Starch synthesis and grain filling in rice*

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The genetic resource of rice plant is so rich that many high yield and disease resistant cultivars adaptable to diversified agroenvironments have been bred, and currently it is the major staple food source for one half of world population. As in other cereals, the main constituent of rice grain is starch, thus the grain filling capacity is determined mainly by the starch-synthesizing capability of endosperm. Among the three loci of starch synthesis, starch granules formed in leaf and stem are transitory in nature. The stem starch serves as a temporary storage of photosynthate from source leaf to sink seed, and sucrose is the main form of photosynthate trafficking. ADPG is the glucose carrier between sucrose and starch, and besides ADPG pyrophosphorylase, an endosperm-specific sucrose synthase isoenzyme is another agent of ADPG synthesis. A futile cycling of sucrose synthesis-degradation is probably the mean of fine-tuning sucrose concentration to regulate metabolic activities. In the growing rice seed, the concerted functioning of multiple forms of granule bound and soluble starch synthases, branching and debranching enzymes, together with the ADPG supplying strength, determine the overall grain-filling capacity. The biochemistry, molecular biology, cell biology and molecular genetics relevant to the functioning of enzymes needed for transforming sucrose into various forms of starch were discussed.

1. INTRODUCTION

1.1 Genetic background

The rice plant belongs to the class Monocotyledonae, the order Glumiflorae, the family Glamineae, and the genus Oryza. The species cultivated in the Asian region is Oryza sativa L., while that cultivated in the savanna of western Africa is O. glaberrima Steud. Besides these two cultivated species, there have been found about 20 wild species belonging to the genus. For the species O. sativa, two subspecies, indica and japonica, and three ecotypes, Indica (continental), Javanica (tropical island) and Japonica (temperate island) are known. The centers of distribution of the three ecotypes are Indian subcontinent and Indochina peninsula, Indonesia, and Japan and Korean peninsula, respectively.

*Abbreviations: A(U)DPG, adenosine (uridine) diphosphate D-glucose; BE, branching enzyme; DAP, day after pollination; DE, debranching enzyme; F6P, D-fructofuranose 6-phosphate; GB, granule bound; G1(6)P, a-D-glucopyranose 1(6)-phosphate; GUS, f-D-glucuronosidase; IT, invertase; PPase, pyrophosphorylase; SDS, sodium dodecyl sulfate; SPPase, sucrose phosphate phosphatase; SPS, sucrose phosphate synthase; SS, starch synthase; SuS, sucrose synthase.
Among five billions of world population, about one-half subsist on rice as the staple food. Together with the other two major cereal crops wheat and maize, rice has a very long history of improvement through selection and breeding by farmers and breeders. Although being the most important carbohydrate supply for the population subsisting on rice-based diet, polished rice grains contain 7 to 10 % of easily digestible proteins closely associated with starch granules. It is thus also an important source of protein when supplies of meat, dairy products, beans, etc., are limited and rice is consumed in quantity. Currently, about 11% of world arable land, or 150 million ha, is used for rice cultivation, and the annual production of hulled grain is over 400 million metric tons. From these figures, we may see that the rice plant has one of the highest productivity among all cereal plants.

Among important cereal plants, rice is endowed with the most versatile properties to be exploited for the development of cultivars for agricultural industry. This is probably due to a wide range of genetic mutability and diversity we find in the rice plant. In spite of genetic diversity, the rice genome is the smallest among main cereal plants. It is natural then, besides being investigated intensively by agronomists as a staple food, the rice has become a model monocotyledonous plant for the basic researches in genetics, cell biology, physiology, biochemistry and molecular biology. Especially the success in establishing the technology for regenerating whole plant from cultured cells or protoplasts has opened up the possibilities of improving the agronomic properties by molecular breeding, and exploiting the rice plant as a reactor for the production of exotic proteins.

1.2. Agronomic properties

From the agricultural point of view, the germination of rice seed is considered as the onset of its life span. Although direct sowing of seeds on the paddy field and allow them to grow until harvest is also practiced, usually seedlings are grown in a seedbed for 20 to 40 days. They are transplanted into a paddy field in a neat pattern for easy management of fertilizer application, weed control, grain harvest, etc., and also for optimizing the growth space to get the highest return from a unit land area. Within a week after transplantation, new roots emerge and the tillering (or branching) starts. The number of tillers reaches the maximum in about a month. The stem of rice plant is consisted of leaf sheath and culm, the latter of which is composed of nodes and internodes. The first stem emerged from seed is the main culm. The branch emerging from the main culm is the primary tiller, the tiller emerging from the primary tiller is the secondary tiller, and sometimes the tertiary tiller may emerge from the secondary tiller. There is a rule in tillering. When the $n$-th leaf emerges from the leaf sheath beneath, the primary tiller emerges from the axil of $(n-3)$th leaf. This rule is also observed when the secondary tiller emerges from the primary tiller. The root system comprises the seed root, which develops from the root emerged from seed, and the crown root, which emerges from node afterward. The rule of periodicity as we find for the leaf and tiller developments is also observed in the crown root and node developments.

As the tillering stage comes to conclusion, the rice plant goes into the reproductive stage by differentiating flag leaf to form panicles. The panicle grows and differentiates to panicle axis and primary and secondary branches. From these branches spikelets grow, each of which has a short rachilla and bears a glumous flower. A glumous flower is consisted of two empty glumes, one each of palea and lemma, six stamens and one pistil. It takes about a month from the onset of flag leaf differentiation to the finished panicle to be pushed out of leaf sheath and start flowering. The flowering takes place in the chronological order of the main culm, the
primary tiller and the secondary tiller, the upper flower on the primary branch in the same panicle, the uppermost flower, and then from the bottom to the second uppermost flower in the same branch. In one panicle, the earliest to the latest may have a week lag in flowering. The pollination ensues almost instantaneously after flowering, and the fertilization completes within 3 to 4 hours. Therefore the flowering and pollination are recorded as to take place in the same day. After fertilization, the differentiation of embryo primordium completes in 3 days, the differentiation of seed root completes in 5 days, and the differentiation of the primordia of up to the third leaf completes. All of the essential parts of an embryo are formed in about 10 days after pollination (DAP). The spikelet after fertilization is called caryopsis. The cell division in endosperm completes in about 10 DAP. By this time, the aleurone layer is formed on the surface of endosperm, and the former tissue starts accumulating proteins and lipid particles, while the latter starch granules. Although most of the starch in endosperm is derived from the photosynthate produced after panicle formation, a part of photosynthate stored as starch in the stem before panicle formation are also transferred into endosperm. In about one and a half months after panicle emergence, the seed formation comes to a conclusion.

