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Gene Expression of the Biosynthetic Enzymes and Biosynthesis of Starch during Rice Grain Development

Jung-Won Hwang, Sung-Kon Kim[1], Jeom-Sig Lee[2], and In-Soo Kim*
School of Life Science and Biotechnology, College of National Sciences,
Kyungpook National University, Daegu 702-701, Korea
[1]Department of Food Science and Nutrition, Dankook University, Seoul 140-714, Korea
[2]National Yeongnam Agricultural Research Institute, NICS, RDA, Milyang 627-803, Korea

Amylose and amylopectin are determinants of the physicochemical properties for starch and grain quality in rice. Their biosynthesis is catalyzed by the interplay of ADP-glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), soluble starch synthase (SSS), a starch branching enzyme (SBE), and a starch debranching enzyme (SDE). In this study, the genes for these enzymes were highly expressed 7 to 28 days after flowering during grain development, and their expression closely matched increases in both starch content and grain weight. Among all the tested cultivars, amylose contents in the rice grains remained essentially constant throughout their development. The AGPase gene was highly expressed in the high-yield cultivars of both glutinous and non-glutinous rice. The SSS gene was actively expressed when mature GBSS mRNA decreased. Genes responsible for amylopectin biosynthesis were simultaneously expressed in the late stage of grain development. We have now demonstrated that the expression patterns of starch biosynthetic genes differ between glutinous and non-glutinous rice, and between Tongil (a Japonica/ Indica hybrid) and Japonica types.

Keywords: amylopectin, amylose, rice grain development, starch, starch biosynthetic genes

Starch is a major plant carbohydrate reserve, and is localized to the chloroplasts in photosynthetic tissues and to the amyloplasts in non-photosynthetic tissues. It comprises both amylose, a linear α-1,4 glucan, and amylopectin, a branched α-1,4: α-1,6 glucan, that has a well-defined structure of multiple clusters in which the amorphous and crystalline lamellae are structurally repeated (Manners, 1989; Su, 2000). The properties of starch are determined by the ratio of amylose and amylopectin, and by the frequency of chain-branching and the chain length of amylopectin (Manners, 1989).

Starch biosynthesis is initiated with a substrate of ADP-glucose that is formed by AGPase (EC 2.7.7.23) from glucose-1-phosphate. Starch synthase (EC 2.4.1.21) adds ADP-glucose to the non-reducing end of the α-1,4 glucan primer to form an elongated, linear α-1,4 glucan chain. It exists in two major forms: GBSS for amylose synthesis and SSS for amylopectin synthesis (Dry et al., 1992). Non-glutinous rice produces both mature, 2.3-kb GBSS mRNA and incompletely spliced immature, 3.3-kb GBSS mRNA with Intron 1; glutinous rice, however, produces only the immature, 3.3-kb mRNA (Wang et al., 1995). The addition of α-1,6 glucosyl branching points for amylopectin is catalyzed by SBE (EC 2.4.1.24), which hydrolyzes the α-1,4 glucosyl bond and the resulting oligosaccharide chain is then added to the α-1,4 glucan chain to form α-1,6 branched linkages. The branch chain after considerable lengthening by SSS is debranched by SDE (EC 3.2.1.41) to yield an elongated α-1,4 glucan chain. The structural regularity of amylopectin is determined by SDE that is classified into two isoforms -- isoamylase and pullulanase (Kubo et al., 1999).

Amylopectin structure, along with the amylose content and non-starch components such as proteins and lipids, determines the physicochemical properties of rice that influence its cooking and eating qualities (Reddy et al., 1993; Ong and Blanshard, 1995; Kim and Rhee, 2004). The length and distribution patterns of the branching chain of amylopectin affect the ther-

*Corresponding author; fax +82-53-943-0401
e-mail ikim@knu.ac.kr

Abbreviations: AGPase, ADP-glucose pyrophosphorylase; DAF, days after flowering; GBSS, granular-bound starch synthase; SBE, starch branching enzyme; SDE, starch debranching enzyme; SSS, soluble starch synthase.

mal, pasting, and rheological properties of starch (Safford et al., 1998; Jane et al., 1999). In barley, reductions in the activity of isoamylase-type SDE convert the amylopectin structure to a phytoglycogen structure (Burton et al., 2002). These results indicate that the fine structure of amylopectin is determined by cooperative interactions among SBE, SSS, and SDE.

