endosperm requires the presence of either starch synthase I or IIIa, 4819–4831, Fig. 1, © 2011, Oxford University Press.

expressed in *E. coli* and the *in vivo* analyses using mutant lines described above (**Fig. 7**). The prototype of this model (Nakamura 2002) was modified with additional new information regarding the function of the isozymes.

The main SS isozymes that accounted for amylopectin biosynthesis in rice endosperm were SSI, SSIIa, and SSIIIa. SSIIIa elongated long chains and connected multiple clusters of amylopectin. The DP 6 and DP 7 chains produced by BEIIb (Nakamura *et al*. 2010) were elongated to form the DP 8 and DP 9 chains, predominantly by SSI in japonica rice amylopectin. In indica rice amylopectin, these chains were further elongated by SSIIa. BEI and BEIIb mainly formed branch points in the amorphous lamellae (black circles) and the crystal lamellae (white circles), respectively. ISA1 homo-oligomers remove improper branches produced by the BEs in the crystal lamellae. Thus, the sequential and close interactions among BE and SS isozymes play an important role in the efficient production of amylopectin molecules in cereal endosperms. The relay reactions of functionally specialized SS and BE isoforms would be an efficient way to multiply the same-sized clusters to form amylopectin molecules. This model will be improved by further studies in the future.

8. The significance of the production of multiple mutant lines

Many studies of starch double mutants in maize were conducted in the 1980s–1990s (Shannon and Garwood 1984; Inouchi *et al*. 1991; Wang *et al*. 1993a, b). However, at that time, the genes responsible for the majority of the starch mutant phenotypes had not been identified. Currently, many of the mutant genes have been identified, and this knowledge can be used to understand the relationships between starch characteristics and starch biosynthesis genes. Recent work reported the possibility of a protein complex of starch biosynthesis isozymes in maize and wheat (Tetlow *et al*. 2004; Hennen-Bierwagen *et al*. 2009). It will be necessary to demonstrate this possibility in rice, and the double-mutant lines will provide a good resource for this research. A number of single rice mutants show phenotypes that are similar to the wild type due to the complementation by other isozymes. This complementation makes it difficult to characterize the function of these isozymes. To elucidate individual isozyme function, the overlapping activity of many other isozymes needs to be eliminated through the production of multiple mutant lines. The japonica rice background has traditionally been used to produce the mutant lines (i.e., SSIIa- and GBSSI-reduced leaky mutants). When double, triple, or quadruple mutants are produced in japonica rice, sterility or low yield are a concern. However, some of our multiple mutant lines produce 80–90% of the seed weight of the wild type, and large-scale cultivation of these mutants is possible. Leaky mutant lines have been useful for avoiding the sterility that could result when the major isozyme-deficient mutants were crossed. Some multiple mutant lines show unique starch properties that are not solely the additive effects of the parent mutants. The recent work on the multiple rice mutant lines will be described below.

doi:10.5047/agbm.2014.00401.0001

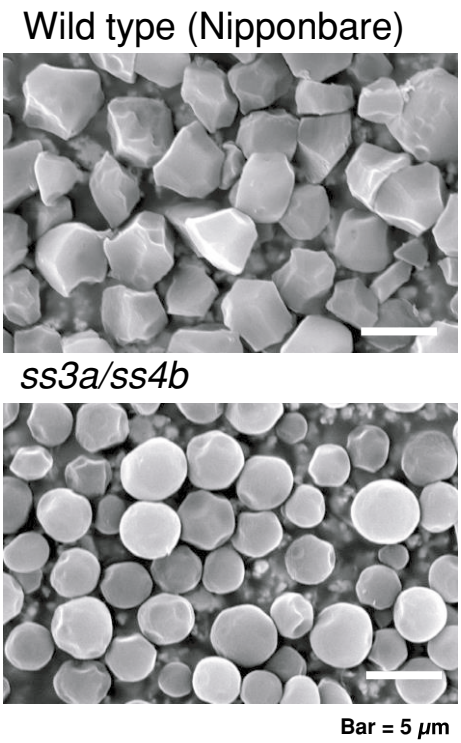

Fig. 11. Spherical starch granules of *ss3a*/*ss4b* double mutant line. Reprinted with permission from *Kagaku to Seibutsu*. **51**(6), Fujita, Denpunhenitaimai no kaiseki to riyou, 400–407, Fig. 3, © 2013, Japan Society for Bioscience, Biotechnology, and Agrochemistry.

8-1. *ss1*/*ss3a* double-mutant lines

The seed weight and accumulation of starch in the endosperm of the *ss1* null mutant line was similar to the wild type, even though the SSI activity, which accounted for more than 60% of the SS activity in the crude extract of rice developing endosperm, was lacking (Fujita *et al*. 2006). This implied that chain elongation was compensated by the other SS isozymes. In addition to the deficiency of SSI, SSIIIa, which is the second major SS isozyme, was also deleted (Fujita *et al*. 2011). In the F_2 developing seeds of crosses between null *ss1* and null *ss3a*, the developing seeds that lost both SSI and SSIIIa activity were not detected, indicating that double-recessive developing seeds became sterile. Opaque seeds, whose phenotype is different from the parent, were present in the F_2 population, and these genotypes were heterozygotes of *SSI* or *SSIIIa* (**Fig. 10**). A double-recessive homozygous mutant line was successfully obtained by crossing a *ss1-leaky* mutant ($ss1^L$) and a *ss3a* mutant (Fujita *et al*. 2011). The amylopectin short chains with DP ≤ 10 and amylose content of $ss1^L$/*ss3a* double-mutant line were decreased and increased (33%), respectively, compared to the wild type. These results suggested that the deficiency of either SSI or SSIIIa resulted in complementation, and either SSI or SSIIIa was necessary to allow starch biosynthesis in rice endosperm. No other SS isozymes could provide this complementation.

