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Differential responses to high temperature during maturation in heat-stress-tolerant cultivars of *Japonica* rice

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ABSTRACT

High-temperature stress during the grain-filling stage reduces grain quality of rice, and this is a serious problem in Japan, especially in the Kyushu region. To solve this problem, various heat-tolerant cultivars have been bred, such as 'Nikomaru', 'Kumasannochikara', 'Genkitsukushi', 'Sagabiyori', and 'Otentosodachi'. When cultivated under high temperature after flowering, these heat-tolerant cultivars had lower percentages of chalky grains than in the heat-sensitive cultivar 'Hinohikari'. All the heat-tolerant cultivars markedly decreased the nonstructural carbohydrate content in the stem under the high temperature compared to control condition during early grain-filling stage, which is considered to be a common trait of heat tolerance. Notably, 'Sagabiyori', 'Genkitsukushi', and 'Nikomaru' maintained a nucellar epidermis at 17 days after flowering (DAF) under high temperature, whereas the nucellar epidermis disappeared in 'Hinohikari'. In addition, the expression of *AGPS2b*, thought to be a rate-limiting enzyme in starch synthesis, in 'Kumasannochikara', 'Otentosodachi', and 'Nikomaru' did not decrease under high temperature, whereas 'Hinohikari', 'Sagabiyori', and 'Genkitsukushi' could not maintain expression of the gene at 17 DAF. Moreover, the expression of *Amy3E*, a starch-degradation-related gene considered to induce grain chalkiness, in 'Kumasannochikara' at 17 DAF was not increased by high temperature. These results suggest that the heat-stress-tolerant cultivars have various mechanisms for dealing with high-temperature stress.

ABBREVIATIONS: DAF, days after flowering; NSC, nonstructural carbohydrate; SEM, scanning electron microscope

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Introduction

High temperature is a major environmental stress that limits the agricultural productivity of plants around the world. Recently, the reduction in grain yield and quality of rice grown under heat stress has become a problem for rice cultivation in Japan (Funaba et al., 2006; Morita & Nakano, 2011; Tanaka et al., 2009). In particular, high-temperature stress during grain ripening facilitates the formation of chalky grains (Ishimaru et al., 2009). Studies have shown that white, immature kernels occurred when the average air temperature for the first 20 days after heading was more than 27 °C and that cultivars differed in the percentage of white immature kernels (Wakamatsu et al., 2007).

When rice plants were exposed to high temperature during the ripening period, the sink–source balance of the carbohydrates was disrupted, and white immature kernels were produced (Morita, 2008). In terms of the carbohydrate

supply from source organs, sink–source manipulation such as shading or panicle clipping during ripening affects grain growth under high air temperatures (Tsukaguchi & Iida, 2008). In addition, the amount of fertilizer top-dressing affects the percentage of chalky grains under high temperature during the grain-maturation stage (Morita et al., 2005b). Nonstructural carbohydrate (NSC) content in the stem of 'Nikomaru' at the full-heading stage much contributed to grain filling than that of the heat-sensitive cultivar 'Hinohikari', especially under high temperature (Morita & Nakano, 2011). Another study reported that heat-stress-tolerant cultivars maintained nucellar epidermis under high temperature (30 °C), although heat-sensitive cultivar showed a clear cessation of the nucellar epidermis under high temperature (Tanaka et al., 2009).

The expression of starch-synthesis-related genes, especially *OsSuSy2*, *OsAGPS2b*, *OsGBSSI*, and *OsBE1b*, decreased under high temperature (Yamakawa et al., 2007). A study

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by Nishi et al. (2001) revealed that the *amylose-extender* mutant of rice, which has a mutation in the *BE11b* gene, specially altered the structure of amylopectin in the endosperm by reducing short chains with a degree of polymerization of 17 or less, resulting in chalky grains. Another study reported that *osagps2* and *osagpl2* mutant had a lesion of one of the two cytosolic isoforms AGPL2 and AGPS2b, the major large subunit and small subunit isoforms in the endosperm, causing a shrunken endosperm because of a marked reduction in starch synthesis (Lee et al., 2007). In addition, gene expression and activation of α -amylase increased under high temperatures during the grain-filling stage, and RNAi-mediated suppression of α -amylase genes resulted in fewer chalky grains under high temperature (Hakata et al., 2012; Yamakawa et al., 2007).