Although some reports have described the gene expression and enzymatic properties of starch biosynthetic enzymes, and others the physicochemical properties of the rice grain, previous research had not elucidated the correlation between gene expression of the starch biosynthetic enzymes and the physicochemical properties of the grain. Therefore, our current study sought to define that relationship by studying gene expression patterns and starch accumulation during grain development in several rice cultivars.

MATERIALS AND METHODS

Materials

From the National Yeongnam Agricultural Experiment Station, Milyang, Korea, we obtained nine rice cultivars with physicochemically different properties: 'Nagdongbyeo', 'Dongjinbyeo', 'Shindongjinbyeo', 'Ilpumbyeo', and 'Junambyeo' (non-glutinous Japonica type); 'Shinshunchalbyeo' (glutinous Japonica); 'Hangangchalbyeo' (glutinous Tongil); and 'Areumbyeo' and 'Namchunbyeo' (non-glutinous Tongil). Their grains were produced from plants that were sown on April 20, 2002, and transplanted into a paddy field on May 20, 2002.

Measurements of Grain Maturity and Weight

At the grain-developing stages of 7, 14, 21, 28, 35, and 42 days after flowering (DAF), 100 rice grains were harvested and their maturity levels and weights were measured. The former parameter was evaluated by visual scoring of color into one of five groups: green (0.2), yellowish-green (0.4), yellow/green (0.6), greenish-yellow (0.8), and yellow (1.0). The grain maturation process was illustrated by plotting their evaluated data against DAF. Grain weight was measured by individually weighing 100 randomly counted, de-hulled grains to the nearest 0.1 mg.

Starch Isolation

Starch was isolated by soaking the powdered rice grains in distilled water at 4°C for 24 h, then centrifuging at 10,000g for 20 min, dispersing the resulting pellet in 0.2% NaOH solution, and, finally, allowing it to stand at 4°C overnight. This alkali treatment was repeated three times. The final pellet was washed with distilled water until its pH became neutral, then air-dried, and passed through a 60-mesh sieve (Kim et al., 1995).

Determination of Starch, Amylose, and Amylopectin Contents

Starch content was determined with a total starch analysis kit (Megazyme Kit, Megazymes International Ireland, Ireland), according to the manufacturer's instructions. Amylose and amylopectin contents were spectrophotometrically measured by iodine, according to the method of Williams et al. (1970), using potato amylase (type III, Sigma, USA) and potato amylopectin (Sigma) as standards.

Preparation of cDNA Probes for Starch Biosynthetic Genes Using RT-PCR

The cDNA clones of AGPase-p, GBSS-p, SSS-p, SBE-p, and SDE-p were amplified by RT-PCR, using the sense and antisense primers of each cDNA (Table 1). Gene amplification was performed by the GeneAmp® PCR system 2400 (Applied Biosystem, USA), beginning with one denaturation cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, and annealing at 60°C (except for SSS, at 65°C) for 1 min and at 72°C for 1 min. Each cDNA was purified by gel electrophoresis, ligated into a pGEM-T Easy vector (Promega, USA), and transformed into *Escherichia coli* (DH5α). The plasmid was isolated and transcribed into [α-^{32}P]dCTP-labeled probes with the Ladderman™ labeling kit (TaKaRa, Japan).

Northern Blot Analysis

Total RNA was isolated from the rice grains by selective precipitation with LiCl, according to the method of Chirgwin et al. (1979). It was separated by formaldehyde-agarose gel electrophoresis at 7 V cm^{-1} for 3 h, and transferred onto a nylon membrane (Hybond TM-N, Amersham, USA) by a modified capillary method for 12 h. The membrane was pre-hybridized for 2 h at 42°C with salmon sperm DNA in hybridization buffer (50% formamide, 5× Denhardt's solution, 0.1% [w/v] SDS, and 5× SSC solution), then hybridized for 18 h with the ^{32}P-

Table 1. Primer sequences used for cDNA probes of rice starch biosynthetic genes.