8-2. *ss3a*/*ss4b* double-mutant lines

The seed phenotype, size, and structure of endosperm starch of the *ss4b* mutant were very similar to those of the wild type (Toyosawa *et al*. submitted). The function of SSIVb was still unknown. The seed morphology of *ss3a*/*ss4b* double-mutant lines was opaque, a phenotype that was different from that of the parents. The starch granules of *ss3a*/*ss4b* lines were completely spherical (**Fig. 11**), although those of the wild type were polygonal. Rice endosperm starches were compound grains, in which several starch granules accumulated in one amyloplast. The endosperm starches of maize, wheat, and barley were simple grains, in which one starch granule accumulated in one amyloplast (Matsushima *et al*. 2010, 2013). At the beginning, we thought that the spherical starch granules of rice *ss3a*/*ss4b* double mutant were changed to the simple grain from compound grains. However, observation of transgenic rice expressing fluorescent-protein markers in the double-mutant lines showed that these mutants contained compound grains. The analyses of the starches of the double-mutant lines suggested that the function of SSIVb was overlapping with that of SSIIIa, which was chain elongation of the long chains connecting multiple clusters of amylopectin. It was assumed that SSIVb was involved in generating the structure of the septum-like sheets (Yun and Kawagoe 2010) between starch granules in the compound grains (Toyosawa *et al*. submitted).

8-3. *ss3a*/*be2b* double mutant

High-amylose starch is useful for producing food materials and biodegradable plastics. The amylose content of the maize *be2b* mutant is 50–70%. This is much higher than that of the rice *be2b* mutant line (30%), which is 1.5 times higher than the wild type. The highest amylose content in rice lines, including our mutant lines (*ss3a*: Fujita *et al*. 2007, $ss1^L$/*ss3a*: Fujita *et al*. 2011, *ss3a*/*ss4b*: Toyosawa *et al*. submitted) and some of the indica rice cultivars (Inouchi *et al*. 2005), is *ca*. 30–34% as measured by way of gel-filtration of debranched starch. We generated the japonica background double-mutant line between *ss3a* (Fujita *et al*. 2007) and *be2b* (Nishi *et al*. 2001). The apparent amylose content of *ss3a*/*be2b* is 46%, which is the highest measured content in rice starches (Asai *et al*. in preparation). The short chains of amylopectin were significantly decreased, and starch showed B-type crystallinity due to the deficiency of BEIIb. Long chains with

doi:10.5047/agbm.2014.00401.0001

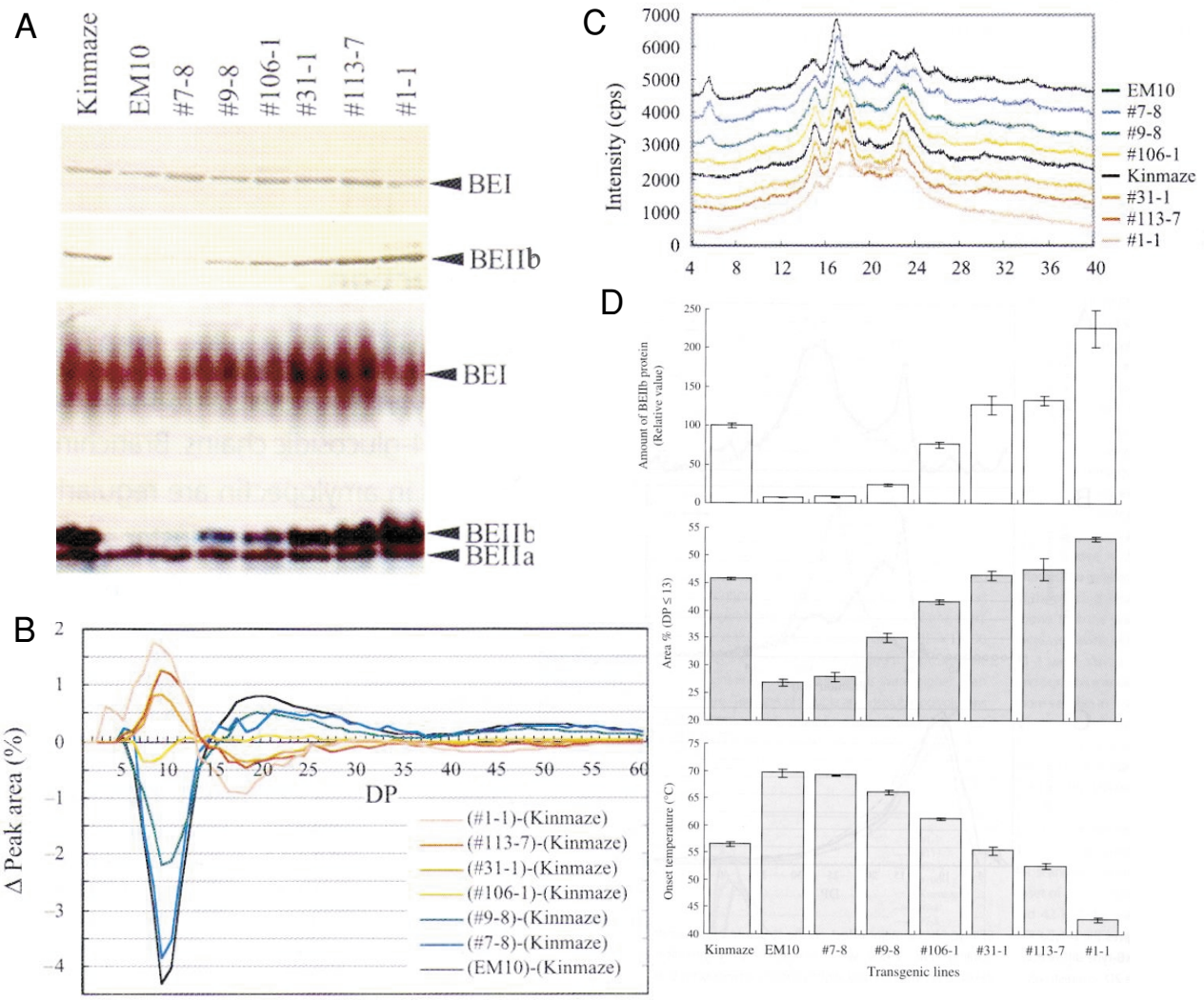

Fig. 12. (A) Western blot analyses and native-PAGE/BE activity staining of BEI and BEIIb in developing rice endosperm of transgenic rice lines and the parents. (B) Differences in chain-length distribution of total α-glucans in transgenic rice lines and the wild-type. (C) X-ray diffraction pattern of starch granules of transgenic rice lines and the parents. (D) Relationship between BEIIb protein level and the phenotypes (the rate of short chains of amylopectin and gelatinization temperature) of transgenic rice lines and the parents (Tanaka *et al*. 2004). Reprinted with permission of John Wiley & Sons, Inc. from *Plant Biotechnol. J*., **2**, Tanaka *et al*., The structure of starch can be manipulated by changing the expression levels of starch branching enzyme IIb in rice endosperm, 507–516, Figs. 1D, 1E, 2D, 3B, 5A, 5B, 5C, © 2004, Wiley-Liss, Inc., a Wiley Company.

