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Original Research Article

Effects of Low Temperature during Ripening on Amylose Content and Enzyme Activities Associated with Starch Biosynthesis in Rice Endosperm

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ABSTRACT The objective of this study was to determine the effects of low temperature on starch accumulation in rice grains. We used four major Japonica-type Korean rice cultivars as materials: *Jinbu* (JB), *Junamjosaeng* (JJ), *Geumyoung* (GY), and *Hwawang* (HW). Rice plants were moved into two phytotrons the day after heading. Temperatures in the two phytotrons were maintained at 19/29℃ (night/day) as the control, and 13/23℃ as the low temperature condition, both under natural daylight with a relative humidity of 65%. The ripening rates of JB and JJ showed no significant difference between the low temperature and control conditions at 45 days after heading (DAH). In contrast, the ripening rates of GY and HW were 86% and 57% lower than those of JB and JJ under the low temperature condition at 45 DAH, respectively. However, the ripening rates of these four varieties at 61 DAH (when accumulated temperature reached 1,100℃) under the low temperature condition were similar to those at 45 DAH under the control condition (JB, 94%; JJ, 97%; GY, 97%; HW, 88%). The total starch contents showed no significant difference between the control and low temperature conditions. However, the amylose contents in the cultivars were higher under the low temperature than under the control condition. The enzyme activities of starch biosynthesis were about 5-10 days slower in cultivars under the low temperature than under the control. The grain-filling rate showed significant correlations with the enzyme activities of SuSase (r^2=0.70***), AGPase (r^2=0.63***), UDPase (r^2=0.36***), StSase (r^2=0.51***), and SBE (r^2=0.59***). In conclusion, although StSase activity was increased at 13/23℃ up to 20 DAH, there might not be enough time for SBE to synthesize amylopectin, thus affecting the amylose content of HW, which had the slowest grain filling rate. Notably, the decreased activity of SuSase and SBE and late increase in AGPase activity under the low temperature during the ripening stage are considered to be disadvantageous, as they delay ripening and increase the amylose content.

Keywords : amylose, low temperature, ripening, starch synthesis

Abnormal temperature events due to recent changes in climate have resulted in loss of yield for crops such as rice by decreasing grain size and number. In addition, they inhibit ripening, consequently leading to decreased yield and degraded rice quality (Peng, Huang *et al*., 2004; Yamakawa *et al*., 2010). Up to date, many studies on the effect of abnormally high temperature on crops have been conducted on damage to rice ripening due to abnormal climate phenomenon known as global warming (Kobata *et al*., 2004; Jagadish, Craufurd *et al*., 2007; Ahmed, Tetlow *et al*., 2015; Bahuguna, Solis *et al*., 2017). On the other hand, the effect of abnormally low temperature on crops is mainly studied by variety selection or at early to hiding state. Studies on the effect of abnormally low temperature on rice ripening are insufficient.

Although global warming is defined only by rising annual average temperature, it is very difficult to predict its exact consequences. It might be far more serious in the event of abnormal weather conditions (Yun *et al*., 2001). Considering recent global warming trend, it is of great importance to understand physiological processes that result in the decrease in seed quantity and quality (Lobell *et al*., 2010; Yamakawa *et al*., 2010).

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Starch is a major component of rice grains. It accounts for about 90% of final dry weight of an unpolished grain (Yang *et al*., 2003). Grain filling is the main process of starch biosynthesis and accumulation. It is generally accepted that five enzymes may play a key role in this process: sucrose synthase (SuSase; EC 2.4.1.13), UDP glucose pyrophosphorylase (UDPase; EC 2.7.7.9), ADP glucose pyrophosphorylase (AGPase; EC 2.7.7.27), starch synthase (StSase; EC 2.4.1.21), and starch branching enzyme (SBE; EC 2.4.1.18) (Ahmadi *et al*., 2001; Hurkman *et al*., 2003; Yang, Zhang *et al*., 2004). Starch biosynthesis primarily involves molecules of amylose and amylopectin. Physicochemical properties of rice endosperm are greatly affected by relative proportions of amylose and amylopectin. Amylose content of rice starch is one factor that determines grain quantity (Nishi *et al*., 2001; Ahmed *et al*., 2015). Thus, starch synthesis in rice endosperm during the ripening stage has a close relationship with the yield and quality of rice (Yamakawa *et al*., 2010).

This study was conducted to determine effects of low temperature on starch accumulation in grains using four major Japonica-type Korean rice cultivars: *Jinbu* (JB), *Junamjosaeng* (JJ), *Geumyoung* (GY), and *Hwawang* (HW). Specifically, changes in enzyme activities, which is related to starch biosynthesis and amylose contents in rice plants, according to low temperature during ripening period were determined.

MATERIALS AND METHODS

Plant materials and growth conditions

Rice cultivars used in this experiment were *Jinbu* (JB), *Junamjosaeng* (JJ), *Geumyoung* (GY), and *Hwawang* (HW). Fourteen days after sowing, three seedlings were transplanted to a 1/5000-a Wagner pot. They were cultivated at 19/29℃ (night/day) under natural daylight with a relative humidity (RH) of 65% in a phytotron of National Institute of Crop Science (NICS) until heading.

Heading date was checked in each panicle. On the next day after heading, they were moved half and half into two phytotrons under natural daylight with RH of 65%. Night/day temperatures during ripening were controlled at two different levels: 19/29℃ as control and 13/23℃ as low temperature treatment.

Sampling

Panicles were sampled at 5 days intervals from 5 to 30 days after heading (DAH) and stored at -80℃ prior to analysis. Grains located directly on the second to fourth upper primary branches (except the two grains from apex) were used in this study (Umemoto *et al*., 1995; Jiang, Dian *et al*., 2003).

Ripening rate (%) was calculated after harvesting: once at 45 DAH for 19/29℃ crops when accumulated temperature reached 1100℃, and twice for 13/23℃ crops at 45 DAH and 61 DAH when accumulated temperature reached 1100℃. Subsequently, hulled grains were analyzed for the percentage of green-kerneled rice (%), grain dry weight, total starch content, and amylose content.