One life span of the rice plant, starting from seed germination to seed maturation, may be from a little less than 100 days to up to 270 days, according to the differences in species variety and growth environment. The major difference in growth rates among rice races with different genetic backgrounds occurs mainly from seed germination to panicle emergence. The rice plant highly tolerates continual cropping on a same piece of land. If one selects appropriate cultivars, it is possible to harvest two to three crops of rice a year from a same piece of land in tropical to subtropical regions. In Taiwan, there are many paddy fields being known to sustain annual double cropping of rice for over a century, and yet the productivity has increased continually because of the improvement in cultivars provided by the agricultural agencies and the advancement in cropping technologies.

1.3. Grain yield

The productivity of a cereal plant is measured by the economic yield, or the productivity of plant part useful as food, while the biological productivity concerns with the biological yield, or the total dry matter produced by the plant. The ratio of economic yield over biological yield is called the harvest index. In order to enhance the productivity, it is of prime importance to improve the net assimilation rate of leaves. Then the increase in harvest index may be achieved by optimizing the partitioning of photosynthates between the source, or photosynthetic leaf, and the sink, or the specific storage organ for human consumption. These have been the foci of researches by many agricultural scientists.

The economic yield of a rice plant is determined by the number of panicle, the number of seed a panicle bears, the rate of seed fertilization and seed weight. Excellent rice cultivars with diversified agronomic properties are available. The difference in length of time span to achieve maturity not only determines the adaptability to agroenvironment but also the economic yield if one considers the spatial and temporal efficiencies of rice cultivation. The early or late maturation of rice varieties is determined by the length of vegetative growth period, which is dependent on the photoperiodic sensitivity and thermosensitivity. After the flower bud initiation, there is not much difference in the length of reproductive phase among different varieties.
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In case of rice plant, the economic yield is measured by the harvest of seed grains. As described above, from fertilization to maturation of rice seeds takes about one and a half months. From 10 to 14 days after pollination (DAP), if one takes a seed at this stage and tweezes it between fingers, a milky juice, the appearance of which is due to the presence of suspended starch granules, is expressed. This stage of seed growth is called the milky, or milk ripe, stage. The rate of soluble sugar incorporation into insoluble polysaccharides, mainly starch, is the highest at this stage, and thus the milky stage seed is the most suitable sample for the study of starch synthesis in rice.

2. CARBOHYDRATE METABOLISM

2.1. Carbon flow from leaf to seed: A general view

In the rice plant, three loci of starch synthesis are known. They are photosynthetic leaf, stem including leaf sheath and culm, and seed. Among them, the starch accumulation in the former two is transient in nature. As in most green plants, the photosynthetic cell of rice assimilates a bulk of primary carbon fixation products as starch granules in chloroplasts during the light phase. In the dark phase, starch is degraded and the products are translocated to cytosol where sucrose is synthesized for the translocation to sink tissues. It has been known that the starch content in stem increases for a period before pollination. This store of starch is directed to seed as it develops. Through the grain filling stage, when the supply of photosynthates from leaf is in excess of the assimilatory capacity of seed, the transient storage in stem is also evident. The function of starch accumulation-degradation in stem seems to serve as an important regulatory role of grain filling in rice. Watanabe et al. (1) analyzed the starch content and activities of enzymes related to starch metabolism in leaf sheaths at different positions. They found a sharp increase in starch content in the 12th and 14th leaf sheath from about 15 days before pollination, which declined at the onset of pollination. A significant correlation between the starch content and the activities of branching enzyme (BE; EC 2.4.1.18), granule bound (GB) and soluble starch synthases (SS; EC 2.4.1.21), and plastidial fructose bisphosphatase was found.

2.2. Sucrose metabolism

After pollination, the seed develops to be the major site of starch depository. The starch synthesis in seed starts from sucrose translocated from leaf cells. In photosynthetic cells, triose phosphates, the glycolytic products of starch, exported from chloroplast, serve as the starting materials for the sucrose synthesis in cytosol. There are two sucrose synthesizing systems in plants, one through the consecutive reactions catalyzed by sucrose phosphate synthase (SPS; EC 2.4.1.14) and a phosphatase specific to sucrose phosphate (SPPase), and the other by sucrose synthase (SuS; 2.4.1.13). Both systems use uridine diphosphate D-glucose (UDPG), a potent glucosyl group transferring agent, as the glucose donor to drive the synthetic reaction. However, SPS and SuS use D-fructose 6-phosphate (F6P) and D-fructose as acceptors, respectively. These two analogous reactions have different equilibrium constants at the same physiological pH for the following reasons. F6P can assume only the furanose form, which is the configuration of fructosyl residue in sucrose, while fructose is in equilibrium of pyranose and furanose forms in favor of the former, thus reducing considerably the activity of acceptor species. So, the SuS catalyzed reaction is freely reversible while the
SPS catalyzed one is much in favor of the synthetic reaction. Besides, sucrose$^{F6}$-phosphate synthesized by SPS is hydrolyzed by SPPase to sucrose and orthophosphate, rendering the overall sucrose synthesizing reaction irreversible. All of these enzyme activities are known to occur in rice plant, and it is generally believed that sucrose for translocation through phloem, or for accumulation in vacuole is synthesized by the irreversible SPS- SPPase system.

