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EFFECT OF HIGH TEMPERATURE ON THE ENZYMATIC ACTIVITIES AND TRANSCRIPTIONAL EXPRESSION OF STARCH DEBRANCHING ENZYME (DBE) MUTIPLE ISOFORMS IN DEVELOPING RICE ENDOSPERMS

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ABSTRACT

This study investigated the influence of high temperature on the expression patterns of multiple DBE isoform genes (PUL, ISA1, ISA2 and ISA3) at RNA transcriptional level in developing endosperm, as well as its relation to the varying total activity of DBE and starch fine structure. Two rice genotypes with different amylose content and palatability were used for research under two temperature treatments (32°C and 22°C for the mean daily temperature, respectively) at filling stage. The temperature treatments were laid out in a completely randomized design. The data were analyzed using SPSS into one-way ANOVA, and means separation was carried out with the LSD method The result showed that high temperature resulted in the decrease of DP5-9 and DP15-22, and increase of DP10-13 and DP>42 of amylopectin in rice endosperm. For enzyme activity, PUL and ISA showed higher activities at initial stage, but substantially lower at later filling stage relative to low temperature. For different DBE isoform genes, ISA1 and ISA2 presented up-regulation pattern at high temperature over the whole sampling stage, while ISA3 and PUL showed the opposite case at the later filling stage in comparison of their corresponding low temperature. The DBEs isozyme activities and their transcriptional expression indicated that ISA2 was one of the dominant DBE isoforms highly expressed at later filling stage. The contribution of ISA3 expression to total ISA activity was mostly at earlier filling stage when rice plants were subjected to high temperature. Further investigations are required to elucidate the coordinated action of multiple key enzymes in starch synthesis at high temperature and their relations to amylopectin fine structure and rice quality.

Key words: Rice (*Oryza sativa* L.), Starch debranching enzyme, Gene expression, Enzyme activity, High temperature

INTRODUCTION

Rice is one of the world's most important crops, particularly in Asia. However, its grain yield and quality often fluctuate due to various environmental stresses (Jia *et al.,* 2008; Quampah *et al.,* 2011). Temperature during reproductive period is one of the crucial factors influencing rice yield and grain quality. In particular, global warming is predicted to increase the frequency and severity of high temperature in tropical and subtropical regions (Qureshi and Ali, 2011) where rice is grown. It will have negative impact on rice productivity by causing yield reduction and grain quality deterioration (Peng *et al.,* 2004; Li *et al.,* 2011; Turner *et al.,* 2011). It has been well documented that grain quality is dependent on both environment and genotype (Tashiro *et al.,* 1991; Akhter *et al.,* 2008), high chalk occurrence in rice grains is mainly attributable to adverse climatic conditions, especially daily mean temperature or episodes of high temperature at filling stage (Prasad *et al*., 2006; Chakrabarti *et al.,* 2010). Another conspicuous injury of high temperature stress during reproductive period is the reduction of pollen fertility, grain weight and plant harvests, in addition to the deteriorating palatability (Jiang *et al.,* 2003; Yamakawa *et al.,* 2007; Li *et al.* 2011). In the past decades, great effort has been made to recognize the effect of high temperature on grain quality, and it has been demonstrated that the deterioration of grain quality for rice exposed to high temperature is closely related to starch granule accumulation, starch components and its biosynthesis metabolism in filling endosperms (Zakaria *et al.* 2002). The rice genotypes with low amylose content and superior palatability generally was more sensitive to high temperature episodes in their starch quality than those with high amylase content and inferior palatability (Cheng *et al*., 2005). High temperature at filling stage could decreases amylose content (AC) in endosperm starches of non-waxy rice plants, as a result of decreased activity of GBSS (Granule bound starch synthase), where Wx gene expression in rice grains is regulated by temperature at the transcriptional and post-transcriptional level (Asaoka *et al*., 1985; Hirano and Sano, 1998; Larkin and Park, 1999). Meanwhile, high temperature at filling stage could also increase the amount of long B chains of amylopectin and decrease short B chains (Asaoka *et al.,* 1985), which is more dominantly attributable to the changing activities of soluble starch synthase (SSS), starch branching

enzyme (SBE) and starch debranching enzyme (DBE) in filling endosperms, rather than the limitation in sucrose supply from photosynthesis tissues and its cleavage metabolism in non-photosynthesis tissues.