Grain weight and grain filling rate

Sampled grains were dried at 70℃ for 72 h to constant weight, dehulled, and weighed for at least 100 grains. Their grain filling and accumulation rates were then calculated using Richard's growth equation (Richards, 1959). Processes of grain filling and grain accumulation in grains were calculated by Richard's (1959) growth equation described by Yiqi, 1988; Yang *et al*., 2003:

$$\mathrm{W} = \frac{A}{(1+Be^{-kt})^{1/N}} \quad (1)$$

Both grain-filling rate and starch accumulation rate (G) were calculated as the derivative of Equation 1:

$$\mathrm{G} = \frac{\mathrm{AK}Be^{-kt}}{N(1+BE^{-KT})^{(N+1)/N}} \quad (2)$$

Where W is the grain/dry weight at time t, A is the final grain/dry weight at harvest (mg), t is the time after heading, and B, k and N are coefficients determined by regression. Active grain-filling duration (days) was defined as days when W was from 5% (t_1) to 90% (t_2) of A. The average grain-filling rate during this period was calculated from t_1 to t_2.

The percentage of ripened grains and grain weight were determined from 30 plants sampled randomly from some pots at maturity.

Starch content and Amylose content in hulled rice

Total starch contents were determined using Megazyme

Total Starch Assay kit (Megazyme International Ireland Limited, Wicklow, Ireland). The method consisted of hydrolyzing starch to glucose by an enzymic procedure. Glucose was measured colorimetrically using glucose oxidase-peroxidase reagent (McCleary *et al*., 1997; AOAC, 2005).

Amylose content was determined by simplified method of Juliano (1985). Briefly, ground rice flour (100 mg) per replicate was put into a test tube. Then 1 ml of rectified spirit and 9 ml of 1 N NaOH were added. The mixture was rigorously shaken and heated in a water bath at 100℃ for 15 min. Its volume was adjusted to 100 ml by adding distilled water. Five ml of this solution was added to a clean 100 ml volumetric flask, to which 1 ml acetic acid and 2 ml I_2-KI (0.2 g iodine + 2.0 g KI dissolved in 100 ml distilled water) were added and the final volume was increased to 100 ml. Its absorbance at 620 nm was then measured. Amylose content (%) was calculated using the standard curve prepared with standard amylose solution (SISCO Research Laboratories Pvt., Mumbai, India).

Extraction and Enzyme assay

Preparation of enzymes

All procedures were performed below 4℃. Endosperms of hulled rice were separated from embryo and pericarp and hand-homogenized with a glass homogenizer in 2 ml of buffer (50 mM HEPES–NaOH (pH 7.4), 4 mM $MgCl_2$, 2 mM EDTA, 50 mM 2-mercaptoethanol and 12.5% (v/v) glycerol). The homogenate was centrifuged at 13,000 x *g* for 10 min. The resulting supernatant was used for the preparation of enzyme (Nakamura *et al*., 1989; Yamanouchi *et al*., 1992; Jiang *et al*., 2003).

Enzyme assays

Sucrose synthase (EC 2.4.1.13)

The assay was conducted in a reaction mixture (140 ㎕) containing 50 mm HEPES-NaOH (pH 7.4), 7.5 mM UDP-glucose, 7.5 mM Fructose, 15 mM $MgCl_2$, and the enzyme preparation. Ten minutes after the start of the reaction at 25℃, the enzyme was deactivated by placing the mixture in a boiling water bath for 2 min. The mixture was then centrifuged at 13,000 x *g* for 5 min at 2℃. The supernatant (100 ㎕) was mixed with a solution of 15 mM HEPES-NaOH (pH 8.0), 0.4 mM phosphoenolpyruvate, 5 mM KCl, 1 mM $MgCl_2$, and 0.1 mM NADH. Enzymatic activity was measured at 340 nm after adding pyruvate kinase (1 ㎕, 2 units) and lactate dehydrogenase (Nakamura *et al*., 1989; Nishi *et al*., 2001).

UDP glucose pyrophosphorylase (EC 2.7.7.9) and ADP glucose pyrophosphorylase (EC 2.7.7.27)

The assay was conducted in a reaction mixture (650 ㎕) containing 100 mM HEPES-NaOH, pH 7.4, 1 mM UDP-glucose, 3 mM Na-PPi, 4.8 mM $MgCl_2$ (for UDPase) or 100 mM HEPES-NaOH, pH 7.4, 3 mM ADP-glucose, 3 mM PPi, 3.7 Mm 3-phosphoglycerate, 5 mM $MgCl_2$, 5 mM DTT (for AGPase), and the enzyme preparation. Twenty minutes after the start of the reaction at 30℃, the enzyme was deactivated by placing the mixture in a boiling water bath for 2 min. The sample was diluted with 350 ㎕ water and then centrifuged at 13,000 x*g* for 5 min. Then 10 ㎕ of 10 mM $NADP^+$ was added to a 500 ㎕ aliquot of the supernatant. Enzymatic activity was measured as increase in absorbance at 340 nm after adding phosphoglucomutase (1 ㎕, 0.4 units) and glucose 6-phosphate dehydrogenase (1 ㎕, 0.35 unit) (1 ㎕, 0.35 unit) (Nakamura *et al*., 1989; Abe *et al*., 2014).

Starch synthase (EC 2.4.1.21)

This assay was conducted in a reaction mixture (280 ㎕) containing 50 mM HEPES–NaOH (pH 7.4), 1.6 mM ADP-glucose, 0.7 mg amylopectin, 15 mM DTT, and enzyme preparation. Twenty minutes after the start of the reaction at 30℃, the enzyme was deactivated by placing the mixture in a boiling water bath for 2 min. A solution (100 ㎕) of 50 mM HEPES–NaOH (pH 7.4), 4 mM PEP, 200 mM KCl, 10 mM $MgCl_2$, and pyruvate kinase (1.2 units) was added to the mixture and incubated at 30℃ for 30 min. The resulting solution was placed in a boiling water bath for 2 min and then centrifuged at 13,000 g for 5 min. The supernatant (300 ul) was mixed with a solution (200 ul) of 50 mM HEPES–NaOH (pH 7.4), 10 mM glucose, 20 mM $MgCl_2$, and 2 mM NADP. Enzymatic activity was measured as increase in absorbance at 340 nm after adding hexokinase (1 ㎕, 1.4 units) and glucose 6-phosphate dehydrogenase (1 ㎕, 0.35 unit) (Nakamura *et al*., 1989; Nishi, Nakamura *et al*., 2001).