Recently, five heat-stress-tolerant *japonica* rice cultivars were bred in the Kyushu region of Japan: 'Nikomaru' at NARO/KARC in 2005, 'Kumasannochikara' in Kumamoto Prefecture in 2009, 'Genkitsukushi' in Fukuoka Prefecture in 2009, 'Sagabiyori' in Saga Prefecture in 2009, and 'Otentosodachi' in Miyazaki Prefecture in 2011. The objective of this study is to investigate the differential responses to high temperature during maturation among heat-stress-tolerant cultivars of *japonica* rice, mainly focusing on sink organs. We examined the five heat-tolerant cultivars and the heat-sensitive cultivar 'Hinohikari' grown under high temperature.

Materials and methods

Plant materials

Seeds of the six *japonica* rice cultivars (*Oryza sativa* L. 'Hinohikari', 'Kumasannochikara', 'Sagabiyori', 'Otentosodachi', 'Genkitsukushi', and 'Nikomaru') were sown on 5 May 2014. Seedlings of each cultivar were transplanted into 1/5000 a Wagner pots on 12 June at a density of 10 plants per pot and grown on in an experimental field of Kyushu University. We used only the main stem in this study, so all tillers were removed after planting. Irrigation was applied as described by Morita (2009). As a basal dressing, compound fertilizers (N-P₂O-K₂O: 4-4-4%) at 0.35 g N and a sigmoid type of controlled-release coated urea at 0.35 g N were supplied to each pot. In addition, 0.1 g N as ammonium sulfate (N: 21%) was top-dressed at the panicle-formation and booting stages. When panicles located on upper primary rachis branches flowered, ('Genkitsukushi' on 15 August, 'Hinohikari' and 'Otentosodachi' on 21 August, 'Kumasannochikara' on 23 August, 'Nikomaru' on 25 August, and 'Sagabiyori' on 27 August), the pots were transferred to a natural lighting growth cabinet, and plants were grown under one of two temperature treatments, control (25 °C), or

high-temperature stress (30 °C), until maturity. Five pots of each cultivar were randomly selected at 10 and 17 days after flowering (DAF) to analyze the necessary parameters. Grains located on upper primary rachis branches were used for the analysis. Statistical analyses were performed using Microsoft Excel 2011 and Statcel3.

Evaluation of grain appearance

The appearance of fully matured grains was evaluated visually. Visual assessment was aided by examining kernels under transillumination (from below), according to the criteria Tashiro & Wardlaw (1991). Normal rice grain is translucent, allowing the transmission of scattered light. In a damaged grain, opaque or chalky areas prevent this transmission resulting in black areas. Grains were classified in five groups according to the criteria of Ishimaru et al. (2009); perfect grain, white-cored or milky-white grain, white-based or white-back grain, opaque grain, or immature grain. For each cultivar, we calculated the percentage of chalky grains.

Percentage of ripened grains and 1000 ripened grain weight

Fully matured grains were harvested at 49 DAF for each of the six cultivars. All grains were evaluated for percentage of ripened grain and 1000 ripen grain weight of the husked grains. Total five replications were allocated. The ratio of ripened grain and 1000-grain weight were assessed on grain width of over 1.8 mm.

Measuring grain dry weight

At 0, 10, and 17 DAF, grains located on upper primary rachis branches were sampled from five pots for each of the six cultivars. Grains were oven-dried at 80 °C for 48 h and weighed.

Scanning electron microscopy

The structure and morphology of the endosperm of perfect grains and chalky grains from the high-temperature treatments were analyzed using a scanning electron microscope (SEM; TM3030, Hitachi, Tokyo, Japan). The SEM samples were cut transversely and then sputter-coated with a gold alloy to facilitate SEM imaging.

Analysis of nonstructural carbohydrates

At 0, 10, and 17 DAF, culms and leaf sheaths (stems) were sampled from five pots for each of the six cultivars. Culms and leaf sheaths were oven-dried at 80 °C for 48 h and

Table 1. Effect of high temperature on percentage of ripened grain and 1000 ripened grain weight of the six cultivars.