Starch debranching enzyme (DBE) is a key enzyme involving in starch metabolism in rice endosperm (James *et al.,* 2003; Jeon *et al.,* 2010). It can hydrolyze the α-1,6-glucosic linkages of polyglucans and play an important role in the starch metabolism, including starch degradation, carbohydrate mobilization, amylopectin synthesis and the structural organization of stored glucan polymers in starch granules. Burton *et al.* (2002) suggested that the synthesis of the correct branching pattern of amylopectin synthesis requires the DBE activity and the glucans are elongated and branched to form phytoglycogen in DBE deficient mutants. DBE in rice endosperm could be classified as isoamylase (ISA, EC: 3.2.1.68) and pullulanase (PUL, also called limit-dextrinase or R-enzyme, EC: 3.2.1.142) in term of their amino acid sequences and the substrate specificities catalyzed enzymatically sites. ISA mainly hydrolyze phytoglycogen and amylopectin, while PUL acts upon pullulan and amylopectin (Zhu *et al.,* 1998; Fujita *et al.,* 2009). Until now, it has been commonly recognized that there are three ISA isoform genes (*ISA1*, *ISA2*, *ISA3*) encoding isoamylase in rice endosperm (Nakamura *et al.,* 1996; Fujita *et al.,* 1999; Zeeman *et al.,* 2010), with only a single *PUL* gene encoding pullulanase identified in cereal plant (Dinges *et al.,* 2003; Hussain *et al.,* 2003; Li *et al.,* 2009). Interestingly, it was generally accepted that the isozymes of ISA1 and ISA2 are mainly responsible for amylopectin synthesis, while the primary role of PUL and ISA3 appears to be mostly in starch degradation except for their involvements in phytoglycogen and amylopectin synthesis (Jeon *et al.,* 2010; Fujita *et al.,* 2009).

Although extensive studies were conducted on the effect of temperature on activities of the enzymes related to starch synthesis metabolism in rice and other cereals, and also focused on the relationship between the lacks of DBE isozyme genes in some mutants and the underlying mechanisms of starch structure formation at the molecular levels, few researches have been done to illustrate the expression response of different DBE isozyme genes, including *ISA1*, *ISA2*, *ISA3* and *PUL*, to ambient high temperature at filing stage and their relations to starch quality of rice grains, with little information on the relationships between the changing activities of different DBE, including pullulanase and isoamylase, in rice filling grain under high temperature and rice genotypes varying in amylose content and palatability.

In this study, two early non-waxy *indica* rice genotypes, J935 with superior palatability and J353 with inferior palatability, were compared for difference in the expression patterns of *PUL* and three *ISA* isoform genes (*ISA1*, *ISA2*, *ISA3*) at RNA transcriptional level in the developing endosperm under different temperatures. Activities of pullulanase and isoamylase in developing endosperm, and starch fine structure in milled grains were also measured with the rice plant grown in the well defined temperature condition. Our aim was to reveal the effect of high temperature on the expression patterns of multiple *DBE* isoform genes (*PUL, ISA1*, *ISA2* and *ISA3*) at RNA transcriptional level in developing endosperm, and their relations to varying total activity of DBE and starch fine structure between two rice genotypes.

MATERIALS AND METHODS

Experimental Materials: Two *indica* rice genotypes with similar growth period and grain weight, namely, J935 and J353, were planted in the experimental field of Zhejiang University, Hangzhou, China. Seeds were sown on 29th March and transplanted on 28th April, 2010. Plants were managed normally in paddy field until late booting stage; then 50 plants with uniform size were selected and transplanted into plastic pots (2 plants per pot) filled with paddy soil. The pots were placed in a greenhouse. At full heading stage, 20-30 panicles with uniformity anthesis day were randomly selected and tagged. The pots were moved into phytotrons (Model PGV-36, CONVIRON, Canada) set at different temperatures. The experimental design was completely randomized, and the treatments were replicated three times. From 5th to 20th day after flowering, the tagged panicles were sampled at a 5-day interval. Grain samples were prepared for the analysis of DBE enzyme activities and RNA transcriptional expression of their isoform genes in rice endosperm. At maturity, the other labeled panicles were harvested simultaneously for measuring starch fine structure.

Two temperature treatments were imposed, daily mean temperature 32°C (HT, high temperature treatment,) and 22°C (LT, optimum temperature putatively for good palatability of *indica* rice), respectively. The diurnal change of daily mean temperature in two phytotrons was designed as shown in Figure 1. The maximum and minimum of daily temperatures were 36ºC and 28ºC in HT and 26ºC and 18ºC in LH, respectively. All the climate conditions were identical in two phytotrons except for temperature, the photoperiod was from 5:30 a.m. to 7:00 p.m. with 100 to 120 J m^{-2} s^{-1} of light intensity, and the relative humidity was maintained around 75-80%.

Preparation and Assay of Enzyme Activities: Twenty de-hulled rice grains were hand-homogenized at 0-4ºC in a mortar and pestle with 5 mL pre-cold extraction buffer containing 50 mmol L^{-1} imidazole-HCl (pH 7.4), 8 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} 2-mercaptoethanol and 12.5% (v/v) glycerol. The homogenate was centrifuged at 4ºC

with 15 000×g for 15 min，then the supernatant solution was collected for enzyme analysis.

The activities of isoamlase and pullulanase were measured according to the procedure of Nakamura *et al.* (1996) with some minor modifications. For isoamylase, 50 μL Hepes-NaOH (pH 7.0) buffer containing 2.5 mg amylopectin (Sigma) was added to 50 μL supernatant solution, then the reaction mixture was run for 2 h at 37°C and terminated by adding 50 μL of 1 mol L^{-1} Na_2CO_3, DNS (3,5-Dinitrosalicylic acid) method was used to determine maltose concentration. For pullulanase, the reaction buffer was substituted by 50 μL citric acid buffer (pH 5.5) and 5 mg pullulan (Sigma), the follow-up steps was the same as isoamylase except for using maltotriose as DNS standard. All samples were conducted by 3~4 duplications.