Branching enzyme (EC 2.4.1.18)

The assay was conducted in a reaction mixture (200 ㎕) containing 50 mM HEPES–NaOH (pH 7.4), 50 mM glucose-

1-P, 2.5 mM AMP, phosphorylase a from rabbit muscle (1.2 units), and the enzyme preparation. Thirty minutes after the start of the reaction at 30℃, the enzyme was deactivated by adding 1 M HCl (50 ㎕). Subsequently, the solution was mixed with dimethylsulfoxide (500 ㎕) and iodine solution (700 ㎕, 0.1% I_2 and 1% KI). Enzymatic activity was measured as increase in absorbance at 540 nm (Nakamura *et al*., 1989; Yamanouchi *et al*., 1992; Sun *et al*., 2013).

To calculate specific activity of each enzyme, protein contents were determined by using Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as standard for quantification.

Statistical analysis

The experiment was arranged in a completely randomized design (CRD) with four replicates. All collected data were subjected to analysis of variance using Statistical Analysis System (SAS 9.1). Treatment means were compared using Duncan's Multiple Range Test or t-test ($\alpha = 0.05$).

RESULTS

Ripening rate (%)

Results of ripening rate (%) are shown in Table 1. At 45 DAH, ripening rates of the four cultivars at control condition (19/29℃) were 90% or higher (JB 96%, JJ 94%, GY 95%, HW 90%). For cultivars at low temperature condition (13/23℃), JB and JJ Showed ripening rates of 93% and 90%, respectively, similar to ripening rates of cultivars at 19/29℃ at 45 DAH. In contrast, GY and HW showed lower ripening rates (86% and 57%, respectively), compared to those under control condition.

However, when cultivars at low temperature (13/23℃) during ripening were harvested at 61 DAH (when accumulated temperature reached 1100℃), the difference in ripening rates compared to the 4 cultivars under control condition (19/29℃) harvested at 45 DAH was not significant (JB 94%, JJ 97%, GY 97%, HW 88%).

Grain weight and filling rate

Grain weight accumulation (GWA) and grain-filling rates affected by low temperature during ripening period are shown in Fig. 1 GWA of the 4 cultivars at control condition (19/29℃) increased rapidly during ripening up to 20 DAH. At 30 DAH, GWA reached 86%, 89%, 89%, and 92% against the final grain weight of JB, JJ, GY, and HW, respectively. It was eventually completed at 45 DAH (A and B). Results of grain filling rate showed the same pattern (C and D).

However, GWA of the 4 cultivars at low temperature (13/23℃) during ripening increased gradually to 78%, 79%,

Table 1. Ripening rate (%) according to harvest season with 19/29℃ as the control and 13/23℃ as the low temperature condition

Ripening temperature	Harvest season (after heading)	Variety†			
		Jinbu	*Junamjosaeng*	*Geumyoung*	*Hwawang*
19/29℃	45 days	96^{a*}	94^{ab}	95^{a}	90^{a}
13/23℃	45 days	93^{a}	90^{b}	86^{b}	57^{b}
	61 days	94^{a}	97^{a}	97^{a}	88^{a}

*Different letters for the same variety indicate a significant difference in means at α = 0.05 by Duncan's multiple range test.

Table 2. Percentage (%) of green-kerneled rice affected by low temperature during ripening (night/day temperatures were maintained at the control or low temperature)

Ripening temperature	Harvest season (after heading)	*Jinbu*	*Junamjosaeng*	*Geumyoung*	*Hwawang*
19/29℃	45 days	0.0^{c}	0.0^{c}	0.0^{c}	0.0^{b}
13/23℃	45 days	8.4^{a*}	9.4^{a}	15.3^{a}	26.3^{a}
	61 days	4.5^{b}	6.5^{b}	8.8^{b}	24.4^{a}

*Different letters for the same variety according to harvest season indicate significant difference in means at α = 0.05 by Duncan's multiple range test.

77%, and 67% of the final grain weight of JB, JJ, GY, and HW at 30 DAH, respectively. GWA results of JB, JJ, and HW at 45 DAH were over 99% of their final grain weight whereas HW showed slow weight accumulation, reaching only 90%. GWA for all 4 cultivars were completed at 61 DAH.

Results of percentage of green-kerneled rice harvested as a measure of incomplete seeds are shown in Table 2. There were no green-kerneled rice for cultivars under 19/29℃ at 45 DAH. However, percentages of the green-kerneled rice under 13/23℃ at 45 DAH were 8.4% in JB, 9.4% in JJ, 15.3% in GY, and 26.3% in HW. Although GWA was complete for GY, the percentage of green-kerneled rice in GY was higher compared to that in JB or JJ. In addition, the percentage of green-kerneled rice in HW, the slowest in grain accumulation, was notably the highest among the four cultivars (Table 2). The percentage of green-kerneled rice in HW (24.4%) harvested at 61 DAH was also the highest of the four cultivars.

Total starch and amylose contents

Total starch contents of these cultivars at 19/29℃ as a control condition harvested at 45 DAH were 19.0 mg/brown rice for JB and JJ, 17.7 mg/brown rice for GY, and 17.1 mg/brown rice for HW. The difference in total starch content of cultivars between control (19/29℃) and low temperature conditions (13/23℃) was not obvious (18.6 mg/brown rice for JB, 18.7 for JJ, 17.1 for GY and 17.1 for HW at 13/23℃) (Fig. 2A). On the other hand, amylose contents were higher in cultivars at low temperature (13/23℃) compared to those at control condition (19/29℃). At 45 DAH, amylose contents for cultivars at control condition (19/29℃) were 3.6 mg/brown