Starch biosynthetic genes*		Primer sequences	GenBank accession no.
AGPase	Sense	5′-GAGTGTGCTTGGGATCATTCT-3′	J04960
	Antisense	5′-GCAATTCTTCCCAATACCAATGGGAATGCC-3′	
GBSS	Sense	5′-CGAGCTACCTGAAGAACAACTAC-3′	AF031162
	Antisense	5′-TCGTCTCTCATCCATATACTGCT-3′	
SBE	Sense	5′-GTTAAATTTCGCTTTAGGCA-3′	D10752
	Antisense	5′-GCTCCTGATCAAGAAATCTG-3′	
SSS	Sense	5′-TTTTCCTTATGCAAAGTCAGGT-3′	D16202
	Antisense	5′-ACGAAATTTATCCCTGTACCCT-3′	
SDE	Sense	5′-ACCTTCAGAGTCCCTAGCTC-3′	D50602
	Antisense	5′-TCGCGGATCCTATCGTTCAAACT-3′	

*AGPase, ADP-glucose pyrophosphorylase; GBSS, granule-bound starch synthase; SBE, starch branching enzyme; SDE, starch debranching enzyme; SSS, soluble starch synthase.

labeled DNA probes of AGPase, GBSS, SSS, SBE, and SDE. Finally, the membrane was washed and exposed to X-ray film for 48 h before development.

RESULTS AND DISCUSSION

Grain Weight and Starch Content

All the rice cultivars examined here developed ears within a week, from August 2 to 9, 2002. Grain weights and starch contents were measured for 'Ilpumbyeo', 'Shinshunchalbyeo', 'Areumbyeo', and 'Hangangchalbyeo' (Fig. 1). The latter two, both high-yield Tongil types, had heavier grains than did the former two, both Japonica rices. Starch accumulation was a major factor in those yield increases (Fig. 1). The grain maturation process, as defined by color, also was closely correlated with these changes in grain weights and starch contents (data not shown).

Starch actively accumulated for two weeks, from 7 or 14 DAF (Fig. 1). This finding agrees with that

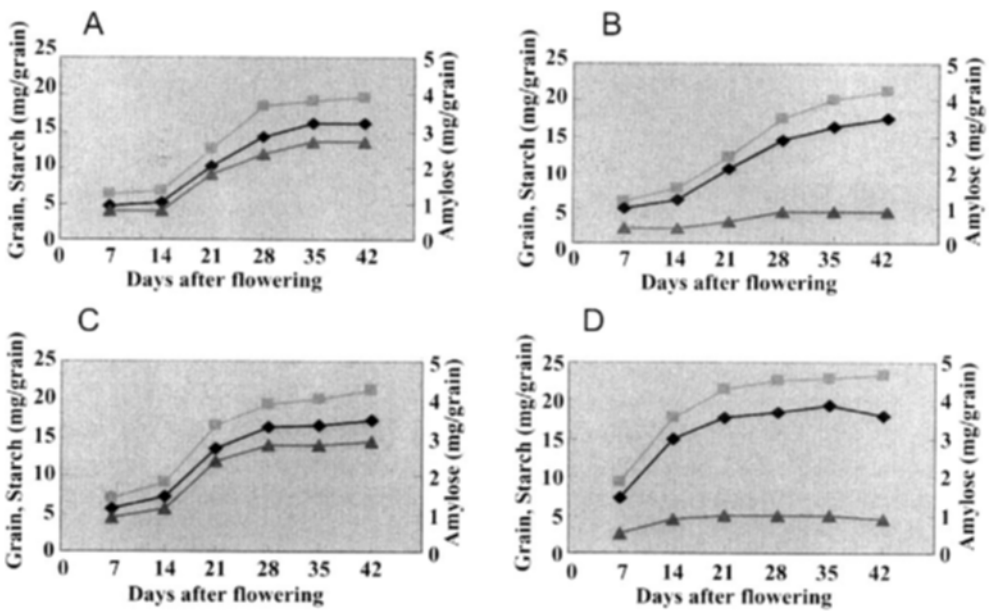

Figure 1. Changes in grain weight, and starch and amylase contents during grain development. Weight values were averaged over 100 grains, and starch and amylose contents were an average of two replications. Rice varieties: **A)** Ilpumbyeo, **B)** Shinshunchalbyeo, **C)** Areumbyeo, **D)** Hangangchalbyeo. ■, grain weight; ◆, starch content; ▲, amylose content.