RNA Extraction and cDNA Preparation: Total RNA of grain samples was extracted with the RNeasy plant mini kit (Qiagen) according to manufacture's instructions. Twenty grains were ground in liquid-nitrogen after removing hull and embryo. The extracts were treated with RNase-free DNase(Qiagen) to completely remove contaminating genomic DNA, then the purified RNAs were measured with formaldehyde denatured agarose electrophoresis and spectrophotometer scan. First-strand cDNA was prepared by a synthesizing reaction of 3 μg total RNA with RevertAid First Strand cDNA Synthesis Kit (Fermentas Company) and Oligo-dT18 primer.

Real-time Fluorescence Quantitative PCR: Aliquots of the first-stand cDNA mixtures corresponding to 5 ng of total RNA served as the templates for quantitative Real-time PCR analysis with EvaGreen™ qPCR Master Mix reagent Kit (Roche Company, Germany). Reactions were carried out on an iQ icycler (BioRad) according to the manufactures protocols. The gene-specific primer pairs used for quantitative PCR are listed in Table-1. To verify the specificity of each primer set and optimize PCR conditions of the annealing temperature and PCR efficiency, the fluorescence signal specificity of PCR amplification was detected for each primer pairs and their melting curve (from 55°C to 94°C) was examined prior to the experimental measurements. The amplification of *Actin* gene was performed as a control and a standard curve was checked. Three replications were conducted for each sample.

Measurement of Total Starch, Amylose Content and Fine Structure of Amylopectin: Total starch contents in rice endosperm were determined according to McCleary *et al.* (1994). Apparent amylose content determined according to Umemoto *et al.* (1995) through measuring 30 de-hulled rice grains. Triplicate measurements were performed for each sample.

The chain length distributions of amylopectin after debranching with isoamylase were determined as previously described (O'Shea *et al.,* 1998; Fujita *et al.,* 2001). Two replicate measurements were performed for each sample.

Data Analysis: Statistical analysis was performed using a statistical software version SPSS 15.0. Determined values were submitted to one-way ANOVA and means separation was carried out with the LSD method.

RESULTS

Starch Accumulation and Amylopectin Structure: As shown in Figure 2, temperature treatments during grain filling had a considerable influence on the total starch accumulation and apparent amylose content in rice endosperms, with high temperature treatment exhibiting lower accumulation of total starch in rice endosperms than low temperature treatment, irrespective of rice genotypes (Figure 2A). However, there was a marked difference in the response of apparent amylose content to high temperature between two rice genotypes, J935 showed lower apparent amylose content at high temperature than at low temperature, while J353 was just opposite (Figure 2B), which was consistent with previous report from Zhong *et al.* (2005). They reported that the influence of high temperature on the ratio of amylose to total starch (RATS) in milled grains was genotype-dependent; the cultivars with low amylose content generally had the reduced RATS under high temperature, while the cultivars with high amylose content increased or kept stable in their RATS under high temperature.

Temperature at filling stage also exerted a remarkable impact on the percentage distribution of different chain-length fractions of amylopectin, thereby affecting the fine structure of starch granules in rice endosperms (Yamakawa *et al.,* 2007). High temperature caused notable decrease of DP5-9 and DP15-22, but significant increase of DP10-13 and DP> 42, irrespective of the genotypes with different palatability (Figure 3). However, the effect of high temperature on the chain-length fraction of DP25-36 was genotype-dependent, the chains-length with DP25-29 increased slightly for J353, but decreased obviously for J935 when the plants were exposed to high temperature. These results confirmed that the genotypic difference in their response of starch quality to high temperature was closely related to the amylopectin structure and their chain-length distribution, in addition to the effect of high temperature on their apparent amylose content.

Activities of Pullulanase (PUL) and Isoamylase (ISA): The total activity of DBE in rice endosperms has previously been reported to be the coordinating result of pullulanase (PUL) and isoamylase (ISA) (Nakamura *et al.,* 1996; Jeon *et al.,* 2010). As shown in Figure 4, the

PUL activity was much higher at high temperature than that at low temperature during early grain filling, while it was contrast or not the case later (Figure 4A and 4B). Hence, PUL activities of both J935 and J353 at high temperature reached their peak levels at 10 day after flowering, and tended to decline thereafter. At late grain filling stage, the PUL activity was significant higher at low temperature than those at high temperature, and two rice genotypes did not show apparent difference in the response pattern of PUL activity to temperature.