Besides SPS and SuS, there is another type of enzyme that involves directly in sucrose metabolism. They are sucrose hydrolyzing enzymes. Although an α-D-glucosidase may hydrolyze sucrose, the enzymes of importance in sucrose metabolism are the isoenzymes of β-D-fructofuranosidase, or invertase (IT; EC 3.2.1.26). In many plants, there are a soluble form and an insoluble cell wall bound form of invertase having an acidic optimal pH, and another soluble form with an alkaline optimal pH. All three types of invertase occur in rice. Sucrose is translocated from photosynthetic leaves to sink organs through phloem, then transferred into sink cells via a membrane factor called sucrose translocator. The presence of the translocator has been shown but not yet well characterized.

2.3. Futile cycling of sucrose synthesis-degradation

When a radioactive glucose is fed to a seed for a short period of time (within an hour), it is assimilated into the two constituent monosaccharides of sucrose while the sucrose level is maintained constant (2). From these findings, we may see that the synthesis and degradation, or turning-over, of sucrose takes place continually without a net gain of sucrose. The pathway of this turning-over can be constructed from the sucrose metabolizing reactions described in the former section, as in Fig. 1. The cyclic operation is at the expense of ATP consumption without net gain of sucrose, so the cycle is "futile" in nature (3). Besides being a major translocated form in plants, sucrose is also known as a metabolic regulator at different functional levels. It is highly probable that such futile cycling is a mechanism of fine tuning the concentration of sucrose as a metabolic regulator.

Overall: $2 \text{Sucrose} + 3 \text{ATP} + \text{UTP} + 4 \text{H}_2\text{O} \rightarrow 2 \text{Sucrose} + 3 \text{ADP} + \text{UDP} + 4\text{Pi},$

if SuS and IT degrade sucrose equally, or

$\text{Sucrose} + \text{ATP} + \text{H}_2\text{O} \rightarrow \text{Sucrose} + \text{ADP} + \text{Pi},$

if only SuS degrades sucrose.

Figure 1. The futile cycle of sucrose synthesis-degradation
2.4. Synthesis of transient and depository starches

A starch synthesizing organelle, either chloroplast or amyloplast, contains two forms of SS; one is soluble and the other starch granule bound. Although both types of SS utilize ADPG as the substrate, the granule-bound one may use UDPG as well, but needing a non-physiologically high concentration. Both enzymes elongate the amylose type \( \alpha-1,4\)-D-glucopyranose chains, which undergo a chain transfer reaction catalyzed by a branching enzyme (BE; EC 2.4.1.18), or Q-enzyme, to amylopectin type molecules containing \( \alpha-1,6\)-branching residues. The branch chain after being elongated to a considerable length may be debranched (by a debranching enzyme, DE, or R-enzyme, RE, or pullulanase; EC 3.2.1.41) to give an amylose chain, which may be further elongated and branched. The granule bound starch synthase (GBSS) is encoded by the waxy gene \((W_x)\) and is responsible for the formation of amylose. These starch-synthesizing enzymes are present in either the stroma of amyloplast, or in close association with starch granules in insoluble forms. The enzyme distributed in stroma may be released into an extraction buffer simply by breaking the plastid membrane. But those of the latter type may be solubilized only when a detergent, such as sodium dodecylsulfate (SDS) is added to the extraction buffer (4). Rice starch granules isolated from grains incorporate these proteins into the granular structure.

Starch granules, when observed under a microscope, show a shape and structure characteristic to the plant species. Under a polarized microscope, when placed between a pair of crossed polarizers, starch granules show a specific cross-shaped pattern. When observed in an X-ray diffractometer, a diffraction pattern characteristic of partial crystalline structure is revealed. The crystalline structure is due to packed amylopectin layers while the amorphous domains are constituted of amylose chains. These physical methods of observations are useful in characterizing starch, yet elucidation of the mode of layering or packing of gucan chains is not easily achieved.

2.5. Synthesis of starch precursor, ADPG

Since the discovery of ADPG and its higher efficiency as the precursor of starch synthesis by Leloir et al., all starch synthesizing reactions have been recognized to use ADPG as the "natural substrate", or the substrate with the least Km value. Consequently, ADPG pyrophosphorylase (PPase) that catalyzes ADPG synthesis from ATP and \( \alpha-D\)-glucose 1-phosphate (G1P) has been regarded as the key enzyme in all starch synthesizing systems, and the rice is no exception. However, there is another enzyme capable of ADPG synthesis and ubiquitously distributed in plants. The enzyme is SuS, and it usually has the same trend of tissue or organ distribution as ADPG-PPase. The main reasons for discriminating SuS but in favor of ADPG-PPase as the sole provider of ADPG in starch synthesis can be that the subcellular localization of the former is in cytosol while the latter in plastids, and also the former uses UDPG as the "natural substrate".

Cardini et al. discovered SuS (5). SuS so far purified from various plants share the same properties as follows (2). It is an enzyme with four protomers in a catalytic entity. It may contain homologous or heterologous protomers, and isoenzymes may be purified. In case of rice plant, a total of five isoenzymes were purified (6), and they all show the highest affinity toward UDP, among other nucleoside diphosphates. However, they also use ADP as the second best substrate, although the Km value for UDP is about 0.1 mM while that for ADP is about 1 mM. The isoenzymes show somewhat different ratios of sucrose synthesis and
breakdown reaction rates. It is noteworthy that four SuS isoenzyme species could be purified from maturing rice seed, but only one from leaf which had properties different from those of seed isoenzymes. These facts indicate that the genes encoding these isoenzymes have distinct differences in temporal and spatial expressions. SuS is abundant in starch accumulating grains and catalyzes a sugar nucleotide synthetic reaction energetically more efficient than a PPase. From these reasons, we thought that SuS could be an agent of starch precursor synthesis as well. We designed a couple of radiotracer experiments to explore the possibility.