amylopectin DP ≥ 40 also were decreased compared with those of the *be2b* parent mutant. This decrease was due to the deficiency of SSIIIa. In *be2b*, the amylopectin structure is quite different from the wild type, and the starch content in the endosperm decreased to 60% of the wild type due to the significant decrease in the non-reduced end of amylopectin (Nishi *et al*. 2001). However, *ss3a*/*be2b* maintained 80% of the seed weight of the wild type even though the amylose content and the structure were dramatically changed. The activities of GBSSI, which is the only isozyme account for amylose synthesis in the endosperm starch, and AGPase, which produce substrate of starch synthase (ADPglc) of *ss3a*/*be2b* developing endosperm were higher than those of the *be2b* (Asai *et al*. in preparation). These results strongly suggested that the amylose synthesis is enhanced in *ss3a*/*be2b* endosperm than that in *be2b* and this lead to the larger seed weight of *ss3a*/*be2b*.

9. Knowledge from studies using transgenic rice lines

Analyses of mutant lines provided information on the function of each isozyme. Transgenic rice lines, in which a specific gene is introduced into the wild-type

doi:10.5047/agbm.2014.00401.0001

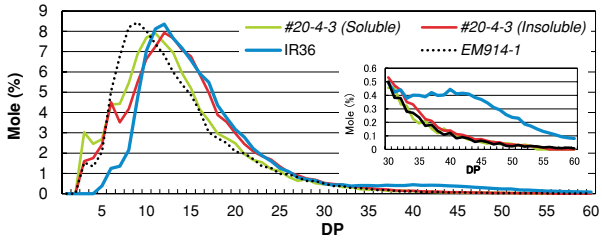

Fig. 13. Chain-length distribution patterns of soluble (*#20-4-3* Soluble) and insoluble (*#20-4-3* Insoluble) α-glucans in the transgenic rice line #20, *isa1* (*EM914*) and wild type (IR36). The inset indicates the magnification of the pattern in the range of chains with DP 30 to 60. Reprinted with permission from *J. Exp. Bot.*, **63**, Fujita *et al.*, Elongated phytoglycogen chain length in transgenic rice endosperm expressing active starch synthase IIa affects the altered solubility and crystallinity of the storage α-glucan, 5859–5872, Fig. 4C, © 2012, Oxford University Press.

or mutant rice lines, also enable *in vivo* studies. It is possible to up- and down-regulate specific genes in transgenic rice, whereas down-regulations occur in the mutants in many cases. In theory, it is possible to introduce any gene from any organism into the host plants, and efficient promoters can be chosen for tissue- or developmental-specific expression. Transgenic plants provide unique experimental insights compared to mutant lines. Because the field-culture of transgenic plants is limited, we focused on development of the mutant lines. However, we also have generated transgenic rice lines for research as described below.

9-1. Anti-ISA1

We generated transgenic rice lines whose ISA1 activity was down-regulated by antisense methods (anti-ISA1) to confirm whether the endosperm of the transgenic lines show the *isa1* phenotype (Fujita *et al.* 2003), the starches of which have changed to the highly branched and soluble α-glucans (Nakamura *et al.* 1997; Kubo *et al.* 1999). This was the first study to modify the structure of amylopectin by gene manipulation. The abundance of short chains with DP ≤ 11 in the endosperm of anti-ISA1 lines suggested that the function of ISA1 was trimming of improper branches in amylopectin molecules. The phenotype of anti-ISA1 was much more moderate than that of the *isa1*, because the residual ISA1 activity of anti-ISA1 was 1/16, whereas that of the *isa1* mutant was less than 1/100. These results implied that there was a threshold of ISA activity to avoid the production of soluble α-glucans.

9-2. *OsBEIIb/be2b*

This work was to confirm whether the *be2b* phenotype, the starch of which is highly resistant to gelatinization, was rescued by the introduction of the *BEIIb* gene. The 18 kb genomic DNA fragment, including the *OsBEIIb* gene and the promoter, was introduced in the *be2b* mutant line (*OsBEIIb/be2b*: Tanaka *et al.* 2004). A wide range of transgenic lines from low to high expression was generated (**Fig. 12A**). Analyses of six transgenic lines showed that the amount of *BEIIb* expression, the percentage of short chains with DP 6–14 of amylopectin, and the gelatinization temperature were closely related (**Fig. 12B**). The B-type starch crystallinity of the *be2b* host mutant returned to the A-type crystallinity in transgenic lines that recovered BEIIb activity (**Fig. 12C**). The starch in the *BEIIb* overexpressing line lost crystallinity due to its excess branched soluble α-glucans. These results suggested that BEIIb formed branch points of short chains in amylopectin crystalline lamellae, and *BEIIb* expression levels accurately regulated the amount of short chains, the gelatinization temperature and the crystallinity (**Fig. 12D**; Tanaka *et al.* 2004).

9-3. *SSIIa^{l}/isa1*

Our mutant lines were in the japonica rice background. Therefore, SSIIa was inactive. The transgenic lines from which active SSIIa was derived were from indica cultivars. Active SSIIa derived from indica cultivars was introduced into the japonica background *isa1* mutant (*SSIIa^{I}/isa1*) that accumulated phytoglycogen instead of starch (Fujita *et al.* 2012). The host *isa1* mutant endosperm contained only 3.1% of insoluble α-glucan, whereas the endosperm of high-expression SSIIa lines (#20) contained more than 60% of insoluble α-glucan. The chains of α-glucan in lines #20 were elongated DP 3–6 compared to the host phytoglycogen chains (**Fig. 13**). Long chains with approximately DP 40 were absent in *SSIIa^{I}/isa1* lines and the host mutant. In *SSIIa^{I}/isa1* lines, the inner structure of α-glucans that had external chains removed by β-amylase was almost the same as that of phytoglycogen. These results suggested that active SSIIa only elongated external chains, if not all, and elongated α-glucan resulted in insolubility. The crystallinity of insoluble α-glucan in *SSIIa^{I}/isa1* line (#20) was weak B-type, although phytoglycogen showed no crystallinity (Fujita *et al.* 2012).

