水稲品種「元気つくし」の高温耐性機構解明と品質改善技術に関する研究

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CONTENTS

GENERAL INTRODUCTION

The Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment Report (AR4) showed that the 100-year (1906-2005) linear trend of a 0.74°C temperature increase is the largest such increase in the last 1000 years (IPCC 2007). In Japan, the temperature has been anomaly rising at a rate of about 1.11°C per century since instrumental temperature records which began in 1898 (Japan Meteorological Agency 2009). The global warming causes reduction in grain yield and quality of rice that is the big problem of rice cultivation in Japan, especially in Kyusyu Region (Funaba et al., 2006b; Tanaka et al., 2009; Morita and Nakano, 2011). In Fukuoka Prefecture, the first inspection grade of rice has been greatly less than 50% since 2002 (Hamachi 2010).

It has been reported that white immature kernels of japonica rice arose when the average air temperature for the first 20 days after heading (DAH) was 27°C and over and that the percentage of white immature kernels changed with cultivars, for example, *Oryza sativa* L. 'Hinohikari', which is cultivated more than 40% per rice planted area in Fukuoka Prefecture, was sensitive to high temperature (Morita et al., 2005a; Wakamatsu et al., 2007). In addition, it has been reported that occurrence of the white immature kernel was caused not only by high temperature but also by an other factors such as low solar radiation and proper management for rice grown under a high temperature condition (Terashima et al., 2001). Kondo et al. (2007) reported that it is important to hold optimum number of spikelet and to maintain the nitrogen nutrient condition of rice to improve the rice grain quality. Therefore, the first objective of this study is to investigate the relationship between the number of spikelet, grain quality and yield of 'Hinohikari' to clarify the optimum number of spikelet for improving grain quality grown under high temperature. In addition, I studied a nitrogen application method for controlling the number of spikelet and improvement of nitrogen nutrient condition of rice cultivar 'Hinohikari' (Chapter 1).

Grain maturation of rice is associated with biochemical and physiological changes in tissues along with dehydration. The dynamic states of water compartments in grain tissues correlate with the organic properties of macromolecular structures related to rice development (Iwaya-Inoue et al., 2001). Nuclear magnetic resonance (NMR) is a useful technique for analyzing water conditions. Spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) has been used as indicators of dynamic states of water in biological tissues as they reflect the motion of water molecules (Farrar et al., 1971, Lenk et al., 1991, Iwaya-Inoue et al., 2004a, Funaba et al. 2006a). Water status of rice (cv. Hinohikari) grains exposed to thermal stress during ripening period was evaluated by NMR relaxation times. This study revealed that the change in T_1 of developing rice grains closely related with the quantity of water until mid-mature stage, while T_2 was more sensitive diagnostic indicator for accumulation of dry matter and quantity of kernel during grain maturity (Funaba et al., 2006b). However, it is not clear about the influence of high temperature treatments for rice plants before heading stage on water status of ripening grains and the quality of rice grains. Therefore, I performed a molecular biologic approach about water transport systems as one of the most important factors of physical properties of water. Membrane permeability for water depends on both the state of the lipid bilayer and the water channels of plant cells (Maurel et al., 1995).

It has been reported that rice has 33 aquaporin genes (Sakurai et al., 2005). Particularly in grains, aquaporins, the proteins forming membrane water channels, comprise 5 to 10% of the total membrane proteins (Maurel et al., 1997). However, detailed expression profiles of the aquaporin genes in rice grains under thermal stress before and after the heading stages is unknown. Therefore, the second objective of this study is to evaluate changes in NMR relaxation times (T_1 and T_2), aquaporin gene expression and quality of grains that was enhanced in response to thermal stress before and after the heading stage of rice plants, especially, focusing on thermal stress before heading at vegetative stage (Chapter 2).

Recently, a number of cultivars showing tolerance against heat stress at ripening stage have been developed in Japan. For example, 'Genkitsukushi' (former named 'Chikushi 64') developed from a cross between 'Tsukushiroman' as a pollen parent and 'Tsukushiwase' as a seed parent at Fukuoka Agricultural Research Center in 2008 (Tanaka et al., 2009; Wada et al., 2010) is one of the heat tolerant cultivars. High air temperatures in summer of 2010 severely damaged an apparent quality of rice kernels in a large area of Japan. Nevertheless, the percentage of the first inspection grade of 'Genkitsukushi' was more than 90% even when the average of air temperature during the 20 DAH was over 28°C, while the average of the first inspection grade of 'Tsukushiroman' and 'Hinohikari' were less than 20 % (Ministry of Agriculture, Forestry and Fisheries, 2011).