		Hinohikari	Kumasannochikara	Sagabiyori	Otentosodachi	Genkitsukushi	Nikomaru
Percentage of ripened grain	C	88.2±9.6	95.9±1.6	95.9±1.1	94.5±1.4	92.0±0.6	87.9±7.0
	H	92.5±5.2	91.5±9.8	94.0±3.8	88.6±7.8	95.6±1.0	91.6±4.4
1000 ripened grain weight (g)	C	20.3±0.4	19.9±0.2	20.7±0.4	20.9±1.0	20.7±0.2	21.6±0.3
	H	19.7±0.5	18.7±0.5*	19.9±0.8	19.8±1.1	20.0±0.4	20.9±0.9

An asterisk (*) indicates a significant difference at the 5% level (Student's test). The reported values are the means and SD of five replications.

weighed. The NSC content in the stem was estimated according to the method of Ohnishi & Horie (1999). The NSC content per spikelet (mg) was estimated as follows:

$$\text{NSC content per spikelet (mg)} = (\text{dried stem weight} - \text{residue weight}) / \text{number of grains per panicle}$$

Morphological features

Grains located on upper primary rachis branches of each cultivar sampled at 17 DAF were fixed in formalin acetic alcohol (80% ethanol: 100% acetic acid: formalin = 90:5:5 v/v/v). Tissues were cut at 20–30 µm in thickness using a cryostat (HM-505E, Thermo Fisher scientific Co. Ltd., Waltham, USA). The morphological features of rice grains of each cultivar were observed with a microscope (ECLISE 80i, Nikon Co. Ltd., Tokyo, Japan). Four or five grains from each treatment were observed.

RNA extraction and real-time PCR analysis

For RNA extraction, grains located on upper primary rachis branches were collected from five pots of each temperature treatment and cultivar at 17 DAF. Immediately after harvest, samples were frozen in liquid nitrogen and stored at –80 °C. Total RNA was isolated from the frozen materials by the SDS/phenol/LiCl method described by Chirgwin et al. (1979). cDNA was synthesized from the extracted RNA by using the ReverTra Ace qPCR RT Master Mix with a gDNA Remover kit (Toyobo, Osaka, Japan). Real-time PCR was performed using the MiniOpticon Real-Time PCR System (Bio-Rad, Hercules, CA, USA), with SYBR Green (Toyobo, Osaka, Japan) as the fluorescent dye, according to the manufacturer's instructions. The primer sequences of *OsActin*, the starch-synthesis-related genes (*OsSuSy2*, *OsAGPS2b*, *OsAGPL2*, *OsGBSSI*, and *OsBEI1b*) and *OsAmy3E* were shown in Table S1. The thermal cycling conditions were as follows: initial denaturation at 94 °C for 2 min; followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at a primer-specific temperature for 20 s, and extension at 72 °C for 20 s; followed by melting and plate reading. The results obtained for the different cDNAs were normalized using the expression level of a rice actin gene. The specificity of the individual PCR amplifications was confirmed by using a heat-dissociation curve protocol following the final cycle of the PCR.

Results

Percentage of ripened grain, 1000 ripened grain weight, and grain quality characteristics

High-temperature stress did not significantly affect the percentage of ripened grain in all six rice cultivars, and it also did not significantly change 1000 ripened grain weight except for 'Kumasannochikara' (Table 1). Grain characteristics were changed under high temperature (Figure 1). Because many grains intermediate between the types of white-cored/milky-white and white-based/white-back were found, the data for these types were pooled as chalky grains. Under control condition, percentage of chalky grain in 'Hinohikari', 'Kumasannochikara', 'Sagabiyori', 'Otentosodachi', 'Genkitsukushi', and 'Nikomaru' were 3.1 ± 0.7 , 1.2 ± 0.6 , 0.5 ± 0.3 , 1.4 ± 1.3 , 0.8 ± 0.5 , and $2.9 \pm 0.3\%$, respectively, and were not significantly different. Under high temperature, approximately 85% of grains of heat-sensitive cultivar 'Hinohikari' had at least some areas of chalkiness. In contrast, the percentage in 'Kumasannochikara' was about 65%, those in 'Sagabiyori' and 'Otentosodachi' were about 55%, and those in 'Genkitsukushi' and 'Nikomaru' were less than 50%. SEM observation of fully matured grains revealed that the amyloplasts of perfect grains were packed without gaps in all six cultivars, including 'Hinohikari', even under high-temperature treatment (Figure 1). In contrast, in chalky grains, single and compound amyloplasts were loosely packed, regardless of cultivar and percentage of chalky grains.