The effect of high temperature on ISA activity was similar to that of PUL, despite a marked difference in the extent of temperature effect and their temporal patterns during filling stage (Figure 4C and 4D). Around 5th after flowering, there was higher ISA activity at high temperature than that at low temperature. Afterward, the ISA activity remained stable or slight decline at high temperature, but gradually increased at low temperature, thereby the remarkable difference in ISA activity was also observed between two temperature treatments at 15th or 20th after flowering, with significant lower ISA activity at high temperature relative to low temperature. For J935, the decreasing ISA activity in high temperature occurred sharply at around 10th day after flowering, corresponding to mostly rapid stage of grain filling. In contrast, J353 showed small difference between two temperature treatments at 10th and 15th after flowering, but the extent of their difference in ISA activity was subsequently enlarged afterward. This result implied that the effect of high temperature on the temporal patterns of ISA activity may be responsible for two genotypic differences in their chain-length distribution and genotype-dependent to high temperature.

Expression of Different DBE Isozyme Genes: High temperature had an apparent impact on their transcriptional expression of DBE isozyme genes, including three *ISA* genes (*ISA1, ISA2* and *ISA3*) and the sole *PUL* gene, in developing endosperm, despite of the influencing pattern and extent being dependent on different gene isoforms and rice genotypes (Figure 5 and 6). The expression levels of *ISA1* and *ISA2* were substantially higher at high temperature than those at low temperature over the whole sampling period, indicting that *ISA1* and *ISA2* genes obviously exhibited an up-regulation expression pattern at their RNA transcriptional levels when rice plants grew at high temperature (Figure 5A-D). On the other hand, the distinct expression pattern was observed for the responses of *ISA3* and *PUL* to high temperature, with higher levels of transcriptional expression induced at the earlier filling stage and lower expression at the later filling stage relative to low temperature (Figures 5E-F and 6A-B). Moreover, the expression of *ISA1* in J353 was detectable at very low level for both high temperature and low temperature treatment, while the expression level of *ISA1* in J935 increased drastically when rice plants were exposed at high temperature treatment (Figure 5A and 5B), implying that the expression level of *ISA1* in J935 was more sensitive to high treatment than that in J353.

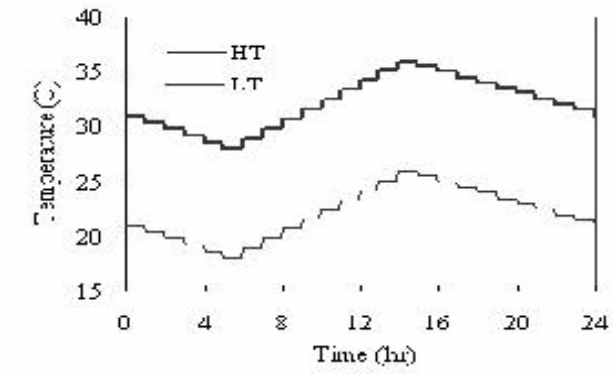

Figure 1: Diurnal regimes of two temperature treatments in phytotrons HT, High temperature regime with the daily mean temperature at 32°C; LT, Low temperature regime with the daily mean temperature at 22°C.

Table-1: The primers sequence of 4 DBE isoforms involving in starch biosynthesis pathway in rice grains

Gene name	Accession No.	Primer pairs [a]	Product size/ bp	Annealing temperature/°C
Actin	XM_469569	F: 5'-CAGCACATTCCAGCAGATGT R: 5'- TAGGCCGGTTGAAAACTTTG	198	58
ISA1	AB093426	F: 5'- TGCTCAGCTACTCCTCCATCATC R: 5'- AGGACCGCACAACTTCAACATA	132	59
ISA2	AC132483.2	F: 5'-CAGTGAGTGCTGCCTTGC R: 5'-TTGGGATTAGATTCGGTTG	106	57
ISA3	AP005574	F: 5'-ACAGCTTGAGACACTGGGTTGAG R: 5'-GCATCAAGAGGACAACCATCTG	100	58
PUL	D50602	F: 5'-AGTGACATTGAGCAAAGGGTTC R: 5'-CACTTCGTGTGGACAGACATTG	168	58

Note: [a.] F = Forward primer, and R = Reverse primer.

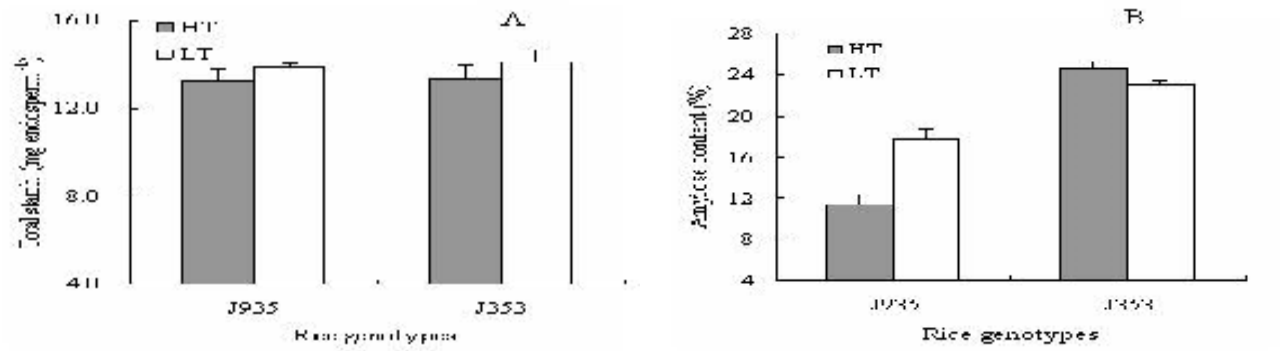

Figure 2: Effect of high temperature on total starch and amylase content in rice endosperm at filling stage. A, Difference in total starch content; B, Difference in amylase content.