reported by Chen et al. (1994), who showed that starch is actively synthesized during the middle stage of grain development. However, the duration of this accumulation period varied among our cultivars. For example, grains of the early-maturing glutinous Tongil type, 'Hangangchalbyeo', drastically increased in grain weight and starch content for one week, starting from 7 DAF and reaching a maximum at 21 DAF (Fig. 1D). 'Areumbyeo', the non-glutinous Tongil type, also increased dramatically in its weight and starch content for one week, from 14 DAF (Fig. 1C). In contrast, 'Shinsunchalbyeo', a glutinous Japonica type, slowly accumulated starch and gained mass over a longer period, three weeks (Fig. 1B). In general, the high-yield Tongil rice accumulated a larger amount of starch and in a relatively shorter period of time (i.e., one week) than did the Japonica type. Therefore, these differences indicate that the increase in weight was largely due to starch accumulation during grain development and that the starch biosynthetic rate

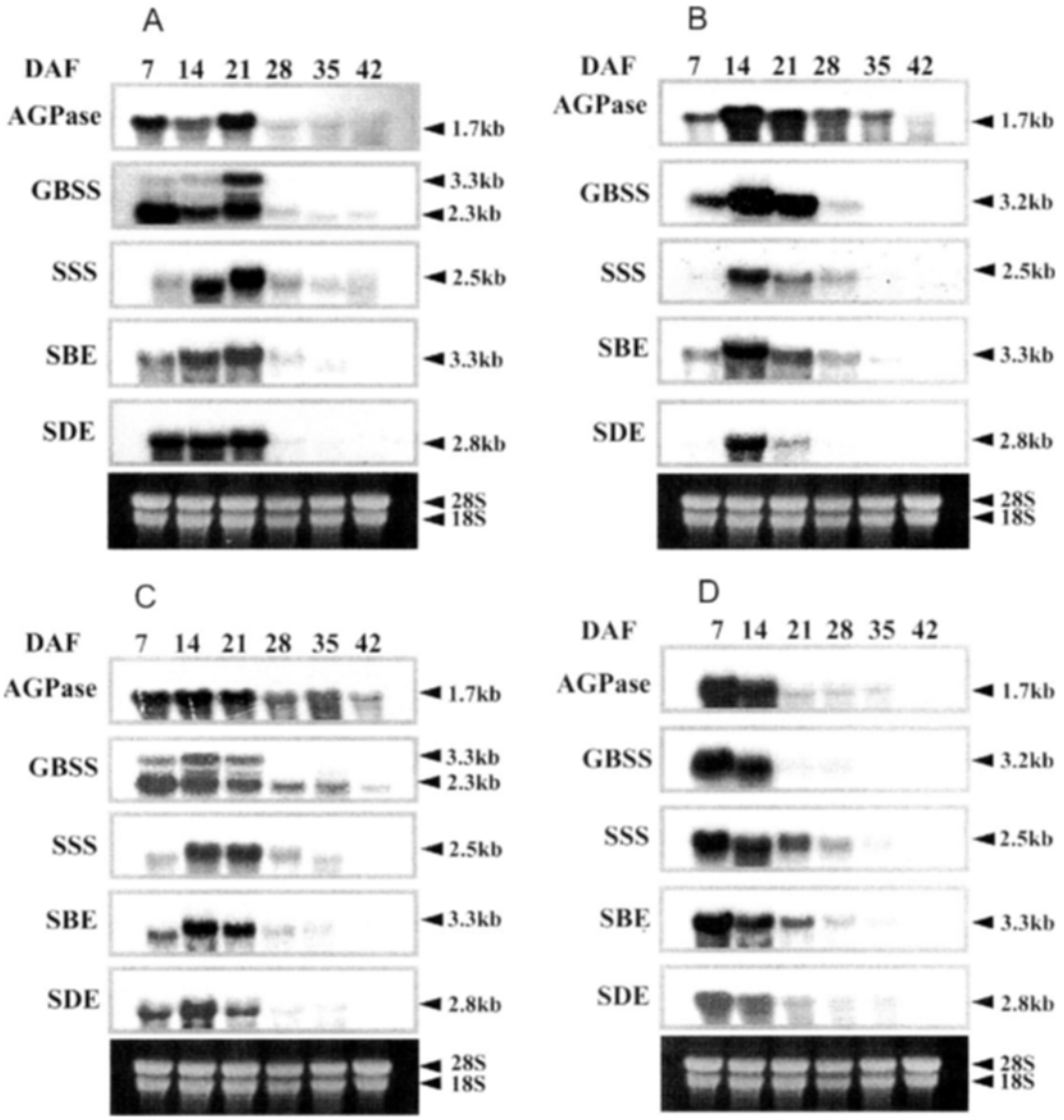

Figure 2. Changes in mRNA levels of starch biosynthetic enzymes during grain development. Total RNAs from developing grains were separated by formaldehyde-agarose gel electrophoresis, transferred onto nylon membranes, and probed by ^{32}P-labeled DNA fragments of ADP-glucose pyrophosphorylase (AGPase), granular-bound starch synthase (GBSS), soluble starch synthase (SSS), starch branching enzyme (SBE), and starch debranching enzyme (SDE). The 'kb' to right of column indicates size of nucleic acid. DAF, days after flowering. Rice varieties: **A)** Ilpumbyeo, **B)** Shinshunchalbyeo, **C)** Areumbyeo, **D)** Hangangchalbyeo.

was higher in the former type than in the latter.

Amylose Content

The percent amylose content from all cultivars remained relatively constant during grain development. Glutinous rice contained 5 to 7% amylose and non-glutinous rice, 15.5 to 18.0% amylose (Fig. 1). However, because the non-glutinous type had a larger amylose fraction per grain, its amylose content drastically increased until 21 DAF, along with the increase in starch content and grain weight, remaining nearly constant thereafter (Fig. 1A and B). In contrast, changes in amylose content for the glutinous rice were relatively small because this type had a smaller amylose fraction per grain (Fig. 1C and D).

Expression of Starch Biosynthetic Genes during Grain Development

We characterized the expression of starch biosynthetic genes in nine rice cultivars with various physicochemical properties. During grain development, those genes were highly expressed at 7 to 21 DAF in all the