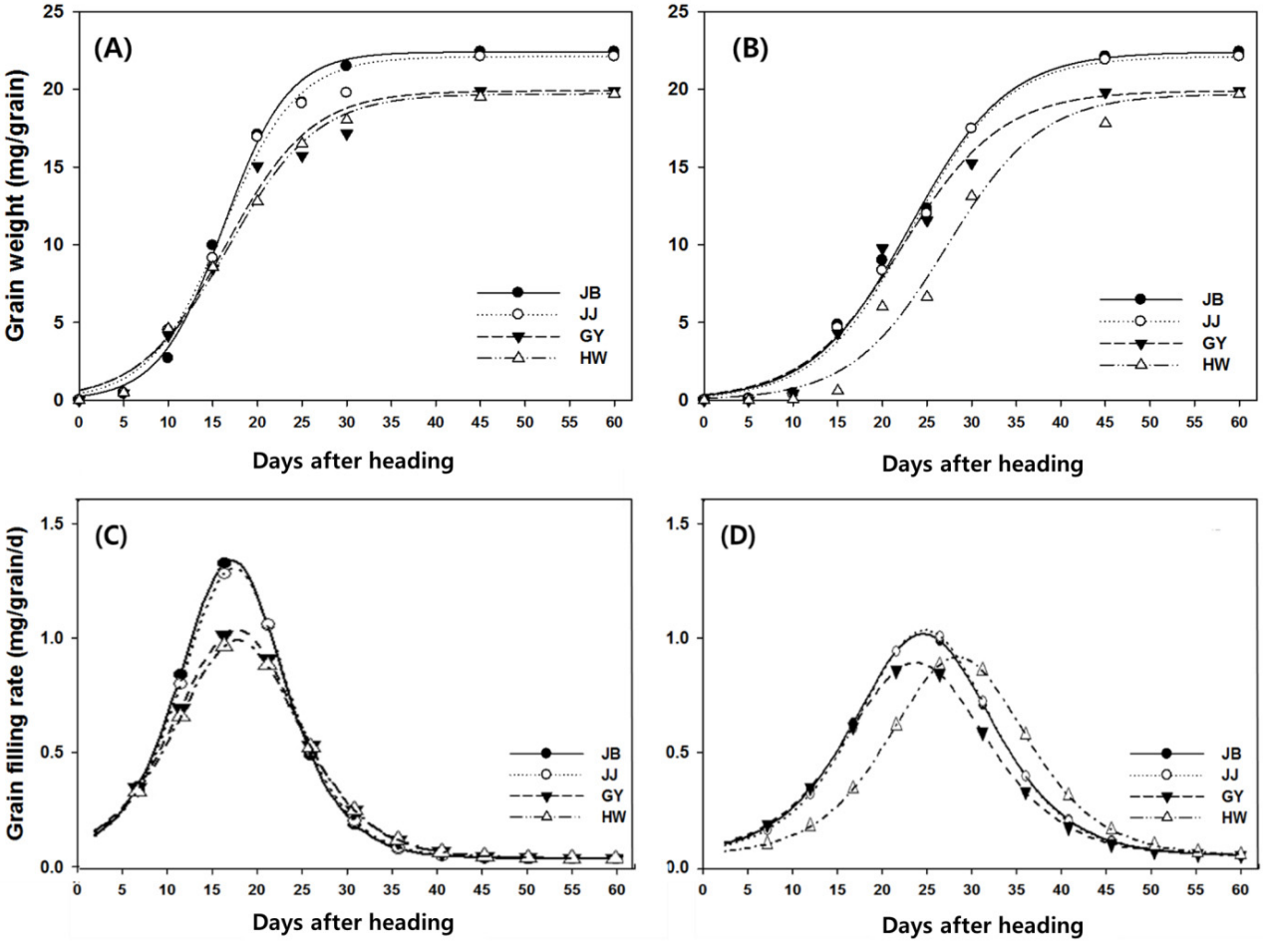
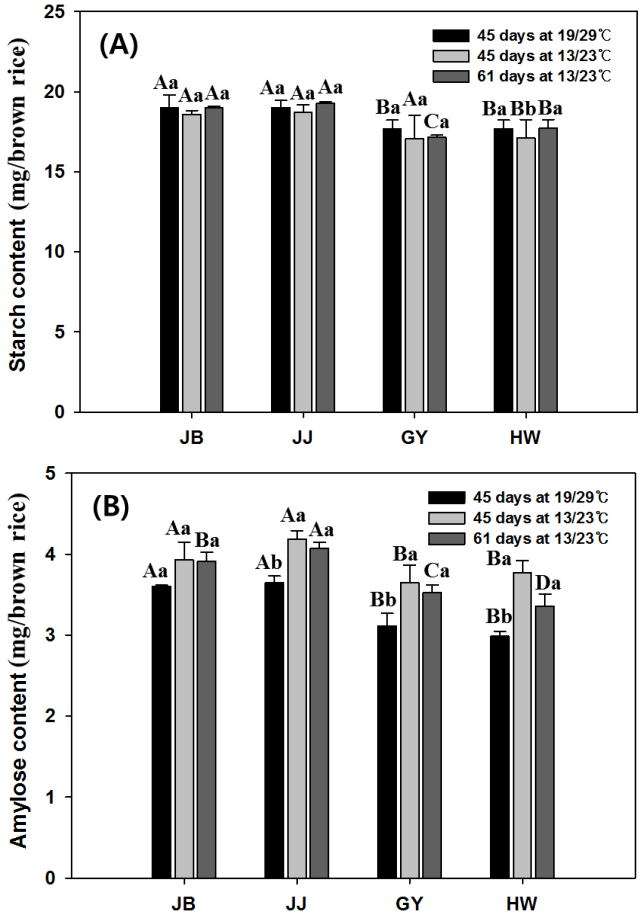


Fig. 1. Changes in grain weight (A, B) and grain filling rate (C, D) of rice affected by low temperature during ripening. Night/day temperatures were maintained at 19/29℃ (A, C) as the control or 13/23℃ (B, D) as the low temperature condition. *Jinbu*, JB; *Junamjosaeng*, JJ; *Geumyoung*, GY; *Hwawang*, HW.

rice for JB and JJ, 3.1 mg/brown rice for GY, and 3.0 mg/brown rice for HW, lower than all 4 cultivars at a low temperature (13/23℃) (JB 3.9 mg/brown rice, JJ 4.2 mg/brown rice, GY 3.7 mg/brown rice, HW 3.8 mg/brown rice). This represented an increase of 9% in JB, 15% in JJ, 17% in GY, and 26% in HW compared to control (19/29℃). At 61 DAH under low temperature (13/23℃), there was no change in amylose content of JB compared to that at 45 DAH. However, amylose contents of other cultivars except JB were increased 12%, 13%, and 13% respectively (JJ 4.1 mg/brown rice, GY 3.5 mg/brown rice, and HW 3.4 mg/brown rice). Thus, the increase rate of amylose content under low temperature was lower at 61 DAH than at 45 DAH.