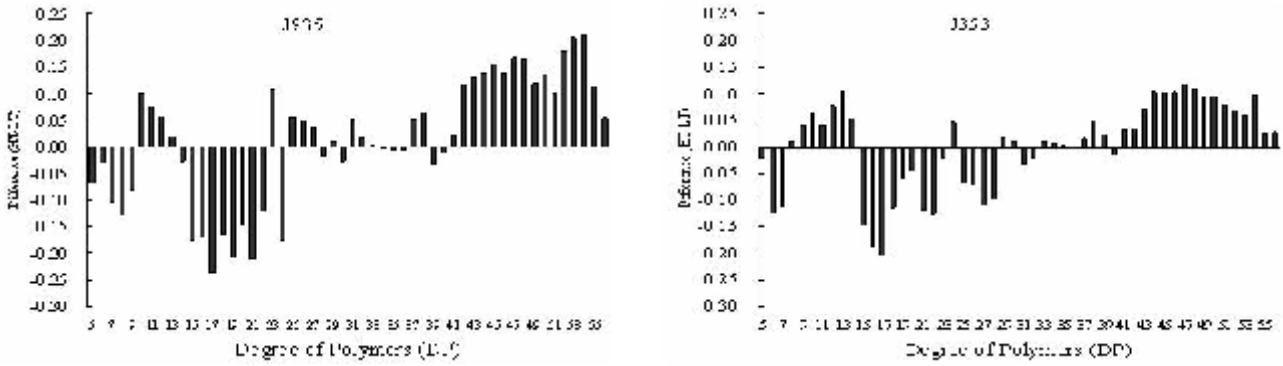

Figure 3: Difference in the chain length distribution of amylopectin in rice endosperm under two temperature treatments.

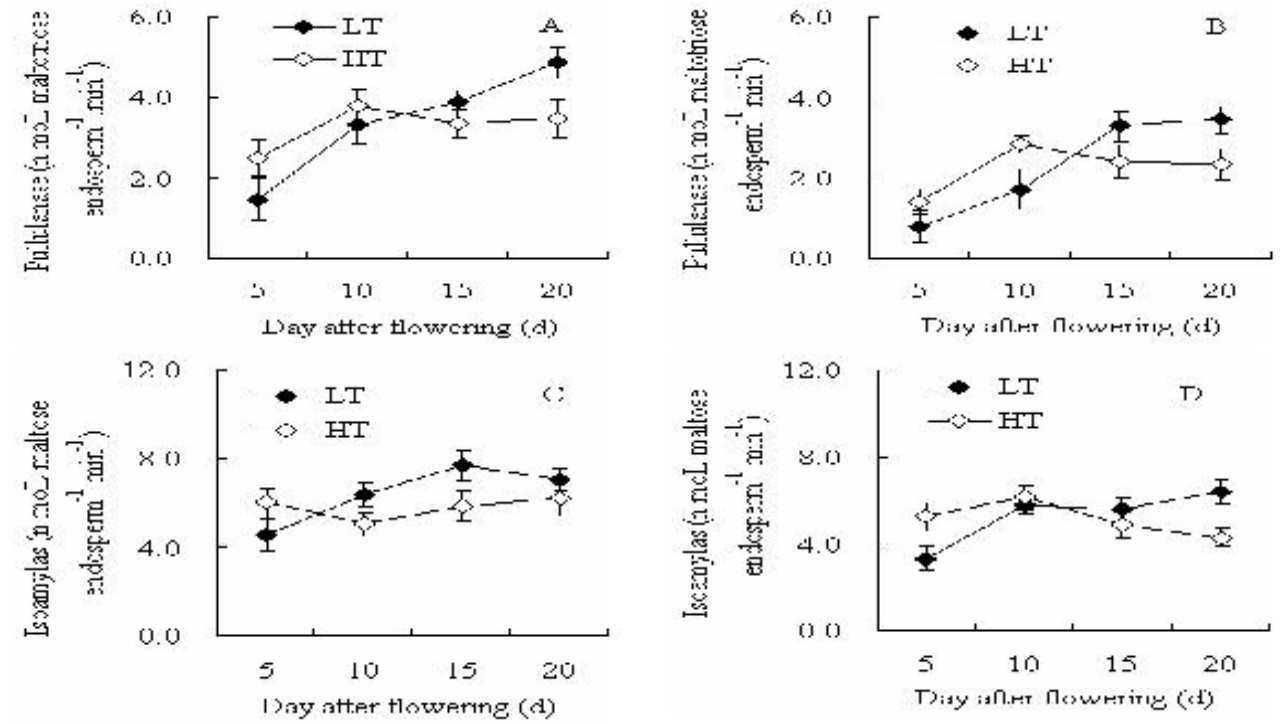

Figure 4: Effect of high temperature on pullulanase and isoamylase activities in rice endosperm at filling stage. A, Pullulanase activity in J935 endosperm; B, Pullulanase activity in J353 endosperm; C, Isoamylase activity in J935 endosperm; D, Isoamylase activity in J353 endosperm.