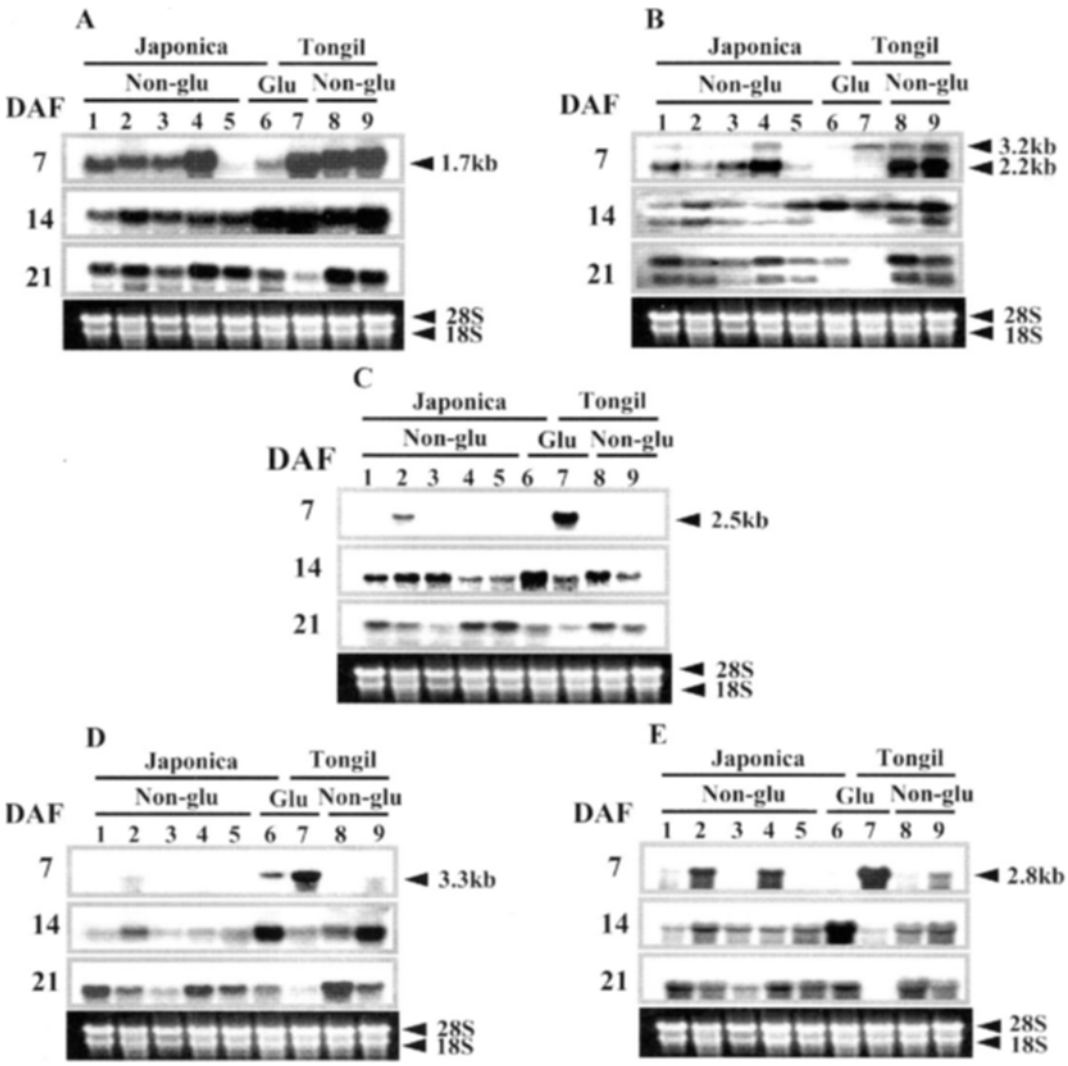

Figure 3. Expression patterns of starch biosynthetic genes. Total RNAs from rice grains harvested at 7, 14, and 21 DAF were separated, transferred, and probed by ^{32}P-labeled DNA probes, which were DNA fragments of **A)** ADP-glucose pyrophosphorylase (AGPase), **B)** granular-bound starch synthase (GBSS), **C)** soluble starch synthase (SSS), **D)** starch branching enzyme (SBE), and **E)** starch debranching enzyme (SDE). Japonica, Japonica-type rice; Tongil, Tongil line of rice; Non-glu, non-glutinous rice; Glu, glutinous rice; DAF, days after flowering; 1, Nagdongbyeo; 2, Dongjinbyeo; 3, Shindongjinbyeo; 4, Ilpumbyeo; 5, Junambyeo; 6, Shinshunchalbyeo; 7, Hangangchalbyeo; 8, Namchunbyeo; 9, Areumbyeo.

cultivars; active expression accompanied large increases in starch accumulation and grain weight (Fig. 2). However, each cultivar also had a characteristic expression pattern, with clear differences between the glutinous and non-glutinous rice, and between the Tongil and the Japonica types (Fig. 2, 3).

AGPase, which supplies ADP-glucose (an activated glucose for starch synthesis), was maximally expressed early in development (7 DAF), except for the late-maturing glutinous cultivar. Shinshunchalbyeo (Fig. 3A). Moreover, the high-yield cultivars, including all the Tongil types plus the