Changes in dry weight of whole grain and NSC content in the stem during grain-filling stage

Dry weight of high-temperature-treated grains increased compared to that of control in 'Hinohikari', 'Kumasannochikara', 'Sagabiyori', 'Genkitsukushi', and 'Nikomaru' at 10 DAF, in 'Kumasannochikara', 'Otentosodachi', 'Genkitsukushi', and 'Nikomaru' at 17 DAF, respectively (Figure 2). Figure 3 shows the NSC content per spikelet in the stem during the initial ripening period. The NSC per spikelet in 'Sagabiyori', 'Otentosodachi', 'Genkitsukushi', and 'Nikomaru' was slightly higher than that in 'Hinohikari' at 0 DAF. That in 'Sagabiyori' was approximately 6 mg per spikelet, which was almost twice that in 'Hinohikari', whereas that in

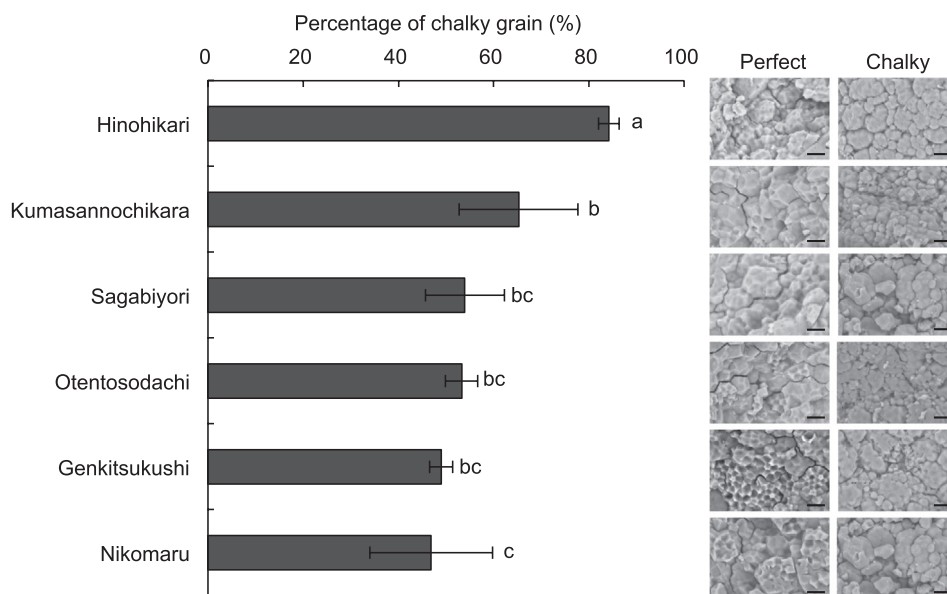


Figure 1. Effect of high-temperature treatment on grain quality of the six cultivars. Left: percentage of chalky grains under high temperature. Different letters indicate significant differences at the 5% level (Tukey's test). The reported values are the means and SD of five replications. Right: amyloplast morphology of perfect and chalky grains exposed to high temperature, respectively. Bar indicates 10 µm.

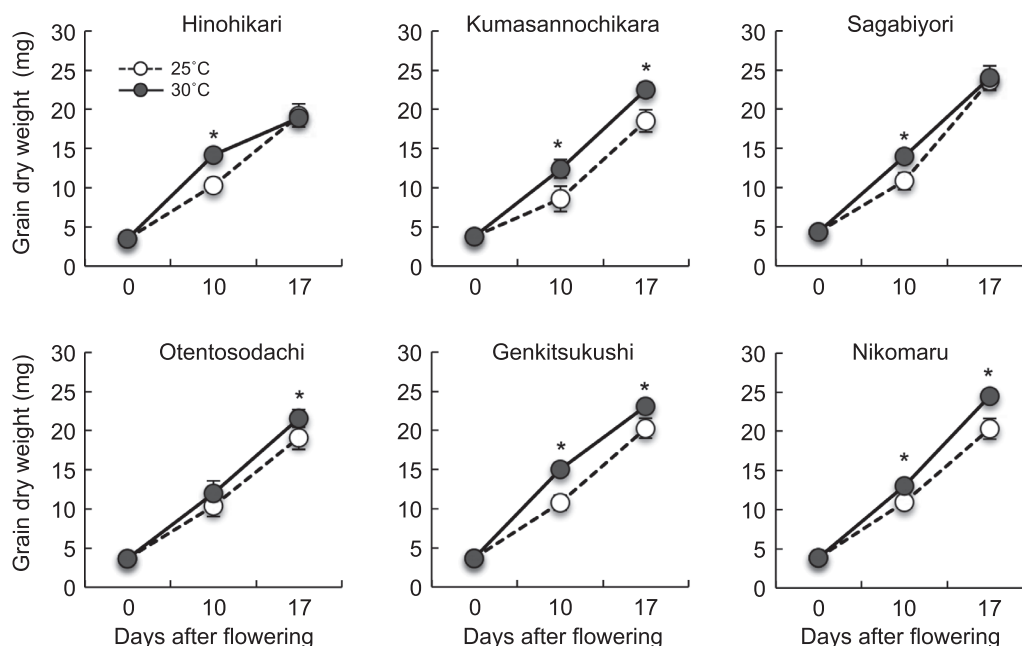


Figure 2. Changes in dry weight in develop grain of the six cultivars under the control (white circle) and high-temperature (black circle) treatments. An asterisk (*) indicates a significant difference between treatments at the 5% level (Student's test). The reported values are the means and SD of five replications.