In the first experiment (7) we fractionated the milk-ripe rice seeds into soluble and insoluble fractions, and titrated the enzyme activities in one fraction with the other. The soluble fraction contained UDPG- and ADPG-PPases and SuS, and the insoluble starch granules had SS activity. Different proportions of the two fractions were combined, to which were added substrates for either ADPG-PPase (1nP plus ATP) or SuS (sucrose plus ADP) at an equimolar level. The insoluble product was characterized as starch by an enzymic hydrolysis technique. From the yield of starch, it was estimated that the soluble fraction contained 4 times more ADPG synthesizing activity derived from SuS than ADPG-PPase. It was further shown that the SuS activity could synthesize either ADPG or UDPG, and the former was more efficiently incorporated into starch in the presence of starch granules.

To further elucidate the role played by SuS in starch synthesis, we designed an experiment using a radioactive sucrose in which the two hexosyl residues were labeled with different radioisotopes (8). The rationale of the experimental design is as follows. As shown in Fig. 1, SuS is the only enzyme that may synthesize a sugar nucleotide directly from sucrose by transferring the glucose residue to a nucleoside diphosphate acceptor. If the sugar nucleotide so synthesized is used directly in starch synthesis, in the pool of hexose phosphates, the glucose moiety of sucrose will not be in equilibrium with the fructose moiety derived from sucrose. Thus the starch synthesized will have more radiotracer derived from the glucose moiety than that from the fructose moiety of sucrose. On the other hand, if sucrose is metabolized via the IT pathway, the two hexose moieties will be metabolized all the way in equilibrium, and starch so synthesized will be derived from equimolar amounts of the two hexoses.

We synthesized a radioactive sucrose in which glucose and fructose residues were labeled with $^{14}$C and $^3$H, respectively. A sample of the sucrose solution was hydrolyzed with a yeast invertase to be used as an equimolar mixture of glucose and fructose. Rice seeds with pedicels were sampled at different DAP. Feeding of the radioactive sugars was done by dipping the pedicel of each seed into 10 μl of the radioactive sugar solution. In an air stream, a seed absorbed the solution in about 5 min, and 10 μl of water was used to chase the residual sugar into the seed. After being incubated at room temperature further, the reaction was terminated at 80 to 120 min from the start of sugar feeding. The seed was crushed in 80 % ethanol and the mixture was boiled. Starch in the ethanol insoluble fraction was hydrolyzed to glucose with a mixture of α-amylase, glucamylase and pullulanase, and glucose was recovered by paper chromatography. The phosphate compounds in the ethanolic extract were separated by paper electrophoresis. The separated compounds were quantitated for $^{14}$C and $^3$H. As shown in Table 1, the glucose moiety of sucrose was the better precursor of starch when sucrose was administered. This tendency is weakened in more mature seeds and when the time for incubation after tracer administration is extended. When the hydrolysate of sucrose was fed, the two monosaccharides were assimilated into starch at about an equal rate. These results verified the rationale of the experimental design as described above. However, as mentioned,
ADPG is the only acceptable substrate of SS while the "natural substrate" of SuS is UDPG. So, many investigators postulated that, if SuS contributes to starch synthesis, UDPG derived from the reaction between UDP and sucrose should be transformed into ADPG by coupling UDPG- and ADPG-PPase catalyzed reactions in which GIP was the intermediate. The presence of both PPase activities in rice grains seemed to support the postulation.

Table 1
Mole ratios of $^{14}$C- and $^3$H-glucose residues in starch. Rice seeds were fed with a double-labeled sucrose, $\alpha$-D-$^{14}$C-glucopyranosyl-$\beta$-$^3$H-fructofuranoside, or its hydrolysate.

<table>
<thead>
<tr>
<th>Incubation (min)</th>
<th>Fed with</th>
<th>Days after pollination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succrose</td>
<td>Hydrolysate</td>
</tr>
<tr>
<td>20</td>
<td>5.76</td>
<td>1.16</td>
</tr>
<tr>
<td>40</td>
<td>5.69</td>
<td>1.09</td>
</tr>
<tr>
<td>60</td>
<td>5.73</td>
<td>1.11</td>
</tr>
<tr>
<td>120</td>
<td>5.44</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Table 2
Mole ratios of $^{14}$C- and $^3$H-hexose in starch and sugar phosphate compounds isolated from rice seeds fed with a double-labeled sucrose or its hydrolysate.

<table>
<thead>
<tr>
<th>Fed with</th>
<th>GIP</th>
<th>G(F)6P</th>
<th>UDPG</th>
<th>ADPG</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>0.15</td>
<td>0.24</td>
<td>4.27</td>
<td>4.05</td>
<td>4.16</td>
</tr>
<tr>
<td>Hydrolysate</td>
<td>1.10</td>
<td>1.07</td>
<td>1.10</td>
<td>1.06</td>
<td>1.07</td>
</tr>
</tbody>
</table>

We considered that, if the coupled PPase reaction should take place, there would be no discrimination of the two hexosyl residues in starch synthesis because the intermediate, GIP, should be in equilibrium with other hexose phosphates in the metabolic pool of starch synthesis. Nevertheless, we isolated UDPG, ADPG and hexose phosphates from the radiotracer-fed grains, analyzed their tracer contents, and obtained results as shown in Table 2. These results indicated that the glucose moieties of the two sugar nucleotides were derived mainly from the glucose moiety while all sugar monophosphates, including GIP, were from
the fructose moiety of sucrose when sucrose was fed. The only plausible explanation was that the sugar nucleotide derived directly from a SuS catalyzed reaction was incorporated into starch. By considering the earlier finding that ADP plus sucrose was a better combination of substrates than UDP plus sucrose in the reconstituted starch synthesizing system, we proposed that, in the growing rice seed, SuS catalyzed reaction would directly provide at least a part of ADPG needed for starch synthesis.