9-4. *GBSSI^{l}/ss3a*

GBSSI expression in japonica cultivars was significantly lower than that in indica cultivars, and this resulted in the different amylose contents in japonica and indica cultivars. To examine the effects of SSIIIa-deficiency and high expression of GBSSI on the amylose content and starch structure, the transgenic rice line (WAB; Hanashiro *et al.* 2008) that *GBSSI* derived

doi:10.5047/agbm.2014.00401.0001

	Cultivars (mutants)	Apparent amylose content (%)
For cooked rice cultivars	Nipponbare	21.5
	Koshihikari	17.9
	Sasanishiki	20.5
	Kirara397	20.1
Mutants	Mutant (*ss3a*)	30.4
	Mutant (*ss3a/be2b*)	46.6

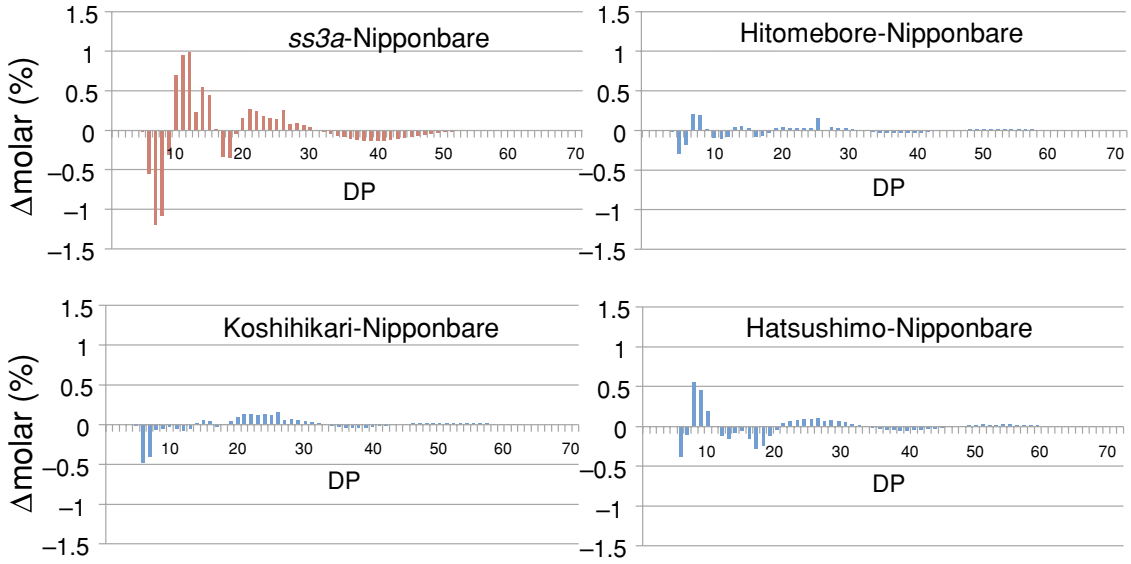

Fig. 14. The traits of starch (apparent amylose content (upper table) and chain length distribution of amylopectin (lower figure)) of cooked rice cultivars (Nipponbare, Koshihikari, Sasanishiki, Kirara397, Hitomebore and Hatsushimo) and mutant lines. Variation of the mutant lines are much larger than that of the cultivars. Reprinted with permission from *Kagaku to Seibutsu*. **51**(6), Fujita, Denpunhenitaimai no kaiseki to riyou, 400–407, Fig. 4, © 2013, Japan Society for Bioscience, Biotechnology, and Agrochemistry.

from indica cultivars (*GBSSI*^{I}) was introduced into the *waxy* (*gbss1*) mutant, was crossed with the *ss3a* mutant line (Crofts *et al*. 2012). The amylose content of WAB and *ss3a* were 25% and 30%, respectively, whereas that of screened *GBSSI*^{I}/*ss3a* lines was significantly increased (41%). The amount of GBSSI protein in WAB was 10 times higher than that of the wild type (japonica cultivars). However, the amount of GBSSI protein in *GBSSI*^{I}/*ss3a* showed no further increase, although AGPase, which produced a substrate of SS, increased compared to the WAB. The chain-length distribution of amylopectin in *GBSSI*^{I}/*ss3a* was similar to that of *ss3a*. These results suggested that high expression of *GBSSI*^{I} derived from indica cultivars and SSIIIa-deficiency synergistically increased the apparent amylose content in rice endosperm (Crofts *et al*. 2012).

10. Future studies

The largest number of starch mutant lines and transgenic lines including multiple mutants are produced in rice. The functions of a large number of isozymes involved in starch biosynthesis expressed in the endosperm have been identified. On the other hand, the isozymes that are not expressed in the endosperm are still unclear. The research of starch biosynthesis in rice is leading that in other plants. The unique starches, which are quite different from those of the wild type, accumulate in the endosperm of mutant lines. Many tasty cultivars for cooked rice were bred in agricultural experiment stations all over Japan. The stations focused on the taste of the cooked rice, and this narrowed the diversity in rice cultivars. For example, the amylose content and the structure of amylopectin are quite similar among cultivars for cooked rice (**Fig. 14**). However, mutant lines lacking specific isozymes involved in starch biosynthesis are diverse and useful for several fields. We are preparing breeding programs to increase agriculturally beneficial traits. In the near future, we hope that unique starches accumulate in the mutant lines that will be useful for industrial applications.