Japonica 'Ilpumbyeo', expressed larger amounts of AGPase mRNA. This observation is very similar to that reported by Smidansky et al. (2003), who found that AGPase activity in developing grains is positively correlated with the increase in seed weight per plant and in total plant biomass.

The glucose α-1,4 glucosidic linkage of the amylose and amylopectin backbone is synthesized by GBSS and SSS. Two forms of GBSS mRNA occur, depending on post-transcriptional modification -- a completely spliced, 2.3-kb mature GBSS mRNA and an incompletely spliced, 3.3-kb immature GBSS mRNA (Wang et al., 1995). Because the mature mRNA is active in amylose biosynthesis, we observed a clear difference in the post-transcriptional modification of GBSS mRNA between glutinous and non-glutinous types. At an early stage of grain development, the non-glutinous rice produced the mature, 2.3-kb GBSS kb mRNA as a major component and the immature, 3.3-kb GBSS mRNA as a minor component (Fig. 3B). Up to the middle stage (14 and 21 DAF), an appreciable amount of the immature GBSS mRNA remained in the non-glutinous rice, suggesting that this mRNA might be a reserved source that could be spliced into the active form when required by the cells. In contrast, the glutinous rice contained only small amounts of this immature GBSS mRNA and no mature GBSS mRNA at all (Fig. 3B).