Besides our demonstration of the importance of SuS in the starch synthesis in rice seeds, other types of work also provided certain evidence in supporting the view. One such work was to correlate the enzyme activities and the status of grain filling on the same rice plant. Flowering of spikelets takes place from the top down to the basal one; and the basal spikelet reaches anthesis a week after the top spikelet on the same branch and achieves a poorer grain filling. A measurement of sucrose translocation showed that the basal spikelet did not receive less sucrose than the top one. However, the activities of SuS and IT were higher and lower, respectively, in the endosperm cells of the top spikelet than those of the basal spikelet. Based on these comparative data, it was concluded that the level of SuS activity was positively correlated with the degree of grain filling, or the biosynthesis of starch (9).

3. GENETICS, ENZYMEOLOGY AND MOLECULAR BIOLOGY OF ENZYMES IN THE PATHWAY FROM SUCROSE TO STARCH

The synthesis of rice starch has been studied in leaf chloroplast and endosperm amyloplast. The synthetic pathways were elucidated mostly by analyzing the levels of carbohydrates and enzyme activities among different tissues or organs at different physiological states. As described earlier, the enzymes ADPG-PPase, soluble and GBSS, BE and DE have been recognized as the enzymes involved in starch synthesis. Besides, we provided evidence to show that SuS was also a good provider of ADPG as described above. Among these enzymes, the genetic, biochemical and molecular biological studies were made most intensively on ADPG-PPase, SuS, GBSS and BE.

3.1. ADPG pyrophosphorylase

In plants, ADPG-PPase is localized in leaf chloroplast and amyloplast of sink tissues. Earlier, ADPG-PPases from potato tuber (10) and maize endosperm (11) were reported to be composed of single subunits of 50 and 54 kDa, respectively. Later, however, the enzyme from these two sources was recognized to have two different protomers distinguishable by size, immunological specificity and specific mutants (12, 13). Similarly, the leaf enzymes from spinach (14), Arabidopsis (15, 16), wheat, etc. (17) were reported to have two different types of protomers.

Anderson et al. cloned a cDNA containing the whole ORF for ADPG-PPase from an expression library constructed from rice endosperm poly(+) RNA (18). The predicted peptide contained 483 amino acid residues and had a molecular mass of 52 kDa. It was believed that the site of enzyme localization was in amyloplast while that of the gene in nucleus, so a putative leader sequence for plastidic transport was assigned. Nakamura et al. (19) purified ADPG-PPase from developing rice endosperm. The final product had a molecular mass of 200 kDa and was resolved into 6 spots by two-dimensional PAGE. These spots all had a molecular mass of about 50 kDa and were positively stained with either one of the antisera
raised against two of the multiple spots. When digested with V8 proteinase, they gave similar but different peptide maps, and the N-termini were not identical. Based on these results, they concluded that the rice endosperm ADPG-PPase had a tetrameric quaternary structure with multiple forms of protomers encoded by a gene family. The rice chloroplast ADPG-PPase has not been studied in detail.

3.2 Sucrose synthase

By enzyme purification, at least 4 isoenzyme species of SuS from rice seed could be characterized (6). These isoenzymes showed different ratios of catalytic activities toward UDP and ADP. This will imply that, as we have proved that the number of SuS genes is no more than 3 (20), the enzymes with a tetrameric quaternary structure could be homotetramers as well as heterotetramers. Since the deduced structures of protomers show high degree of sequence homology and have identical or very little difference (less than 1%) in size, it is very difficult if not impossible to decipher their quaternary structures by ordinary enzyme chemical methods. According to the histochemical and Western analytical methods as described later, rice tissues could be classified according to the protomer expression patterns, and the presence of at least one heterotetrameric enzyme in seed could be revealed.

We cloned and established the whole structures of three each of cDNA and genomic DNA which encoded rice SuS (20-22). It is noteworthy that so far the rice is the only plant that has been shown to have three independent and active genes encoding SuS. They all have an untranslated short stretch of exon 1, which is followed by a long intron 1. The lengths of intron 1 in the three genes were approximately 1 to 3 to 2 in the order of gene numbering, and the longest one had 2.9 kbp.

We studied the expression of three isogenes encoding SuS1, SuS2 and SuS3 at the transcriptional level at different stages of seed maturation and in different rice organs (22). It has revealed that, although all of them are expressed mainly in seed, the expression of Sus1 is ubiquitous while that of Sus3 is exclusive in seed, and the expression sites and strengths of Sus2 seem to complement those of Sus1, especially under the stress conditions such as anaerobiosis. The expression of Sus1 is quite unique; it is upregulated by the availability of sucrose. These findings imply that Sus1 and Sus2 are house-keeping genes while Sus3 bears a specific role in the grain filling with starch.

In order to elucidate the expression of three genes at the translational level, we raised monospecific antibodies against SuS2 and SuS3 in mice by using keyhole limpet hemocyanin conjugated with protomer-specific synthetic peptides as antigens. A maize monoclonal antibody specific to ShS protein (a kind donation of Dr. Chourey of Florida State University) specifically recognized rice SuS1. The three SuS proteins were expressed in E. coli and used as standards for characterizing the monospecificity of the antibodies. The quantitative distribution of three SuS proteins in the seed and etiolated seedling of rice were analyzed in detail by the immunohistochemistry and quantitative Western analysis.