Acknowledgments

These studies were performed in Professor Yasunori Nakamura's laboratories at NIAS (1997–1999) and Akita Prefectural University (1999–2012). The author is grateful

doi:10.5047/agbm.2014.00401.0001

to many people and would like to especially thank Prof. Yasunori Nakamura for his financial support during the author's youth and for his continuing guidance. The author would like to thank many collaborators, Professor Hikaru Satoh and his laboratory members at Kyushu University for providing many great mutant lines, Dr. Hirohiko Hirochika and Dr. Akio Miyao at NIAS for providing the *Tos17* mutant stocks, Dr. Naoko Crofts (Akita Prefectural University), Dr. Akiko Kubo (Glico Co., Ltd.), Dr. Perigio B. Francisco (Phillipine), Dr. Yoshinori Utsumi (RIKEN), and Dr. Yoshiko Toyosawa (Kyushu University) for collaboration as post docters. The author also thanks many researchers, Ms. Naoko Fujita Oitome, Ms. Mayumi Yoshida, Miss Rumiko Ito, Ms. Satomi Aihara and Ms. Yuko Nakaizumi for technical support and graduate students, Mr. Hiroki Asai and Natsuko Abe and many undergraduate students of the laboratory in Akita Prefectural University for their support. Moreover, the staff of NIAS, Dr. Naoki Tanaka, Ms. Kazuko Kimura and Ms. Yumiko Inaba for their support. The author also thanks collaborators at other universities or institutes, Dr. Yasushi Kawagoe at NIAS, Dr. Ryo Matsushima at Okayama University, Dr. Takayuki Umemoto at NARO, Dr. Isao Hanashiro and Professor Yasuhito Takeda at Kagoshima University, Dr. Kimiko Itoh at Niigata University, Prof. Sayuri Akuzawa at Tokyo University of Agriculture, Prof. Jay-Lin Jane and laboratory members at Iowa State University, for sharing projects and providing advice. Finally this work was supported by Akita Prefectural University, Rice Genome Project at NIAS, CREST in JST, Science and technology research promotion program for agriculture, forestry, fisheries and food industry, the Program for the Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry and a Grant-in-Aid for Scientific Research (B) (19380007).

References

Baba T, Arai Y. Structural characterization of amylopectin and intermediate material in amylomaize starch granules. *Agric. Biol. Chem.* 1984; **48**: 1763–1775.

Baba T, Nishihara M, Mizuno K, Kawasaki T, Shimada H, Kobayashi E, Ohnishi S, Tanaka K, Arai Y. Identification, cDNA cloning, and gene expression of soluble starch synthase in rice (*Oryza sativa* L.) immature seeds. *Plant Physiol*. 1993; **103**: 565–573.

Ball SG, Morell MK. From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. *Annu. Rev. Plant Biol*. 2003; **54**: 207–233.

Bertoft E. Investigation of the fine structure of alpha-dextrins derived from amylopectin and their relation to the structure of waxy-maize starch. *Carbohydr. Res*. 1991; **212**: 229–244.

Bertoft E. One the nature of categories of chains in amylopectin and their connection to the super helix model. *Carbohydr. Polym*. 2004; **57**: 211–224.

Bertoft E, Kallman A, Koch K, Andersson R, Aman P. The cluster structure of barley amylopectin of different genetic backgrounds. *Int. J. Biol. Macromol*. 2011; **49**: 441–453.

Cai XL, Wang ZY, Xing YY, Zhang JL, Hong MM. Aberrant splicing of intron 1 leads to the heterogeneous 5′ UTR and decreased expression of waxy gene in rice cultivars of intermediate amylose content. *Plant J*. 1998; **14**: 459–465.

Cao H, Imparl-Radosevich J, Guan H, Keeling PL, James MG, Myers AM. Identification of the soluble starch synthase activities of maize endosperm. *Plant Physiol*. 1999; **120**: 205–215.

Cao H, James MG, Myers AM. Purification and characterization of soluble starch synthases from maize endosperm. *Arch. Biochem. Biophys*. 2000; **373**: 135–146.

Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henkoff S. High-throughput screening for induced point mutations. *Plant Physiol*. 2001; **126**: 480–484.

Colleoni C, Dauville D, Mouille G, Morell M, Samuel M, Slomiany MC, Liénard L, Wattebled F, d'Hulst C, Ball S. Biochemical characterization of the chlamydomonas reinhardtii alpha-1,4 glucanotransferase supports a direct function in amylopectin biosynthesis. *Plant Physiol*. 1999; **120**: 1005–1014.

Commuri PD, Keeling PL. Chain-length specificities of maize starch synthase I enzyme: studies of glucan affinity and catalytic properties. *Plant J*. 2001; **25**: 475–486.

Craig J, Lloyd JR, Tomlinson K, Barber L, Edwards A, Wang TL, Martin C, Hedley CL, Smith AM. Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. *Plant Cell* 1998; **10**: 413–426.

Crofts N, Abe K, Aihara S, Itoh R, Nakamura Y, Itoh K, Fujita N. Lack of starch synthase IIIa and high expression of granule-bound starch synthase I synergistically increase the apparent amylose content in rice endosperm. *Plant Sci*. 2012; **193–194**: 62–69.

Davis JH, Kramer HH, Whistler RL. Expression of the gene du in the endosperm of maize. *Agron. J*. 1955; **47**: 232–235.

Delatte T, Trevisan M, Parker ML, Zeeman SC. *Arabidopsis* mutants Atisa1 and Atisa2 have identical phenotypes and lack the same multimeric isoamylase, which influences the branch point distribution of amylopectin during starch synthesis. *Plant J*. 2005; **41**: 815–830.

Edwards A, Fulton DC, Hylton CM, Jobling SA, Gidley M, Rossner U, Martin C, Smith AM. A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. *Plant J*. 1999; **17**: 251–261.

Eriksson G. Radiation induced reversions of a *waxy* allele in barley. *Rad. Bot*. 1962; **2**: 35–39.

Fujita N, Nakamura Y. Distinct and overlapping functions of starch synthase isoforms. *In*: Tetlow I (ed). *Essential Reviews in Experimental Biology. Volume 5, Starch: Origins, Structure and Metabolism*. Society for Experimental Biology, London. 2012; 115–140.

Fujita N, Kubo A, Francisco PB Jr, Nakakita M, Harada K, Minaka N, Nakamura Y. Purification, characterization, and cDNA structure of isoamylase from developing endosperm of rice. *Planta* 1999; **208**: 283–293.

Fujita N, Hasegawa H, Taira T. The isolation and characterization of a waxy mutant of diploid wheat (*Triticum monococcum* L.). *Plant Sci*. 2001; **160**: 595–602.

Fujita N, Kubo A, Suh DS, Wong KS, Jane JL, Ozawa K, Takaiwa F, Inaba Y, Nakamura Y. Antisense inhibition of isoamylase alters the structure of amylopectin and the physicochemical properties of starch in rice endosperm. *Plant Cell Physiol*. 2003; **44**: 607–618.

doi:10.5047/agbm.2014.00401.0001

Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, Hirochika H, Nakamura Y. Function and characterization of starch synthase I using mutants in rice. *Plant Physiol*. 2006; **140**: 1070–1084.