Mature GBSS is responsible for amylose biosynthesis while SSS functions in the elongation of amylopectin branching chains (Dry et al., 1992; Baba et al., 1993). Here, we also showed that SSS mRNA was abundant in the glutinous rice that contains a larger amount of amylopectin (Fig. 3C). The SSS mRNA was maximally expressed at 14 DAF in all our cultivars except the early-maturing 'Hangangchalbyeo', which expressed it one week earlier. This active expression of SSS mRNA also matched the time when starch biosynthesis was very high. In the non-glutinous rice, the SSS mRNA was actively expressed after when the mature GBSS mRNA disappeared (Fig. 3B and C), suggesting that amylopectin is synthesized at a relatively later stage of grain development. Several isoforms of SSS determine the fine structure of amylopectin. For example, a structural difference in amylopectin between Indica and Japonica rices is determined by SSS II_a, an isoform of SSS (Umemoto et al., 1999; Nakamura et al., 2002). Active expression of the AGPase gene closely matched the maximal expression of the mature GBSS or SSS genes. Because van den Koornhuyse et al. (1996) have also noted that the reduction in ADP-glucose supply greatly decreases the amylose content in starch, we believe that our results support the finding that expression levels of mature GBSS and AGPase activities are important factors in determining the amylose content of starch (Lloyd et al., 1999).

The physicochemical properties of starch are determined mainly by amylose content, the structure and distribution of amylopectin in the rice grain (Ong and Blanshard, 1995; Wang et al., 2002). The interplay of SSS, SBE, and SDE determine the fine structure of amylopectin (Preiss, 1991). These genes were expressed in the later stage of grain development (Figs. 3C, D and E), and maturation was closely related to their expression. 'Hangangchalbyeo', which matures relatively earlier than the others, expressed the amylopectin biosynthetic enzymes a week sooner; 'Ilpumbyeo', a later-maturing cultivar, exhibited delayed expression. SBE and SDE were actively expressed at the same time, except for some of the non-glutinous Japonica types, which showed slightly earlier expression for SDE (Fig. 3D and E). SSS, SBE, and SDE mRNA were simultaneously expressed in glutinous rice, which contains a larger amount of amylopectin. This supports the fact that the amylopectin structure is determined by the interplay of these enzymes (Preiss, 1991). All were maximally expressed at 7 DAF in the early-maturing 'Hangangchalbyeo', but at 14 DAF in the later-maturing 'Shinshunchalbyeo' (Figs. 3C, D and E). Although Mizuno et al. (1992) have reported that SBE mRNA is actively expressed at an early stage of rice grain development, our study results suggest that the timing of active SBE expression depends on the maturation process.

SBE was highly expressed in the high-yield Tongil type, both glutinous and non-glutinous, as well as in the glutinous Japonica rice (Fig. 3D). The non-glutinous, Japonica type usually expressed relatively smaller amounts of both SBE and SDE in the late grain-development stage (Fig. 2D and E), except for

the high-yield 'Dongjinbyeo' and 'Ilpumbyeo', which expressed SDE earlier (7 DAF). These results suggest that amylose branching and debranching occur at an earlier stage in high-yield cultivars.

Our study indicates that rice cultivars with different physicochemical properties have characteristic expression patterns for their starch biosynthetic genes. The ultimate structure of amylopectin must be determined by the isoforms of those biosynthetic enzymes. Kubo et al. (1999) have reported that the fine structure of amylopectin is determined by the SDE isoforms of isoamylase and pullulanase, and that the former is the predominant component while the latter is a compensable component in the rice grain. In addition, an isozyme of SDE IIb that catalyzes synthesis of the short-length branching chain of amylopectin plays important roles in determining the structural difference in amylopectin between Indica and Japonica rice (Umemoto et al., 1999), as well as the rheology of starch (Jane et al., 1999). Although the present results provide an overview of expression patterns of the starch biosynthetic genes, further research is warranted to elucidate the expression of each isoform in order to define the correlation between starch biosynthesis and the physicochemical properties of rice grains.

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