*Different capital letters in the same treatment according to temperature and small letters for the same variety indicate (for vertical comparison) significant difference in means at α = 0.05 by Duncan's multiple range test.

Fig. 2. Starch (A) and amylose (B) contents (mg/hulled rice) in hulled rice grain affected by low temperature during ripening. Night/day temperatures were maintained at 19/29℃ (A, C) or 13/23℃ (B, D). *Jinbu*, JB; *Junamjosaeng*, JJ; *Geumyoung*, GY; *Hwawang*, HW.

Change in starch biosynthesis

Developmental patterns of enzymes related to starch biosynthesis during ripening stage are presented in Fig. 3 and 4 SuSase activity in cultivars at 19/29℃ as a control condition was increased rapidly over time. It was the highest at 15 DAH (JB 38.9, JJ 35.4, GY 39.3, HW 33.1 U/mg protein). In contrast, SuSase activity at a low temperature (13/23℃) was increased gradually, reaching its peak at 20 DAH, about 5 days later compared to control condition at 19/29℃. SuSase activity in both temperature conditions was decreased from its peak until 30 DAH during the ripening stage. At 13/23℃ (low temperature) during ripening, SuSase activity was decreased rapidly from 25 DAH after reaching its peak at 20 DAH, representing a decrease of 6.2% in JB, 15.9% in JJ, 16.5% in GY, and 18.9% in HW.

UDPase activity showed the same pattern as SuSase activity. It was the highest at 10 DAH for cultivars at control condition (19/29℃) (JB 25.8, JJ 29.7, GY 23.8, HW 29.7 U/mg protein). UDPase activities of JJ, GY, and HW were decreased rapidly after 10 DAH. For JB, its UDPase activity maintained at a high level up to 15 DAH (25.8 U/mg protein). For cultivars at low temperature (13/23℃) during ripening, UDPase activity was the highest at 15 DAH (JB 22.6, JJ 23.9, GY 21.7, HW 26.7 U/mg protein), but decreased rapidly thereafter up to 30 DAH. However, similar to cultivars at 19/29℃, UDPase activity of JB maintained at a high level up to 20 DAH (23.0 U/mg protein).

AGPase in these cultivars at control condition (19/29℃) also showed the highest activity at 10 DAH (JB 4.7, JJ 4.7, GY 4.8, HW 4.5 U/mg protein), after which its activity was decreased gradually. For JB and JJ, AGPase activity maintained at a high level up to 30 DAH (JB 4.9, JJ 4.1, GY 3.2, HW 3.3 U/mg protein).

For cultivars at a low temperature (13/23℃) during ripening, AGPase activities in JB, JJ, GY, and HW at 5 DAH were 0.8, 0.7, 0.8, and 0.8 U/mg protein respectively. Among these 4 cultivars, AGPase activities in JB and JJ were increased rapidly by about 5.5~5.7 times (4.4 and 4.1 U/mg protein) from 5 DAH. On the other hand, AGPase activities in GY and HW were increased gradually. Notably, AGPase activity of