Fujita N, Yoshida M, Kondo T, Saito K, Utsumi Y, Tokunaga T, Nishi A, Satoh H, Park JH, Jane JL, Miyao A, Hirochika H, Nakamura Y. Characterization of SSIIIa-deficient mutants of rice: the function of SSIIIa and pleiotropic effects by SSIIIa deficiency in the rice endosperm. *Plant Physiol*. 2007; **144**: 2009–2023.

Fujita N, Goto S, Yoshida M, Suzuki E, Nakamura Y. The function of rice starch synthase I expressed in *E. coli*. *J. Appl. Glycosci*. 2008; **55**: 167–172.

Fujita N, Toyosawa Y, Utsumi Y, Higuchi T, Hanashiro I, Ikegami A, Akuzawa S, Yoshida M, Mori A, Inomata K, Itoh R, Miyao A, Hirochika H, Satoh H, Nakamura Y. Characterization of pullulanase (PUL)-deficient mutants of rice (*Oryza sativa* L.) and the function of PUL on starch biosynthesis in the developing rice endosperm. *J. Exp. Bot*. 2009; **60**: 1009–1023.

Fujita N, Satoh R, Hayashi A, Kodama M, Itoh R, Aihara S, Nakamura Y. Starch biosynthesis in rice endosperm requires the presence of either starch synthase I or IIIa. *J. Exp. Bot*. 2011; **62**: 4819–4831.

Fujita N, Hanashiro I, Suzuki S, Higuchi T, Toyosawa Y, Utsumi Y, Itoh R, Aihara S, Nakamura Y. Elongated phytoglycogen chain length in transgenic rice endosperm expressing active starch synthase IIa affects the altered solubility and crystallinity of the storage α-glucan. *J. Exp. Bot*. 2012; **63**: 5859–5872.

Gao M, Wanat J, Stinard PS, James MG, Myers AM. Characterization of dull1, a maize gene coding for a novel starch synthase. *Plant Cell* 1998; **10**: 399–412.

Hanashiro I, Itoh K, Kuratomi Y, Yamazaki M, Igarashi T, Matsugasako J, Takeda Y. Granule-bound starch synthase I is responsible for biosynthesis of extra-long unit chains of amylopectin in rice. *Plant Cell Physiol*. 2008; **49**: 925–933.

Hanashiro I, Higuchi T, Aihara S, Nakamura Y, Fujita N. Structures of starches from rice mutants deficient in the starch synthase isozyme SSI or SSIIIa. *Biomacromolecules* 2011; **12**: 1621–1628.

Hennen-Bierwagen TA, Lin Q, Grimaud F, Planchot V, Keeling PL, James MG, Myers AM. Proteins from multiple metabolic pathways associate with starch biosynthetic enzymes in high molecular weight complexes: a model for regulation of carbon allocation in maize amyloplasts. *Plant Physiol*. 2009; **149**: 1541–1559.

Hirochika H. Contribution of the *Tos17* retrotransposon to rice functional genomics. *Curr. Opin. Plant Biol*. 2001; **4**: 118–122.

Hirose T, Terao T. A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). *Planta* 2004; **220**: 9–16.

Hizukuri S. Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydr. Res*. 1986; **147**: 342–347.

Hovenkamp-Hermelink JHM, Jacobsen E, Ponstein AS, Visser RGF, Vos-Scheperkeute GH, Bijmolt EW, de Vries JN, Witholt B, Feenstra WJ. Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). *Theor. Appl. Genet*. 1987; **75**: 217–221.

Hussain H, Mant A, Seale R, Zeeman S, Hinchliffe E, Edwards A, Hylton C, Bornemann S, Smith AM, Martin C, Bustos R. Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. *Plant Cell* 2003; **15**: 133–149.

Inouchi N, Glover DV, Sugimoto Y, Fuwa H. DSC characteristics of gelatinization of starches of single-, double-, and triple-mutants and their normal counterpart in the inbred Oh43 maize (*Zea mays* L.) background. *Starch* 1991; **43**: 468–472.

Inouchi N, Hibiu H, Li T, Horibata T, Fuwa H, Itani T. Structure and properties of endosperm starches from cultivated rice of Asia and other countries *J. Appl. Glycosci*. 2005; **52**: 239–246.

International Rice Genome Sequencing Project. The map-based sequence of the rice genome. *Nature* 2005; **436**: 793–800.

Isshiki M, Morino K, Nakajima M, Okagaki RJ, Wessler SR, Izawa T, Shimamoto K. A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5′ splice site of the first intron. *Plant J*. 1998; **15**: 133–138.

James MG, Robertson DS, Myers AM. Characterization of the maize gene sugary1, a determinant of starch composition in kernels. *Plant Cell* 1995; **7**: 417–429.

Jane J-L, Wong K-S, McPherson AE. Branch-structure difference in starches of A- and B-type X-ray patterns revealed by their Naegeli dextrins. *Carbohydr. Res*. 1997; **300**: 219–227.

Jenkins PJ, Cameron RE, Donald AM. A universal feature in the structure of starch granules from different sources. *Starch* 1993; **45**: 417–420.

Kossmann J, Abel GJW, Springer F, Lloyd JR, Willmitzer L. Cloning and functional analysis of a cDNA encoding a starch synthase from potato (*Solanum tuberosum* L.) that is predominantly expressed in leaf tissue. *Planta* 1999; **208**: 503–511.

Kubo A, Fujita N, Harada K, Matsuda T, Satoh H, Nakamura Y. The starch-debranching enzymes isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. *Plant Physiol*. 1999; **121**: 399–409.

Kubo A, Rahman S, Utsumi Y, Li Z, Mukai Y, Yamamoto M, Ugaki M, Harada K, Satoh H, Konik-Rose C, Morell M, Nakamura Y. Complementation of *sugary-1* phenotype in rice endosperm with the wheat isoamylase1 gene supports a direct role for isoamylase1 in amylopectin biosynthesis. *Plant Physiol*. 2005; **137**: 43–56.