'Kumasannochikara' was significantly lower than that in 'Hinohikari'. Moreover, under high-temperature stress, the NSC content in the stem of all five heat-stress-tolerant cultivars decreased notably more than under the control conditions from 0 to 17 DAF. In most cultivars, the difference in NSC content between the control and high-temperature conditions at 17 DAF was smaller than at 10 DAF, and only in 'Nikomaru' was the difference greater at 17 DAF. Changes

in the NSC content of 'Otentosodachi' were similar to those in 'Hinohikari'.

Morphological features of rice grains

We observed the development of a nucellar epidermis in the grains of 'Hinohikari' and the heat-tolerant cultivars at 17 DAF (Figure 4). 'Hinohikari', 'Otentosodachi', and

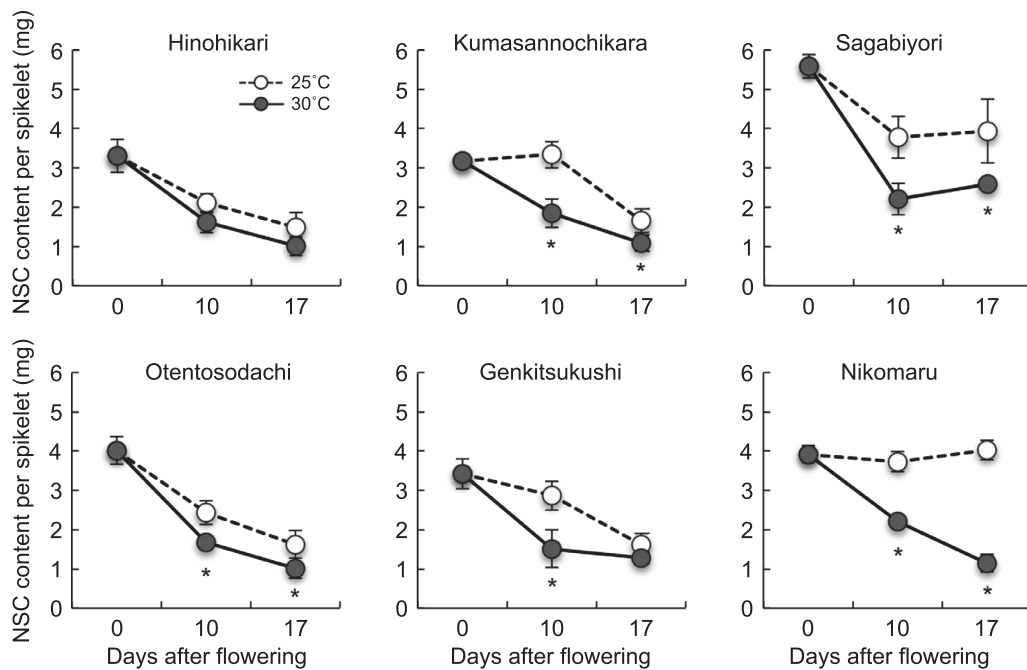


Figure 3. Nonstructural carbohydrate (NSC) content in stems of the six cultivars under the control (white circle) and high-temperature (black circle) treatments. An asterisk (*) indicates a significant difference between treatments at the 5% level (Student's *t* test). The reported values are the means and SD of five replications.

'Kumasannochikara' grown under high-temperature treatment for 17 DAF clearly showed cessation of the development of the nucellar epidermis. In contrast, we found no influence of high temperature on the development of rice grains, especially of the nucellar epidermis, in 'Sagabiyori', 'Genkitsukushi', and 'Nikomaru'.