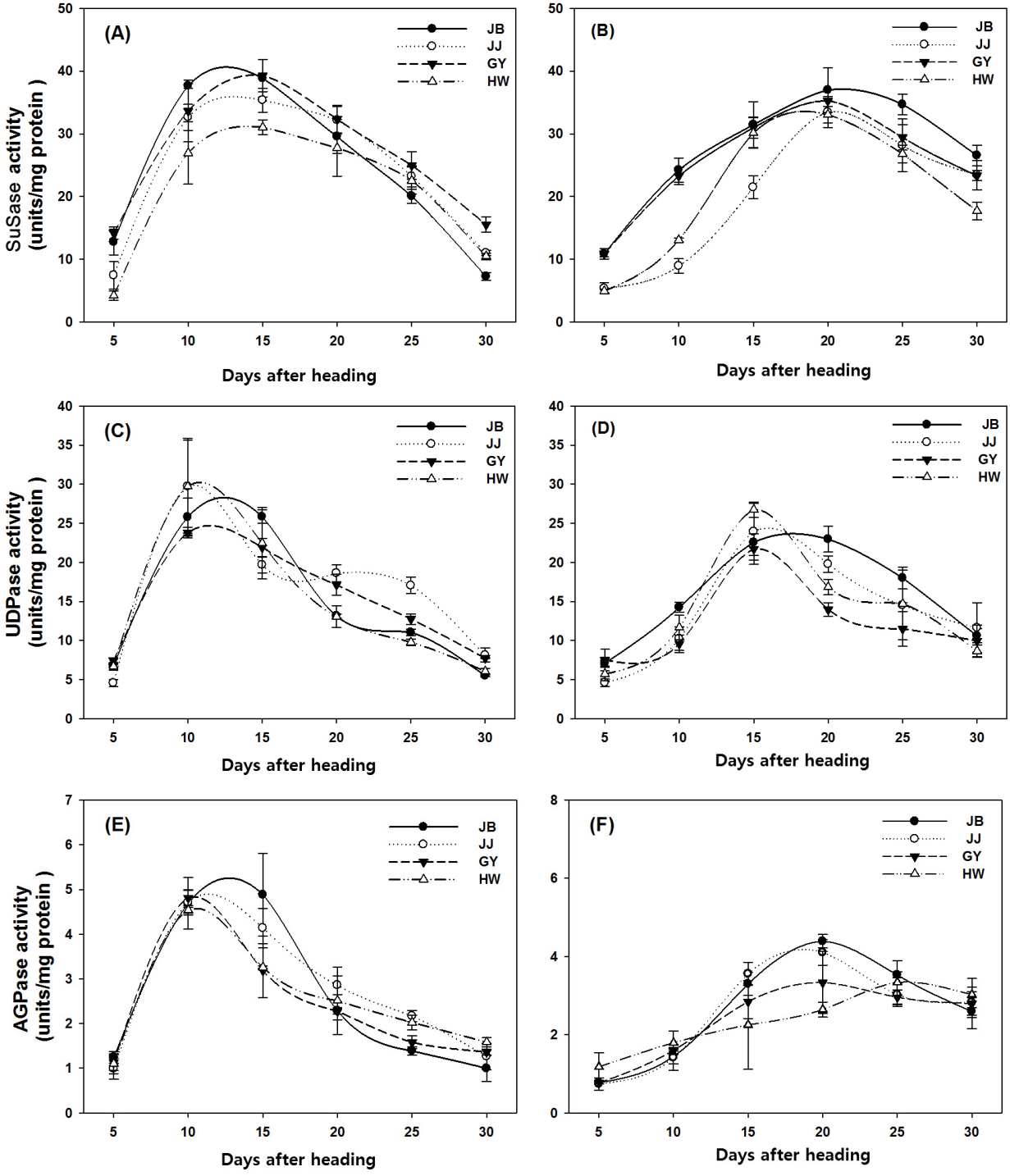


Fig. 3. Changes in activities of sucrose synthase (SuSase), UDP-glucose pyrophosphorylase (UDPase), and ADP-glucose pyrophosphorylase (AGPase) in rice endosperm at 19/29℃ (A, C, and E) as the control and 13/23℃ (B, D and F) as the low temperature condition during ripening. *Jinbu*, JB; *Junamjosaeng*, JJ; *Geumyoung*, GY; *Hwawang*, HW.

HW was the highest at 25 DAH. It showed the slowest rate of increase (rate of increase: GY 4.3 times, HW 2.8 times).

StSase activities of the four cultivars at control condition (19/29℃) were increased rapidly to their highest level at 10 DAH (JB 4.2, JJ 4.0, GY 4.0, HW 4.1 U/mg protein), after which their activities decreased gradually up to 30 DAH (Fig. 4).

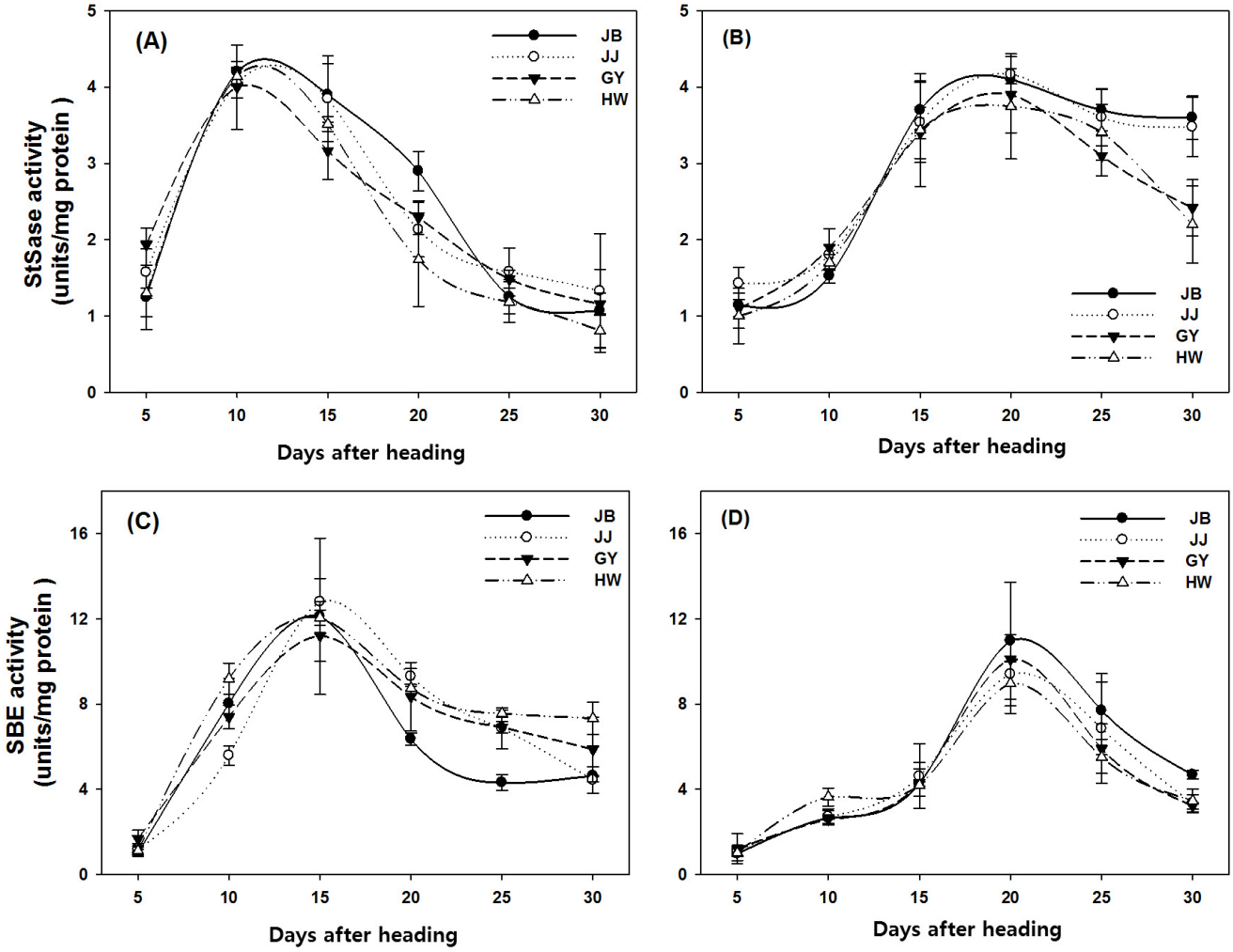


Fig. 4. Changes in activities of starch synthase (StSase) and starch branching enzyme (SBE) in rice endosperm under conditions of 19/29℃ (A, C and E) as the control and 13/23℃ (B, D and F) as the low temperature condition during ripening. *Jinbu*, JB; *Junamjosaeng*, JJ; *Geumyoung*, GY; *Hwawang*, HW.