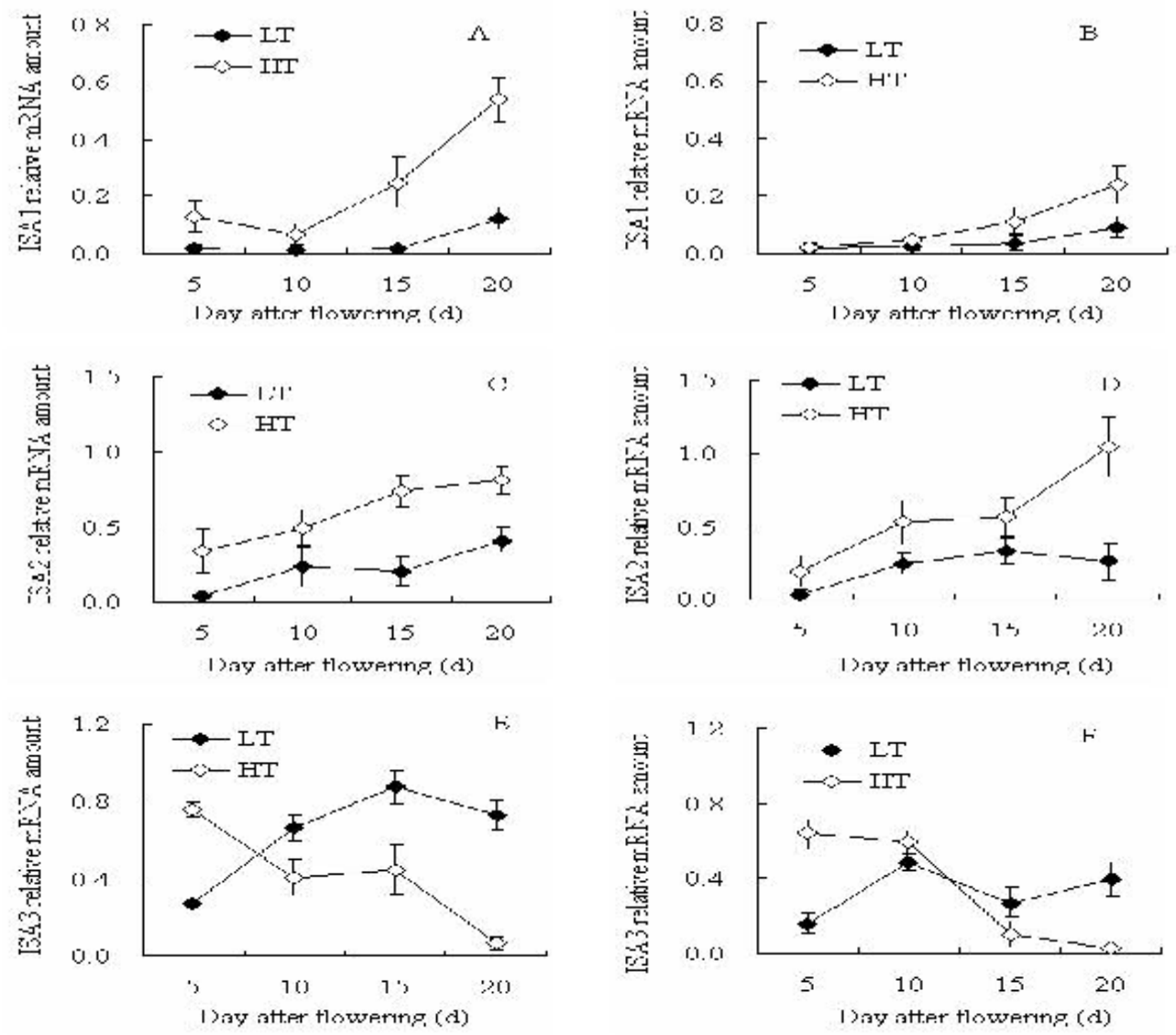

Figure 5: Difference in the relative expression amount of the three *ISA* isoform genes in rice endosperm under two temperature treatments at filling stage. A, C, E: Difference of expression pattern of *ISA* isoform genes in J935; B, D, F: Difference of expression pattern of *ISA* isoform genes in J353.