Expression of starch-synthesis- and degradation-related genes

We investigated the transcript levels of starch-synthesis- and degradation-related genes in rice grains of the six cultivars under high temperature (Figure 5). The expression of most starch-synthesis-related genes (i.e. *OsSuSy2*, *OsGBSS1*, and *OsBE1b*) decreased under high temperatures in all six cultivars. Interestingly, the expression of *OsAGPS2b* in 'Hinohikari', 'Sagabiyori', and 'Genkitsukushi' decreased under high temperature, but there was no significant difference in the expression of *OsAGPS2b* in 'Kumasannochikara', 'Otentosodachi', and 'Nikomaru' between the two temperature treatments. In addition, the expression pattern of *OsAGPL2* also differed among the six cultivars: it decreased in 'Hinohikari', 'Otentosodachi', and 'Genkitsukushi', but was maintained in 'Kumasannochikara', 'Sagabiyori', and 'Nikomaru'. Furthermore, in 'Kumasannochikara', there was no significant difference in the expression of *OsAmy3E* between the two treatments, although the expression of the gene increased in response to high temperature in the other cultivars.

Discussion

In this study, we investigated the resistance mechanisms of heat-tolerant rice cultivars bred in the Kyushu region. Although the percentage of ripened grain was not significantly affected by high-temperature treatment, the 1000 ripened grain weight of each cultivar, except for 'Kumasannochikara', was prone to decrease under high temperature conditions (Table 1). The grain quality in all heat-tolerant cultivars was clearly superior to that of 'Hinohikari', a heat-stress-sensitive cultivar (Figure 1). These results were the same as reported previously (Fujii et al., 2009; Miyazaki et al., 2013; Sakai et al., 2007; Wada et al., 2010), suggesting that these heat-tolerant cultivars have one or more mechanisms that let them tolerate high-temperature stress.

A reduced carbohydrate supply to the panicle increases the percentage of milky white kernels (Kobata et al., 2004; Nakagawa et al., 2006; Tsukaguchi et al., 2011). Cultivars with a high NSC content in the stem at full heading, even under adverse conditions such as low radiation, were thought to better resist high temperatures, because the NSC content in the stem can adjust the balance of supply and demand of carbohydrate (Nagata et al., 2001; Rawson & Evans, 1971). In this study, dry weight of high-temperature-treated grains increased faster than control in all six cultivars during early grain maturing stage (Figure 2), indicating that demand of assimilates in sink might be higher under high temperature than under control in all