For cultivars at 13/23℃, StSase activity was increased rapidly from 5 to 15 DAH, reaching the highest level at 20 DAH (JB 4.1, JJ 4.2, GY 3.9, HW 3.7 U/mg protein). Thereafter, its activity was decreased gradually up to 30 DAH.

SBE activity was the highest at 15 DAH for cultivars at control condition (19/29℃) (JB 12.1, JJ 10.8, GY 11.2, HW 12.1 U/mg protein) and at 20 DAH for cultivars at 13/23℃ (JB 11.0, JJ 9.4, GY 10.1, HW 9.0 U/mg protein). It was lower for the 4 cultivars at a low temperature (13/23℃) during ripening compared to that at control condition (19/29℃). Rates of decrease in SBE activity were 10.9% in JB, 8.9% in JJ and GY, and 20.5% in HW.

Analysis results on the correlation between grain-filling rate and enzyme activities in starch biosynthesis are shown in Fig. 5 and 6 Grain-filling rate showed highly significant correlations with enzyme activities of SuSase (r^2=0.70***), AGPase (r^2=0.63***), UDPase (r^2=0.36***), StSase (r^2=0.51***), and SBE (r^2=0.59***). Among these enzymes, SuSase activity had the highest correlation coefficient with grain-filling rate.

DISCUSSION

This research was performed to analyze the effect of low temperature on starch and amylose contents in rice grain. The difference in ripening rate of JB or JJ between 19/29℃ and 13/23℃ was not obvious. However, ripening rates of GY and

HW were lower at 13/23℃ compared to those at 19/29℃. HW had a lower ripening rate than GY. These results were closely related to the fact that under control ripening temperature (19/29℃) condition, all tested varieties exhibited the highest grain filling rate at 16 DAH which was extended to 23 DAH (JB, JJ, and GY) or 27 DAH (HW) under low temperature (13/23℃) condition.

Starch contents showed little difference between low temperature and control conditions. However, amylose contents were higher at low temperature (13/23℃) compared to those at control condition (19/29℃). This suggests that during grain development phase, low temperature has a profound impact on starch accumulation and composition, but not on starch quantity (Umemoto, *et al*., 1995; Ahmed *et al*., 2015). Many studies have reported that temperature differences during the ripening stage have a significant effect on the ratio of amylose and amylopectin as well as starch accumulation (Keeling *et al*., 1993; Matsue, 1995; Jiang, Dian *et al*.,. 2003; Ahmed *et al*., 2015). Therefore, investigation was carried out to confirm

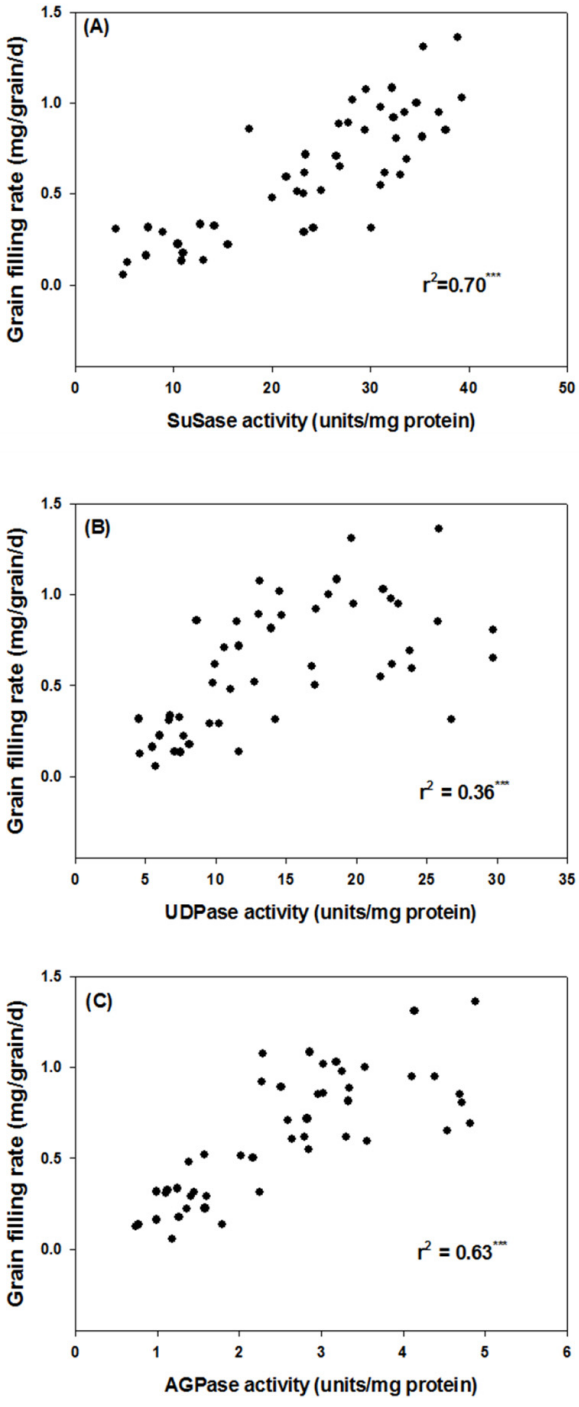


Fig. 5. Relationship of grain filling rate with activities of sucrose synthase (SuSase), UDP-glucose pyrophosphorylase (UDPase), and ADP-glucose pyrophosphorylase (AGPase).