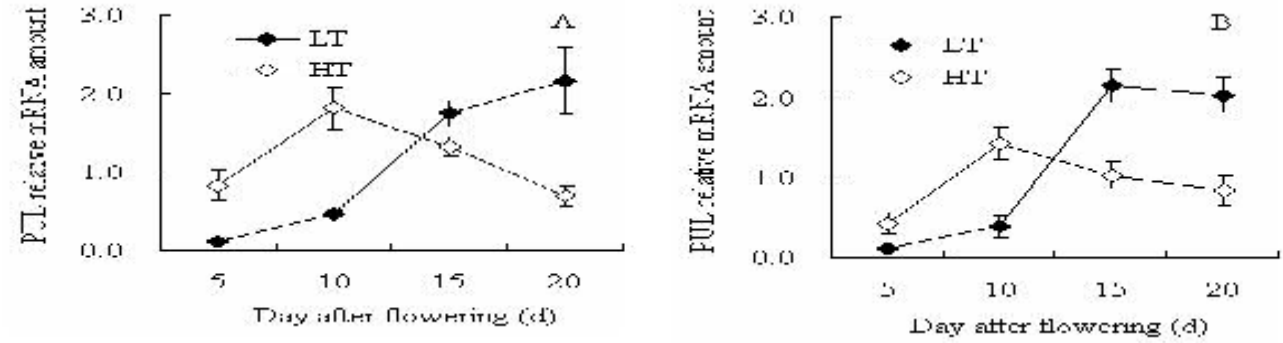

Figure 6: Difference in the relative expression amount of *PUL* in rice endosperm under two temperature treatments at filling stage. A, Difference of expression pattern of *PUL* in J935; B, Difference of expression pattern of *PUL* in J353.

From Figure 5, it could be also found that the expression levels of *ISA2* and *ISA3* generally were much higher than that of *ISA1* in rice endosperms, particularly at early filling stage, indicting that the contribution of individual *ISA1* expression to total isoamylase activity might be relatively trivial in early filling endosperms, particularly for J353. On the other hand, there were a considerable difference in the temporal-pattern between *ISA2* and *ISA3* in term of their expressions during filling period. For *ISA2*, the lowest expression level generally occurred at earlier filling stage and tented to increase thereafter, while the opposite was true for *ISA3* at high temperature, irrespective of rice genotypes. Therefore, it could be presumed that *ISA2* was one of dominant *DBE* isoforms highly expressed at later filling stage, while the contribution of *ISA3* expression to total ISA activity was mostly at earlier filling stage when rice plants were subjected to high temperature.

DISCUSSION

It has been well reported that higher temperature during grain filling stage results in the decreased starch (Keeling *et al.,* 1993; Umemoto *et al.,* 1995) and as well as starch components and fine structure of amylopectin chain in the endosperms of non-waxy rice (Takeda and Sasaki, 1988; Jiang *et al.,* 2003). Higher environmental temperature could cause the increase long B chains (58<DP<64) and intermediate B chains (23<DP<58), and also the decrease of short chains (short B chains and A chains, CL<22) in amylopectin distribution (Asaoka *et al.,* 1985; Cheng *et al.,* 2005). However, it was found that the effect of high temperature on AAC (apparent amylose content) was genotype-dependent (Normita *et al.,* 1989; Zhong *et al.,* 2005). In the current study, we found a distinct reduction of total starch in rice endosperm under high temperature relative to the control (low temperature), but the extent of the difference between the two temperature treatments was not as great as that of apparent amylose content for both genotypes, with much larger difference also displayed in J935 than J353, which was agreement with our previous report (Cheng *et al.,* 2005). The present results also showed that high temperature resulted in the decrease of DP5-9 and DP15-22, but the increase of DP10-13 and DP>42 at high temperature with the short and intermediate (DP<58) chains being further focused, However, the effect of high temperature on the chain-length fraction of DP25-36 varied greatly with genotype. These results confirmed that the genotypic difference in their response of starch quality to high temperature was closely related to the amylopectin structure and their chain-length distribution, in addition to its difference in apparent amylose content.

In the past, the metabolic role of debranching enzymes was initially thought to be the degradation of amylopectin in storage starch during germination, or in transitory starch. However, it had been extensively demonstrated that starch debranching enzymes (DBE) also played essential roles on amylopectin biosynthesis by mutations of deficient isoamylase or pullulanase in many species (Nakamura *et al.,* 1996; Jeon *et al.,* 2010). This is because the DBE can trim and remove the misplaced, loosely branched chains to allow starch biosynthesis normally and the formation of crystalline amylopectin (Kubo *et al.,* 1999; Fujita *et al.,* 2009). According to Nakamura *et al*. (1996) and Kawagoe *et al.* (2005), the activity of isoamylase-type DBE was specifically reduced in rice *sugary* mutants, and the number and size of starch granule were also altered in endosperms. The *ISA* deficient mutant in *Arabidopsis* caused an 80% of decrease in the starch content and the accumulation of water-soluble polysaccharides, In addition, accompanied by a strong modification of amylopectin structure relative to its wild type (Wattebled *et al*., 2005). Moreover, it was suggested that the role of isoamylase partially overlapped with Pullulanase for the trimming of pre-amylopectin chains during starch synthesis (Fujita *et al.,* 2009). In our present study, the temporal patterns of both isoamylase-type and pullulanase-type DBEs in grain filling process as affected by high temperature were investigated at their enzymatical activity and RNA transcriptional levels, the results revealed that the effect of high temperature to ISA activity was similar to that of PUL, with the highest activities of PUL and ISA at later filling stage, which was consistent with the results of Umemoto *et al.* (1995) and Cheng *et al.* (2005), who concluded that the peak activity of DBE, without taking DBE-type distinction into consideration, was relatively later than those of AGPase and SSS. In addition, Smrcka and Szarek (1986) described that the reduction of starch accumulation in cereal grain at high temperature was attributable to the increased starch degradation and the decreased starch synthesis. Stahl *et al.* (2004) clarified that the deficient PUL and ISA activity in barley endosperm could cause a reduction in the small (B-type) granules, and also an alteration of starch content and amylopectin chain-length distribution. Our present results indicted that high temperature resulted in higher activities of PUL and ISA at initial stage, but substantially lower at later filling stage relative to low temperature, implying that the decline of total starch content at high temperature was mostly caused by the decreased starch synthesis, rather than by the increased starch degradation, due to much higher PUL and ISA activities detected for low temperature treatment, although PUL and ISA also played crucial roles in catalyzing starch degradation.