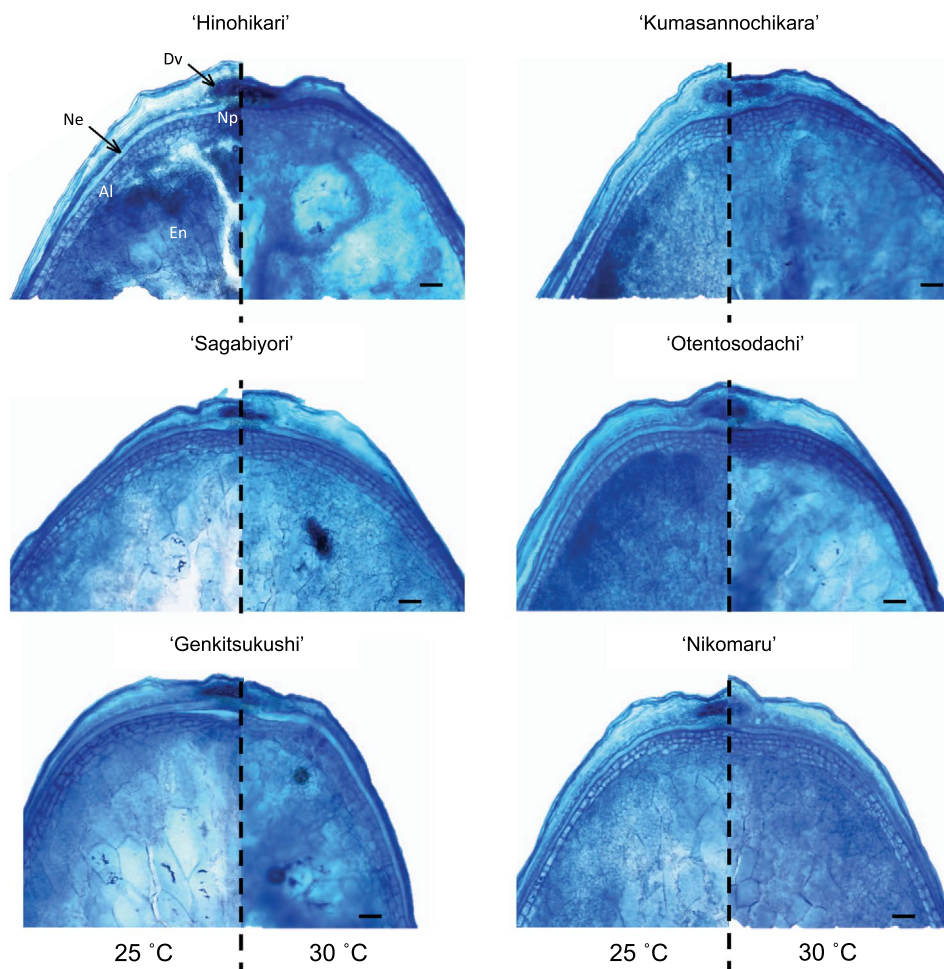


Figure 4. Influence of high temperature on morphological features of rice grains of each cultivar at 17 DAF. Left: Rice grains under control condition; right: rice grains under high-temperature conditions. Samples were stained with 0.1% toluidine blue. About five grains from each treatment were observed. Al, aleurone; Dv, dorsal vascular bundle; En, endosperm; Ne, nucellar epidermis; Np, nucellar projection. Bar indicates 100 μm .

six cultivars. Previous studies have shown that the heat-stress tolerance of rice cultivars is positively related to the NSC content in the stem at the full heading (Miyazaki et al., 2013; Morita & Nakano, 2011). As expected, NSC content per-spikelet in the stem of most heat-tolerant cultivars (except 'Kumasannochikara') was higher than that of 'Hinohikari' at 0 DAF (Figure 3). Notably, NSC content in the stem of all the heat-tolerant cultivars decreased during early grain-filling stage under high-temperature conditions as compared to the control conditions, though that of 'Hinohikari' did not change between two treatments (Figure 3). This result indicates that the decrease of NSC might contribute to high-performance ripening under high temperature. However, it was known that plant maintenance respiration increases with increasing temperature (Amthor, 2000; Long, 1991), and that a greater rate of maintenance respiration reduces the amount of assimilates available for growth and yield (Monteith, 1981). Therefore, following research is needed to understand the

relationship between the decrease of NSC and high-performance ripening under high temperature during grain-filling stage.

Seed development and accumulation of storage products depend on the delivery of sucrose from the maternal to the filial tissues (Melkus et al., 2011), and morphological studies of endosperm development in rice grains revealed the transport of assimilates and water into the caryopsis during this stage (Hoshikawa, 1993). In a previous study, high temperatures caused clear cessation of development of the nucellar epidermis at 14 DAF in 'Hinohikari', whereas it did not affect grain development, especially with respect to the nucellar epidermis, in 'Nikomaru' and 'Genkitsukushi' (Tanaka et al., 2009). In this study, 'Sagabiyori' in addition to 'Nikomaru' and 'Genkitsukushi' also maintained nucellar epidermis under high temperature (Figure 4). However, 'Otentosodachi', and 'Kumasannochikara' grown under high-temperature conditions for 17 DAF showed cessation of nucellar epidermis development, even though