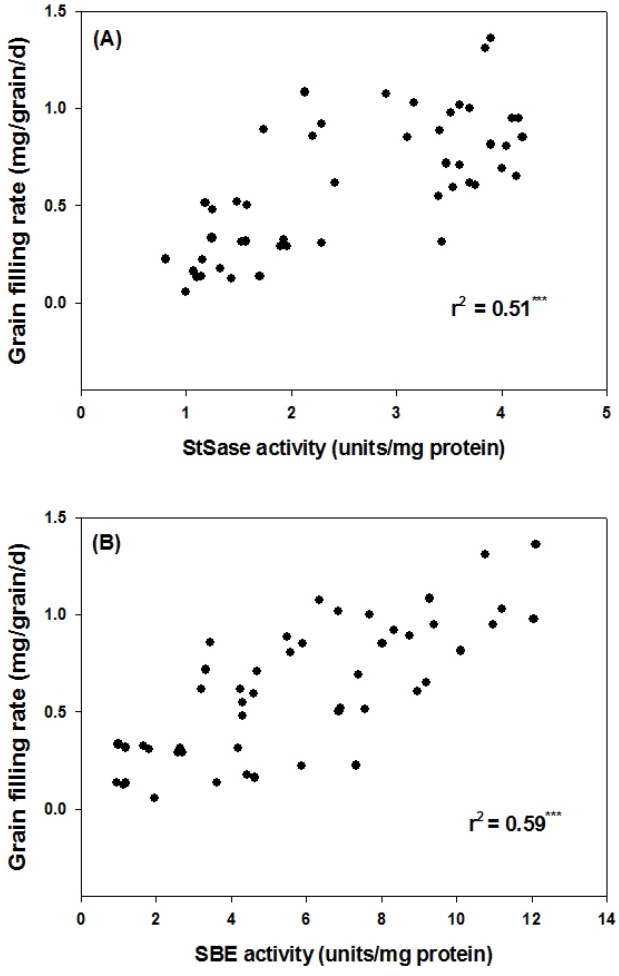


Fig. 6. Relationship of grain filling rate with activities of starch synthase (StSase) and starch branching enzyme (SBE).

the correlation between grain filling rate and amylose content in starch biosynthesis.

Enzyme activities of starch biosynthesis were about 5~10 days slower in cultivars at low temperature (13/23℃) compared to those at control condition (19/29℃). Initially, SuSase showed the highest activity at 20 DAH at low temperature (13/23℃). After which, it decreased in the order of JB < JJ < GY < HW. Notably, SuSase activity had the highest correlation coefficient with GWA rate ($r^2 = 0.70^{***}$).

In sucrose-to-starch conversion, the formation of UDP-glucose and fructose is believed to be the first phase of the process. This happens when SuSase catalyses the main transported form of assimilates in wheat plants which is a cleavage of sucrose. Furthermore, in the developing of rice, this process is linked to its sink strength (Yang *et al*., 2003; Yang *et al*., 2004; Schmölzer *et al*., 2016). Therefore, maintaining high enzyme activity or slowly decrease with low temperature condition can affect energy metabolism which controls the ripening speed.

Although UGPase plays an important role in sucrose synthesis/breakdown, it is often overlooked compared to other enzymes involved in sucrose metabolism. Conditions resulting in phosphorus deficiency in *Arabidopsi*s and low temperature in potato tubers have a strong up-regulating effect on UGP expression and UGPase activity/protein (Gupta *et al*., 2003; Kleczkowski *et al*., 2004). In addition, UGP expression and UGPase activity/protein are down-regulated by drought and flooding conditions (Ciereszko *et al*., 2001), suggesting that UGPase is closely related to stress and essentially involved in sucrose metabolism (Kleczkowski *et al*.,. 2004). Our results also suggest that it will be advantageous to maintain high activity of UDPase at 10~15 DAH under low temperature condition such as at 13/23℃.

AGPase had a high correlation ($r^2=0.63^{***}$) with GWA rate. It showed rapid increase in activity in JB and JJ, resulting in relatively fast grain filling up to 20 DAH. In contrast, GY and HW showed slow increase in AGPase activity which resulted in relatively slow grain filling up to 20 DAH. The activity of AGPase in HW was the slowest among the 4 cultivars. Consequently, HW was the slowest in grain filling.

Research efforts on AGPase activity have achieved increased starch contents in crops such as rice, wheat, corn and potatoes (Hendriks *et al*.,. 2003; Ballicora *et al*., 2004; Kim *et al*., 2010). In starch biosynthesis, ATP and glucose-1-phosphate are used to control AGPase activity which produces ADP-glucose as a precursor of starch synthesis. If AGPase activity increases, starch synthesis speed known as the "rate-limiting step" will increase (Smith, 2008). Thus, volume and rate of AGPase activity might be closely related to improved starch biosynthesis during ripening stage. In addition, amylose content was increased in all four cultivars at low temperature during ripening stage with rate of increase as follows: JB < JJ < GY < HW. The higher the amylose content, the higher the GWA rate. However, when the harvesting time was 1100℃ (61 DAH), amylose content of all four cultivars was increased without significant difference between cultivars. This result is consistent with previous reported result showing that amylose content is increased at low temperature during ripening stage (Umemoto *et al*., 1995; Ahmed *et al*., 2015)

StSase catalyzes chain-elongation reaction of α-1,4-glucosidic linkage by transferring a glucose moiety from ADP-glucose to the non-reducing end of the linkage in plants (Nakamura, 2002). SBE is the only enzyme that catalyzes the production of α-1,6-linked branches on already synthesized starch. It is key to the ratio of amylose and amylopectin as it can catalyze the formation of branch points by breaking α-1,4-glycosidic bonds in the starch and creating α-1,6-glycosidic branches within linear α-1,4 segments (Tian *et al*., 2016). Amylose content in rice endosperm is also decided by ambient temperature at the early days during ripening stage (Asaoka, *et al*., 1985; Ahmed *et al*., 2015). StSase activity was increased while SBE activity was decreased in all four cultivars from 5 to 15 DAH at 19/29℃ and from 5~20 DAH at low temperature (13/23℃). As a result, although StSase activity was increased at low temperature (13/23℃) up to 20 DAH, there might not be enough time for SBE to synthesize amylopectin, thus affecting amylose content of HW which was the slowest in grain filling. The increase in amylose content at low temperature was different according to grain filling rate (harvesting at 45 DAH). However, there was no significant difference in amylose content among cultivars at 61 DAH when accumulated temperature reached 1100℃. In this regard, further studies on amylopectin synthesis by amylose content and SBE activity are needed.

In conclusion, the activity of enzymes such as SuSase,

UDPas, AGPase, StSase, SBE in starch biosynthesis is proven to be highly related to grain filling process. Notably, decrease in activity of SuSase and SBE and late increase in AGPase activity at low temperature in the ripening stage are considered to be disadvantageous as they delay ripening and increase amylose content.

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