SuS1 was poorly expressed in seed issues including husk, pericarp, testa and endosperm, but at a higher level in embryo. The expression of SuS2 was contrary to that of SuS1; it was expressed distinctly in all tissues but slightly in embryo. Embryos isolated from 12 DAP seeds were allowed to germinate in water for 8 days. The level of SuS1 declined to nil but SS2 was induced to a high level on germination. In an etiolated seedling, SuS1 and SuS2 were found in both shoots and roots, but with SuS1 predominating over SuS2. In sections of leaves on etiolated seedlings, SuS1 was localized in the mesophyll but not in epidermis and vascular
tissues. SuS2 was localized at the same sites as SuS1 and also in the phloem, especially in the phloem of sheath. In the roots of etiolated seedlings, SuS1 was localized only in the phloem where SuS2 was not observed. SuS3 was exclusively expressed in the starch-storing parenchymatous tissue and testa containing aleurone layer, but not even in trace in green tissues or embryo. These findings confirm the transcriptional level data that SuS3 was endosperm-specific, and SuS1 and SuS2 complementing each other to serve a house-keeping role. The endosperm specific expression of SuS3 should confer this protein an important role in the starch synthesis in filling grains.

Sucrose is transported into sink organs from leaves through phloem. Enzymes located in phloem might regulate the sucrose concentration to force the sucrose flow from source to sink. Immunoblotting of SuS1 and SuS2 in the extract of either shoot or root of etiolated seedling showed that they were present in larger quantities than in seed. As mentioned above, SuS2 was located in phloem of shoot and SuS1 in phloem of root of etiolated seedlings. The tissues were at a rapid growth phase in 8 day seedlings, and SuS could play roles in enhancing sucrose unloading and providing substrates for complex saccharides biosynthesis. The same situation was found in seed where SuS1 and SuS2 were found in vascular tissues in a large quantity. So, we may conclude that, in the grain filling, SuS1 and SuS2 contribute to the enhancement of a pulling power of the sink organ, and SuS3 contributes to the supply of ADPG for starch synthesis (23). For further proving this conclusion, the expression of endosperm specific Sus3 was knocked out by the antisense technique. The seeds borne on the mutant plant had a shrunken phenotype. The first generation progeny also had the shrunken phenotype with a reduction of about one half of starch content (24). It was thus concluded that Sus3 in rice was analogous to Shl of maize.

The biochemical role that SuS may play in rice was also studied by a comparative method. Kato (25) analyzed four rice cultivars with different grain sizes and grain filling rates with respect to their activity profiles of SuS, acid IT and UDPG-PPase in the developing endosperm. He found that only SuS showed a significant difference in activity per seed or per seed fresh weight among different cultivars, and the activity was higher in seeds on primary branches that had a higher grain-filling rate. These results were taken to suggest that SuS plays a role in the regulatory system of grain development, but does not have any relations to genetic variations in grain size and grain filling rate.

3.3. Granule bound starch synthase

This enzyme from rice endosperm, encoded by \( W_x \) gene, has drawn much attention from both agronomic and biochemical standpoints. The \( W_x \) allele determines the synthesis of amylose, and the loss of \( W_x \) protein, or GBSS, results in the glutinous phenotype, or loss of amylose from endosperm. A \( W_x \) locus of rice was cloned (26). The primary structure of the gene having 12 introns was established. The elucidated polypeptide structure had a putative transit peptide of 77 amino acids and a mature protein region of 532 amino acids. By the Northern and Western analyses, the sites of gene expression were identified only in the endosperm and pollen, and the gene was active in the early and middle stages but not in the late stage of seed development. However, the protein accumulates linearly in the seed. Two \( W_x \) alleles have been found at the \( waxy \) locus in rice chromosome 6, and their gene products \( W_x^a \) and \( W_x^b \) were predominant in indica and japonica, respectively (27). In the mature grain, indica and japonica rice varieties contained about the same amount of amylose. Yet the latter contained less \( W_x \) protein than the former but the GBSS activity at the midmilky grain was...
much higher in the latter. Therefore, Wx\(^a\) appeared to have a much less specific activity than that of Wx\(^b\) (28). Taira et al. (29) observed that the leaf starch from a glutinous rice still contained 3.6 % amylose, and none of proteins bound to leaf starch granules cross-reacted with an antiserum raised against a Wx protein isolated from a nonglutinous rice. It is evident that the leaf starch amylose is not encoded by Wx.

An antisense construct corresponding to exons 4 to 9 (including the introns) was introduced into a wild type japonica rice, and some of the seeds regenerated from the transformants showed remarkable decreases in amylose content (30). When a Wx gene was introduced into rice, the silencing of Wx in pollen and endosperm ensued in different patterns (31). In pollen grains of transgenic wild type rice, there were two gene silencing patterns; one had 100% and the other 50% wx phenotype. The gene transmission analysis showed that Wx silencing was transmitted meiotically and the Wx transgene had a paramutagenic effect on the endogenous Wx. When a wx mutant was transformed with the same Wx gene construct, the transgenic Wx behaved as a dominant Mendelian factor. It was thus concluded that the endogenous Wx activity influenced the silencing phenomenon.

The effect of intron 1 (1126 bp) on the expression of Wx was first indicated by the transgenic rice and tobacco plants transformed with a chimeric construct containing the intron sequence fused to a β-D-glucuronosidase (GUS) reporter gene (32). The chimeric construct with the intron sequence strongly enhanced the expression of the reporter gene, yet the intron sequence was accurately spliced out to form a mature messenger. The essentiality of accurate splicing of intron 1 in the Wx expression was revealed by analyzing the contents of endosperm amylose, Wx protein and Wx mRNA on 31 rice cultivars (33). Accurate intron 1 splicing for obtaining a mature Wx mRNA needed for Wx protein synthesis was revealed by comparing the splicing signals in Wx\(^a\) and Wx\(^b\) (34). The activity of Wx\(^a\) was 10 times of that of Wx\(^b\) at the level of both protein and mRNA. Sequence analysis of Wx\(^a\) transcripts revealed that splicing occurred at the mutant AG/UU (wild type AG/GT) and two cryptic sites, one of which was A/GUU, one base upstream of the original site, and the other was located at about 100 bases upstream of the original site. The effect of point mutation on intron 1 of Wx\(^a\) and Wx\(^b\) was analyzed by using chimeric constructs containing a GUS reporter. The 5' end splicing site of intron 1 in Wx\(^a\) was mutated from G to T, and that of Wx\(^b\) from T to G. These mutations resulted in switching GUS activity from high to low and from low to high, respectively. These results demonstrated that the low level expression of Wx\(^b\) was resulted from a single base mutation at the 5'-end splice site of intron 1. Similar results were obtained by a comparative study on a collection of varieties and breeding lines currently used in the USA; these samples had a range of amylose contents from 0 to 27% (35).