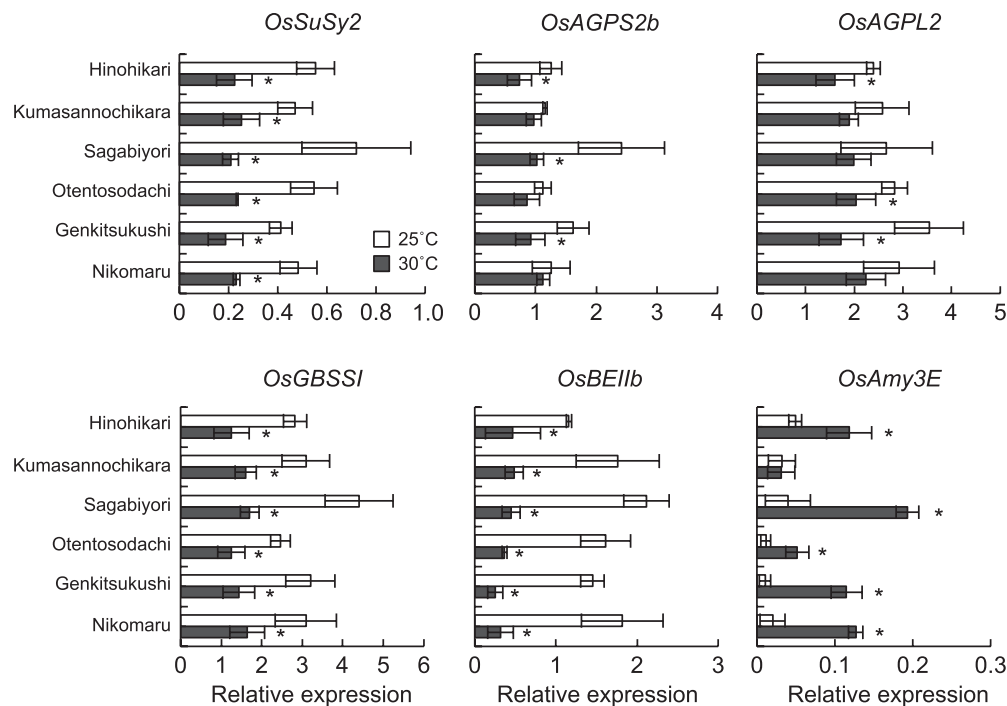


Figure 5. Expression profiles of starch-synthesis-related genes and *OsAmy3E* of six cultivars grown under the control (white bar) and high-temperature (black bar) treatments. The expression levels were determined by quantitative real-time PCR analysis and normalized to the expression of *OsActin*. An asterisk (*) indicates a significant difference at the 5% level (Student's test). The reported values are the means and SD of five replications.

these are heat-tolerant cultivars. 'Otentosodachi' and 'Kumasannochikara' have 'Hinohikari' as a grandparent and a parent, respectively, suggesting that this characteristic trait was inherited from 'Hinohikari'.

Yamakawa et al. (2007) reported that several starch-synthesis-related genes, such as *GBSSI* and *BE11b*, and a cytosolic pyruvate orthophosphate dikinase gene were down-regulated by high temperature, whereas those for starch-consuming α -amylases and heat shock proteins were up-regulated. Another study reported that high-temperature stress suppressed the expression of the starch-synthesis-related genes *GBSSI*, *BE11b*, *SuSy2*, and *AGPS2b* to about 50 and 80% of that in the control conditions throughout grain filling (Phan et al., 2013). In the present study, the high-temperature treatment suppressed the expression of *OsGBSSI*, *OsBE11b*, and *OsSuSy2* in all six rice cultivars, but there was no significant difference in the expression of *OsAGPS2b* in 'Kumasannochikara', 'Otentosodachi', and 'Nikomaru' as compared to the control. Changes in the expression of *OsAGPS2b* due to the *shrunk* mutation can greatly affect the patterns of gene expression of other members of ADP-glucose pyrophosphorylase (AGP) (Ohdan et al., 2005). This report indicates that maintaining the expression of *OsAGPS2b* under high temperatures plays an important role in maintaining grain quality in 'Kumasannochikara', 'Otentosodachi', and 'Nikomaru'. Hakata et al. (2012) also reported that

activation of α -amylase by high temperature was a crucial trigger for grain chalkiness. Another study had also shown that *japonica* rice cultivar, 'Yukinkomai', which is tolerant to high temperature during grain development, maintained α -amylase activity under high temperature (Mitsui & Fukuyama, 2005; Shiraya et al., 2015). Based on the result of the expression analysis at 17 DAF, 'Kumasannochikara' did not increase the expression of *OsAmy3E*, which may play a crucial role in maintaining rice grain quality under high temperature. However, it is necessary to investigate a further analysis of the expression level of *Amy3E* during other grain-filling stage in this cultivar.

In conclusion, our study revealed various characteristic traits in heat-tolerant rice cultivars, including high NSC content in the stem at the heading stage, maintaining a nucellar epidermis, and normal expression of starch-synthesis-related genes under high-temperature conditions. All the heat-tolerant cultivars significantly decreased the NSC content in the stem under high temperature compared to control condition during early grain-filling stage, which may therefore be a common trait in heat tolerance. Notably, 'Sagabiyori', 'Genkitsukushi', and 'Nikomaru' maintained a nucellar epidermis under high-temperature stress. In addition, the expression of *OsAGPS2b* in 'Kumasannochikara', 'Otentosodachi', and 'Nikomaru' did not decrease under high temperature. Moreover, only 'Kumasannochikara' did not increase the expression of

OsAmy3E under high temperature. These results suggest that the heat-stress-tolerant cultivars have different mechanisms to deal with high-temperature stress, meaning that it may be possible to breed rice cultivars with even stronger heat tolerance by crossing these heat-tolerant cultivars.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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*In Japanese with English abstract.

**In Japanese with English summary.

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