Kubo A, Colleoni C, Dinges JR, Lin Q, Lappe RR, Rivenbark JG, Meyer AJ, Ball SG, James MG, Hennen-Bierwagen TA, Myers AM. Functions of heteromeric and homomeric isoamylase-type starch-debranching enzymes in developing maize endosperm. *Plant Physiol*. 2010; **153**: 956–969.

Kudo M. Genetical and thremmatological studies of characters, physiological or ecological, in the hybrids between ecological rice groups. *Series D (ed) Bull. Natl. Inst. Agric. Sci*. 1968; **19**: 1–84.

Maddelein M-L, Libessart N, Bellanger F, Delrue B, D'Hulst C, van den Koornhuyse N, Fontaine T, Wieruszeski J-M, Decq A, Ball S. Toward an understanding of the biogenesis of the starch granule. *J. Biol. Chem*. 1994; **269**: 25150–25157.

Mangelsdorf PC. The inheritance of amylaceous sugary

doi:10.5047/agbm.2014.00401.0001

endosperm and its derivatives in maize. *Genetics* 1947; **32**: 448–458.

Marshall J, Sidebottom C, Debet M, Martin C, Smith AM. Identification of the major starch synthase in the soluble fraction of potato tubers. *Plant Cell* 1996; **8**: 1121–1135.

Matsushima R, Maekawa M, Fujita N, Sakamoto W. A rapid, direct observation method to isolate mutants with defects in starch grain morphology in rice. *Plant Cell Physiol*. 2010; **51**: 728–741.

Matsushima R, Yamashita J, Kariyama S, Enomoto T, Sakamoto W. Special Issue "Starch Metabolism, Structure and Properties" A Phylogenetic Re-evaluation of Morphological Variations of Starch Grains among Poaceae Species. *J. Appl. Glycosci*. 2013; **60**: 37–44.

Morell MK, Kosar-Hashemi B, Cmiel M, Samuel MS, Chandler P, Rahman S, Buleon A, Batey IL, Li Z. Barley *sex6* mutants lack starch synthase IIa activity and contain a starch with novel properties. *Plant J*. 2003; **34**: 173–185.

Myers AM, Morell MK, James MG, Ball SG. Recent progress toward understanding biosynthesis of the amylopectin crystal. *Plant Physiol*. 2000; **122**: 989–997.

Nakamura T, Yamamori M, Hirano H, Hidaka S, Nagamine T. Production of waxy (amylose-free) wheats. *Mol. Gen. Genet*. 1995; **248**: 253–259.

Nakamura Y. Some properties of starch debranching enzymes and their possible role in amylopectin biosynthesis. *Plant Sci*. 1998; **121**: 1–18.

Nakamura Y. Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. *Plant Cell Physiol*. 2002; **43**: 718–725.

Nakamura Y, Umemoto T, Ogata N, Kuboki Y, Yano M, Sasaki T. Starch debranching enzyme (R-enzyme or pullulanase) from developing rice endosperm: purification, cDNA and chromosomal localization of the gene. *Planta* 1996a; **199**: 209–218.

Nakamura Y, Umemoto T, Takahata Y, Komae K, Amano E, Satoh H. Changes in structure of starch and enzyme activities affected by *sugary* mutant in developing rice endosperm: possible role of starch debranching enzyme (R-enzyme) in amylopectin biosynthesis. *Physiol. Plant*. 1996b; **97**: 491–498.

Nakamura Y, Kubo A, Shimamune T, Matsuda T, Harada K, Satoh H. Correlation between activities of starch debranching enzyme and α-polyglucan structure in endosperms of *sugary-1* mutants of rice. *Plant J*. 1997; **12**: 143–153.

Nakamura Y, Sakurai A, Inaba Y, Kimura K, Iwasawa N, Nagamine T. The fine structure of amylopectin in endosperm from Asian cultivated rice can be largely classified into two classes. *Starch* 2002; **54**: 117–131.

Nakamura Y, Takahashi J, Sakurai A, Inaba Y, Suzuki E, Nihei S, Fujiwara S, Tsuzuki M, Miyashita H, Ikemoto H, Kawachi M, Sekiguchi H, Kurano N. Some Cyanobacteria synthesize semi-amylopectin type alpha-polyglucans instead of glycogen. *Plant Cell Physiol*. 2005a; **46**: 539–545.

Nakamura Y, Francisco PB Jr, Hosaka Y, Sato A, Sawada T, Kubo A, Fujita N. Essential amino acids of starch synthase IIa differentiate amylopectin structure and starch quality between japonica and indica rice varieties. *Plant Mol. Biol*. 2005b; **58**: 213–227.

Nakamura Y, Utsumi Y, Sawada T, Aihara S, Utsumi C, Yoshida M, Kitamura S. Characterization of the reactions of starch branching enzymes from rice endosperm. *Plant Cell Physiol*. 2010; **51**: 776–794.

Nishi A, Nakamura Y, Tanaka N, Satoh H. Biochemical and genetic analysis of the effects of *amylose-extender* mutation in rice endosperm. *Plant Physiol*. 2001; **127**: 459–472.

Pan D, Nelson OE Jr. A debranching enzyme deficiency in endosperms of the *sugary1* mutants of maize. *Plant Physiol*. 1984; **74**: 324–328.

Peat S, Whelan WJ, Thomas GJ. Evidence of multiple branching in waxy maize starch. *J. Chem. Soc*. 1952; **886**: 4546–4548.

Regina A, Bird A, Topping D, Bowden S, Freeman J, Barsby T, Kosar-Hashemi B, Li Z, Rahman S, Morell M. High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. *Proc. Natl. Acad. Sci. USA* 2006; **103**: 3546–3551.

Regina A, Kosar-Hashemi B, Ling S, Li Z, Rahman S, Morell M. Control of starch branching in barley defined through differential RNAi suppression of starch branching enzyme IIa and IIb. *J. Exp. Bot*. 2010; **61**: 1469–1482.

Sano Y. Differential regulation of waxy gene expression in rice endosperm. *Theor. Appl. Genet*. 1984; **68**: 467–473.

Satoh H, Omura T. Induction of mutation by treatment of fertilized egg cell with *N*-methyl-*N*-nitrosourea in rice. *J. Fac. Agric. Kyushu Univ*. 1979; **24**: 165–174.