3.4. Soluble starch synthase

In spite of the wealth of accumulated information on soluble SS from other plants, the enzyme from rice has been little studied. Two types of soluble SS are known, one requiring a primer and the other not. Baba et al. (36) purified three SS proteins from a soluble extract of immature rice seeds. One of them had a molecular mass of 55 kDa and the other two 57 kDa. They all cross-reacted with an antiserum raised against rice GBSS. Their N-terminal amino acid sequences were identical, except that the 55 kDa protein lacked 8 terminal residues, thus they were considered as the products of a same gene. They cloned a cDNA the ORF of which predicted a protein of 626 amino acid residues including a putative transit peptide of 113 residues. A consensus sequence of ADPG binding sequence was present. It is a single copy
gene in the rice genome and is expressed in leaves and immature seeds, indicating that it plays a role distinct from that of GBSS. The same group of workers further isolated and sequenced a genomic clone of the gene (37). They compared the gene structures of the soluble and GBSS and found that they had a significant but low sequence identity and the gene organizations were divergent. However, the two genes were closely located to each other on chromosome 6 at a map distance of about 5 centimorgans. The availability of the genomic structure of the gene will permit studies on the regulatory mechanism of gene expression.

3.5. Starch branching enzyme (Q-enzyme)

The reaction catalyzed by BE modifies the starch structure from an amylose type linear chain form to an amylpectin type branched form. This change in structure also increases the number of non-reducing end groups of α-1,4-glucose chain, or the concentration of one of the substrates for SS, for enhancing starch synthesis. It is then natural that the increase of BE activity in the developing rice endosperm is specifically correlated with the increase in starch synthesis (38). The branching enzyme in rice has multiple forms. From developing rice endosperm, two isoforms, BE1 and BE2, were purified (39), the latter of which was further resolved into BE2a and BE2b (40). The molecular masses of BE1 and BE2 were 80 and 85 kDa, respectively. The two isoforms were immunologically not cross-reactive, and their peptide maps prepared by V8 protease digestion were substantially different. Rice organs other than seed, such as leaf blade, leaf sheath, culm and root all had two isoforms, but their BE2 could not be resolved into BE2a and BE2b as the endosperm isoenzyme. BE is apparently endosperm specific because the enzyme activity in that tissue is 100- to 1000-fold higher than those in others irrespective of being estimated on soluble protein or fresh tissue weight basis. And BE1 should be more important because the activity of BE1 in endosperm is about 6-fold of that of BE2. The mobility on native PAGE of BE2 from organs other than seed coincided with that of BE2b of endosperm, but, although the antiserum raised against BE2a cross-reacted with BE2b, it did not recognize any of BE2 isoforms from non-seed organs. These data were complemented with another piece of purification work (41) in which were obtained four apparent isoforms. Three of these isoforms were characterized as products of a gene analogous to maize Be1 and had molecular masses from 82 to 85 kDa, while the remainder which had a molecular mass of 87 kDa was distinguished from gene products of Be1. These enzyme data were further elaborated by the molecular cloning data as described in the following.

So far, three genes, Be1, Be2 and Be3, encoding BE isoenzymes are known. The ORF in a cDNA clone isolated from a λgt11 library prepared from developing rice seeds predicted a BE1 protein consisting of 820 amino acids and having a Mᵣ of 93,258 (42). Although abundant in endosperm, a purified BE1 could not be sequenced because of N-terminus blocking, so the prediction of a transit leader sequence could not be done. Two genomic clones encoding BE1 and BE2 were isolated from a commercial genomic library (43). The ORF of Be2 was analogous to that of bacterial glycogen BE but that of Be1 showed an extreme divergence. Be1 is a single copy gene in the rice genome. The expression pattern of Be1 completely coincides with that of Gbss, implicating the concerted functioning of the two in starch synthesis. Intron 2 of Be1, which is 2.2 kbp in length, precedes the boarder between the regions encoding a leader sequence and mature protein, and contains a high G/C with several repeated sequences in its 5' half.
The third gene, Be3, is involved in the *amylose-extender* (high amylose) mutation, which is characterized by an increased amylose content in the storage starch. The *amylose-extender* mutation in maize changes the physical properties of starch granules in shape, x-ray diffraction pattern and solubility in chemical reagents, and the chemical properties such as susceptibility to enzyme digestion. Besides, the mutant starch contains an amylepectin with longer internal and external branches than the normal, and an intermediate material having four or five branches of an average chain length of 50 glucosyl residues that are linked to a main linear chain of 100 to 150 glucosyl residues. Satoh et al. chemically induced high amylose mutants in rice (44, 45). Characterization by unit chain profile, x-ray diffractometry, photopastegraphy and scanning electron micrography gave data consistent with those of maize mutants. The mutant lines were further characterized by protein analysis and molecular cloning (46). Western blot analysis indicated that two out of five mutant lines lacked an isoform of BE (BE3), although the levels of GBSS and BE1 were normal. Three other mutant lines and the wild type had an 87 kDa protein corresponding to BE3. However, all five mutant lines showed significant decrease in BE activity, indicating that the BE3 protein in the three mutant lines was an inactive form of the enzyme. From a normal rice seed cDNA library, a clone encoding BE3 was isolated. It encoded a protein of 825 amino acid residues including a 65-residue transit peptide. Sequences of catalytic domains of amylolytic enzymes are highly conserved in the BE3 sequence, indicating that it is a member of the amylase family. When compared with BE1, it has an extra stretch of 70 residues at the N-terminus but 50 residues less at the C-terminus, and the overlapping portions had a noticeable degree of sequence identity. These differences in structural features may be the reason why the two enzymes play distinct roles in starch synthesis. The chromosomal position of BE3 was mapped to a single locus on chromosome 2 flanked by *CDO715* and *RG157*, possibly in tandem-repeated fashion (47).