In this study, high temperature was found to influence the expression of DBE genes at their transcriptional levels, despite of the impacting pattern being dependent on different isoforms. For example, higher expression amount and largely up-regulated

pattern at high temperature was observed for *ISA1* and *ISA2* over the whole sampling stage, while the opposite was true for *ISA3* and *PUL* at the later filling stage in comparison of their corresponding low temperature. According to Utsumi *et al.* (2006), *ISA1* could form a homomultimer and heteromultimer complex with *ISA2* in developing rice endosperm. Some experiments on the mutants of deficient *ISA1* and transgenic plant materials clarified that *ISA1* participated in the form of amylopectin chain of DP≤12, with the increasing distribution of DP≤12 chains and the decreasing percentage of DP >40 chains in *sugary* mutants (Kubo *et al.*, 1999; Kubo *et al.*, 2005; Fujita *et al.*, 2009). In our present study, The response of *ISA1* and *ISA2* to high temperature were very similar but different from those of ISA3 and PUL, with much higher expressions of *ISA1* and *ISA2* at high temperature than those at low temperature (Figure 5A-D), implying an coordinate impact pattern between *ISA1* and *ISA2*. On the other hand, it was found that high temperature at filling stage significantly increased the percentage of DP>42 amylopectin chains and apparently decreased the distribution of DP5-9 chains (Figure 3). Therefore, it could be deduced that high expression amount and largely up-regulated pattern of *ISA1* and *ISA2* at high temperature were responsible for the increasing DP>42 and decreasing DP5-9 distribution when rice plants exposed at high treatment. However, the effect of high temperature on *ISA1* expression was rice genotype dependent, the expression levels of *ISA1* drastically increased at high temperature for J935 (Figure 5A), while it was not this case for J353 with slight difference and low level in *ISA1* expression between two temperature treatments (Figure 5B), implying that the response of *ISA1* expression at high temperature probably was closely related to their difference in grain quality and palatability to high temperature sensitivity among different rice genotypes.

It should be noted that DBE is not the sole enzyme controlling the chain length distribution and fine structure of amylopectin, although it plays a crucial role on the normal amylopectin biosynthesis and formation of crystallizing starch granules, in addition to the degradation of amylopectin (Nakamura *et al.,* 1996; Jeon *et al.,* 2010) . Soluble starch synthases (SSS), starch branching enzymes (SBE) were also responsible for starch biosynthesis and controlling its chain length fine structure of amylopectin (Mizuno *et al.,* 2001; Yandeau-Nelson *et al.,* 2011). Fujita *et al.* (2006) reported that the degree of change in amylopectin chain-length distribution was significantly correlated with the extent of decrease in SSSI activity in the mutants, which suggested that SSSI affects the starch structure (Fujita *et al.*, 2006). In *Zea mays*, the loss of SSIIa increases the amount of amylose and increases the proportion of short glucan side chains in amylopectin and decreases intermediate chain length in amylopectin (Zhang *et al.*, 2004). Satoh (2003) isolated a starch mutant that was deficient in SBE1 from the endosperm mutant and indicated that the genetic modification of amylopectin fine structure is responsible for changes in amylopectin chain-length distribution. James *et al.* (2003) and Tetlow (2006) concluded that amylose content in cereal was mostly controlled by granule-bound starch synthase (GBSS), whereas amylopectin generally was synthesized by the coordinated actions of soluble starch synthases (SS), branching enzymes (BE), and debranching enzymes (DBE). Moreover, the different DBE isoforms might interact together or was partially substituted in catalyzing role on starch biosynthesis metabolism also exalted the complexity to recognize the relationship between DBEs metabolism and grain starch quality as affected by high temperature. For this reason, it was difficulty to explain the causal effect of high temperature on starch content and amylopectin structure, including the variations of DP15-23 and DP 25-36 amylopectin, and the genotype-dependent of amylose content between two temperature, by DBEs isozyme activities and their transcriptional expression presented in our current result. Therefore, further investigation is required to elucidate the coordinated actions of multiple DBEs, SBEs and SSSs isoforms at high temperature and their relations to amylopectin structure, so as to take genetic and agronomical efforts for the improvement of grain quality and palatability to resistance or tolerance high temperature.

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