Satoh H, Nishi A, Yamashita K, Takemoto Y, Tanaka Y, Hosaka Y, Sakurai A, Fujita N, Nakamura Y. Starch-branching enzyme I-deficient mutation specifically affects the structure and properties of starch in rice endosperm. *Plant Physiol*. 2003; **133**: 1111–1121.

Satoh H, Shibahara K, Tokunaga T, Nishi A, Tasaki M, Hwang SK, Okita TW, Kaneko N, Fujita N, Yoshida M, Hosaka Y, Sato A, Utsumi Y, Ohdan T, Nakamura Y. Mutation of the plastidial alpha-glucan phosphorylase gene in rice affects the synthesis and structure of starch in the endosperm. *Plant Cell* 2008; **20**: 1833–1849.

Shannon JC, Garwood DL. Genetics and physiology of starch development. *In*: Whistler RL, BeMiller JN, Paschal EF (eds). *Starch 2nd ed*. Academic Press. 1984; 25–86.

Shimonaga T, Konishi M, Oyama Y, Fujiwara S, Satoh A, Fujita N, Colleoni C, Buléon A, Putaux JL, Ball SG, Yokoyama A, Hara Y, Nakamura Y, Tsuzuki M. Variation in storage alpha-glucans of the Porphyridiales (Rhodophyta). *Plant Cell Physiol*. 2008; **49**: 103–116.

Smith AM, Denyer K, Martin C. The synthesis of the starch granule. *Annu. Rev. Plant Physiol. Mol. Biol*. 1997; **48**: 67–87.

Szydlowski N, Ragel P, Hennen-Bierwagen TA, Planchot V, Myers AM, Mérida A, d'Hulst C, Wattebled F. Integrated functions among multiple starch synthases determine both amylopectin chain length and branch linkage location in Arabidopsis leaf starch. *J. Exp. Bot*. 2011; **62**: 4547–4559.

Takeda Y, Hizukuri S, Juliano BO. Structures of rice amylopectins with low and high affinities for iodine. *Carbohydr. Res*. 1987; **168**: 79–88.

Tanaka N, Fujita N, Nishi A, Satoh H, Hosaka Y, Ugaki M, Kawasaki S, Nakamura Y. The structure of starch can be manipulated by changing the expression levels of starch branching enzyme IIb in rice endosperm. *Plant Biotechnol*.

doi:10.5047/agbm.2014.00401.0001

J. 2004; **2**: 507–516.

Tetlow IJ, Wait R, Lu Z, Akkasaeng R, Bowaher CG, Esposito S, Kosar-Hashemi B, Morell MK, Emes MJ. Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein-protein interactions. *Plant Cell* 2004; **16**: 694–708.

Tsai CY. The function of waxy locus in starch synthesis in maize endosperm. *Biochem Genet*. 1974; **11**: 83–96.

Umemoto T, Nakamura Y, Satoh H, Terashima K. Differences in amylopectin structure between two rice varieties in relation to the effects of temperature during grain-filling. *Starch* 1999; **51**: 58–62.

Umemoto T, Yano M, Satoh H, Shomura A, Nakamura Y. Mapping of a gene responsible for the difference in amylopectin structure between japonica-type and indica-type rice varieties. *Theor. Appl. Genet*. 2002; **104**: 1–8.

Utsumi Y, Nakamura Y. Structural and enzymatic characterization of the isoamylase1 homo-oligomer and the isoamylase1-isoamylase2 hetero-oligomer from rice endosperm. *Planta* 2006; **225**: 75–87.

Utsumi Y, Utsumi C, Sawada T, Fujita N, Nakamura Y. Functional diversity of isoamylase oligomers: the ISA1 homo-oligomer is essential for amylopectin biosynthesis in rice endosperm. *Plant Physiol*. 2011; **156**: 61–77.

Wang YJ, White P, Pollak L, Jane J-L. Characterization of starch structures of 17 maize endosperm mutant genotype with Oh43 inbred line background. *Cereal Chem*. 1993a; **70**: 171–179.

Wang YJ, White P, Pollak L, Jane J-L. Amylopectin and intermediate materials in starches from mutant genotypes of the Oh43 inbred line. *Cereal Chem*. 1993b; **70**: 521–525.

Wattebled F, Dong Y, Dumez S, Delvallé D, Planchot V, Berbezy P, Vyas D, Colonna P, Chatterjee M, Ball S, D'Hulst C. Mutants of *Arabidopsis* lacking a chloroplastic isoamylase accumulate phytoglycogen and an abnormal form of amylopectin. *Plant Physiol*. 2005; **138**: 184–195.

Wong K-S, Kubo A, Jane J-L, Harada K, Satoh H, Nakamura Y. Structures and properties of amylopectin and phytoglycogen in the endosperm of *sugary-1* mutants of rice. *Journal of Cereal Science* 2003; **37**, 139–149.

Yamamori M, Fujita S, Hayakawa K, Matsuki J, Yasui T. Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. *Theor. Appl. Genet*. 2000; **101**: 21–29.

Yano M, Isono Y, Satoh H, Omura T. Gene analysis of sugary and shrunken mutants of rice, *Oryza sative* L. *Jpn. J. Breed*. 1984; **34**: 43–49.

Yoo S, Jane J-L. Molecular weights and gyration radii of amylopectins determined by high-performance size-exclusion chromatographyequipped with multi-angle laser-light scattering and refractive index detectors. *Carbohydr. Polym*. 2002; **49**: 307–314.

Yun MS, Kawagoe Y. Septum formation in amyloplasts produces compound granules in the rice endosperm and is regulated by plastid division proteins. *Plant Cell Physiol*. 2010; **51**: 1469–1479.

Yun MS, Umemoto T, Kawagoe Y. Rice debranching enzyme isoamylase3 facilitates starch metabolism and affects plastid morphogenesis. *Plant Cell Physiol*. 2011; **52**: 1068–1082.

Zhang X, Colleoni C, Ratushna V, Sirghie-Colleoni M, James MG, Myers AM. Molecular characterization demonstrates that the *Zea mays* gene *sugary2* codes for the starch synthase isoform SSIIa. *Plant Mol. Biol*. 2004; **54**: 865–879.

Zhang X, Myers AM, James MG. Mutations affecting starch synthase III in Arabidopsis alter leaf starch structure and increase the rate of starch synthesis. *Plant Physiol*. 2005; **138**: 663–674.

doi:10.5047/agbm.2014.00401.0001