3.6. Starch debranching enzyme (R-enzyme)

Although RE is regarded as an enzyme that contributes to the complete hydrolysis of starch in seed germination, the role it may play in starch synthesis has been implicated by the finding that a marked activity of the enzyme is present in the developing endosperm of rice (48). This view was greatly substantiated by a study on the structures of glucans synthesized in the endosperm of rice mutants induced by N-methyl-N-nitrosourea which had a *sugary-1* phenotype (49). In the *su-1* mutant, the cells in the inner part of endosperm contained phytoamylopectin while those located in outer part had numerous starch granules. The phytoamylopectin had more of short branch chain (DP 5-12) while much less of longer ones (DP ≥ 37) than amylepectin. Analyses on activities of BE1, BE2a, ADPG-PPase and RE revealed that only the activity of RE was positively correlated with the proportion of the starch region to whole endosperm. This finding suggests that the reduction in RE activity is related to the development of the *su-1* phenotype and that the enzyme plays an essential role in determining the fine structure of amylepectin molecules.

RE was purified from developing rice endosperm and its cDNA was cloned (50). The molecular mass of the enzyme was about 100 kDa and the ORF of the cDNA predicted a protein of 912 amino acids with a molecular mass of 102,069 Da. The amino acid sequence was substantially similar to that of bacterial pullulanase. The gene was identified to be a single copy in the rice genome and located in chromosome 4.
3.7. Trans regulation of starch synthesis by *Floury-2* locus (51)

The recessive *floury-2* (*flo-2*) locus, which is located in chromosome 4, causes a strong reduction in expression of *Bel*, which is located in chromosome 6, in immature seeds on 10 DAP. Moreover, the reduction in expressions of *Be3* and *Gbss* was also found in *flo-2* seeds. However, the expression level of *Bel* in the leaves of *flo-2* plant was as high as in the wild type. These data imply that *Flo-1* gene regulates expression of some starch synthesis-related genes in *trans* and developing-seed-specific manner.

4. CONCLUDING REMARKS

Among cereal crops, rice is unique in that it is planted in the paddy field. The submerged condition of the root system confers it with a tolerance to anoxia. However, an enhanced growth of the root system during the tillering stage by draining water will bring about an enhanced growth of the root, which in turn enhances the grain yield. The importance of a well grown root system is also demonstrated in the practice of transplanting rice from nursery to paddy field. Besides establishing an arrangement of rice plants to optimize the growing space and achieve a better condition for crop management, the practice also confers the seedling with the vigor for growth. In the process of transplanting, the extended roots on seedlings are cut, but the new growth of abundant roots ensures a better colonization of the paddy soil. These facts show that the cultured rice is a highly domesticated cereal plant and its optimum life cycling by attentive farming is needed to obtain a high grain yield from a unit land area. Besides selecting cultivars suitable for a certain agroecosystem, such as the use of dwarf cultivars that tolerate strong wind better in areas where typhoon conditions may prevail, attentive farming with appropriate technology is always the key to assure a high productivity.

The genetic, biochemical and molecular biological elements that may influence the grain yield, or more precisely, the grain filling with starch, were mentioned where appropriate in this paper. The starch molecule is composed of only one type of hexose, glucose, and only two types of glucosidic linkages. However, the biosynthesis of a seemingly simple starch molecule is a complex process, and we know only fragmented knowledge for the time being. The modern biochemistry and molecular biology have revealed the fact that, even one type of catalytic reaction may be catalyzed by multiple forms of isoenzymes specifically in certain tissue or organ at certain time frame of plant growth. In the fermentation technology, it has been known that breeding of a microbe harboring multiple copies of a gene, or a high gene dose, may bring about the enhancement of the gene activity. However, as exemplified in the silencing of endosperm *Wx* by a transgenic *Wx*, microbial experiences may not apply to higher plants always. Whether the molecular breeding of rice may surpass the brilliant outcomes that the traditional rice breeders worldwide have achieved is a big challenge to the new breed of rice breeders. The writer has the feeling that no remarkable achievements may be forthcoming in the near future even though much effort is being dedicated to achieve such goal.

A new environmental concern on the rice productivity is the effect of increasing carbon dioxide in the atmosphere, the emission from the paddy field of green house gases other than carbon dioxide, and the global warming. Work on the monitoring of green house gas emission under different agroenvironments has been carried out intensively. The obtained data may be applicable to modify the rice cropping practices to make the rice cultivation more
environment-friendly. The effects of increased carbon dioxide concentration or temperature were studied. Rice cultivars were grown under the atmospheric concentrations of carbon dioxide at 340 and 650 µL L\(^{-1}\) (52), or 350 and 700 µL L\(^{-1}\) (53) levels. The midday canopy photosynthetic rate was enhanced by 18 to 34\% under a normal water supply, and 5 to 12\% at a water deficit condition in the higher carbon dioxide concentration. However, the enrichment of carbon dioxide enhanced neither starch accumulation nor the activities of ADPG-PPase, UDPG-PPase, SuS and SS in the seed. An interesting observation is that, when rice was cultivated under 4, 18, 28 and 35 °C, only the 18 °C treated one had a higher \(W_x\) activity, and hence an increase of amylose content (54). A transgenic study by transforming rice with a chimeric construct of \(W_x\) promoter and GUS reporter revealed that the response was due to the response of the promoter.

The rice biology has entered a new era of deciphering the molecular mechanisms of very richly developed biological phenomena. Undoubtedly, all of the outcome of such research will contribute to the development of plant science in general and the crop science in particular.

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