When rice plants were exposed to high temperature during the ripening period, the sink-source balance of carbohydrates was disrupted, and white immature kernels were produced (Morita, 2008). In fact, the reduction of carbohydrate supply increased the percentage of white immature kernels (Morita et al., 2005a; Nakagawa et al., 2006; Tsukaguchi et al., 2011). Recently, it was suggested that the level of high-temperature tolerance of rice was related to the nonstructural carbohydrates (NSC) content in the stem at the full-heading stage (Morita and Nakano, 2011). The supply of the carbohydrate grain for the ripening consists of two components: NSC in the stem at full heading and the newly assimilated carbohydrate after heading of rice (Matsushima 1957; Weng et al., 1982). In addition, a sucrose transporter gene of rice, *SUT1*, was highly expressed in leaf sheaths, stems, grains after heading, and also in germinating seedlings, but very low levels in roots (Aoki et al., 2003; Scofield et al., 2007). These findings suggest that SUT1 plays an important role in maintaining the supply of photoassimilates to the filling grains (Scofield et al., 2002). Recently, our group reported that high-temperature repressed the expression of *SUT1* in 'Hinohikari' and starch-synthesis-related genes in sink and source organs at the milky ripening stage caused chalky grains (Phan et al., 2013).

As reported in Phan et al (2013), the grain quality in 'Genkitsukushi' was remarkably superior to that of 'Tsukushiroman' in especially hot summer of 2010, although 'Tsukushiroman' is a pollen parent of 'Genkitsukushi'. Therefore, the final objective of this study is to gain insights into heat tolerance through the analyses of physiological differences between heat-tolerant cultivar 'Genkitsukushi' and heat-sensitive cultivar 'Tsukushiroman'. Accordingly I especially focused on assimilate translocation and gene expression of *SUT1* during the ripening period under heat stress and to reveal the mechanism of heat tolerance in 'Genkitsukushi' as a leading heat-tolerant rice cultivar for the purpose of extending a cultivation area and acceleration of breeding for new rice cultivars producing high grain quality and yield even under high temperature conditions (Chapter 3, 4).

Chapter 1

A cultivation method for improving grain quality of rice cultivar 'Hinohikari' grown under high temperature conditions

1.1 Introduction

High temperature causes severe reduction of grain yield and quality of rice cultivated in Japan. In particular, the occurrence of white immature kernels causes a reduction of the grain quality of rice (Morita 2008). Especially, high air temperature during summer of 2010 damaged the grain quality in a large area of Japan (Hamachi et al., 2012).

It has been reported that the occurrence of white immature kernel increased when average temperature during 20 days after heading (DAH) was 27°C and over. Especially, *Oryza sativa* L. 'Hinohikari', which is cultivated more than 40% per rice planted area in Fukuoka Prefecture, was sensitive to high temperature (Wakamatsu et al., 2007), thus the first inspection grade of rice was less than 50% in Fukuoka Prefecture since 2002 (Hamachi 2010).

A cultivation method for improving grain quality grown under high temperature conditions can be classified two types; 1) avoidance against heat temperature and 2) tolerance against heat temperature (Morita 2008). The former type has been reported to be applied effectively by changing transplanting time later than conventional planting time (Yamaguchi et al., 2004, Takahashi, 2006, Miyazaki et al., 2008). The later strategy is important to improve or maintain ability for the assimilates supply in source organ, such as leaf blade, culm and leaf shelf, because white immature kernels were caused by a deficiency of assimilation during the initial half period of grain filling stage (Tsukaguchi and Iida, 2008).

In addition, it has been reported that occurrence of white immature rice kernels caused not only by simple high temperature but also by an other complicate factors such as low solar radiation and nitrogen application level (Terashima et al., 2001). Occurrence of milky white kernels and white based kernels was observed at lower levelof sunshine (Yoshida et al., 1991, Sato et al., 2002） and abundance of the number of spikelet (Kobata et al., 2004), white based kernels and white-back kernels was decreased by applying nitrogen top-dressing (Nakagawa et al., 2006, Miyazaki et al., 2013). Therefore, it is important to hold optimum number of spikelet and to maintain the nitrogen nutrient condition of rice to improve the rice grain quality (Kobata et al., 2004, Kondo et al., 2007).

In this study, *Oryza sativa* L. 'Hinohikari' cultivated in 2009-2011 was used. I investigated the relationship among the number of spikelet, grain quality and yield for the purpose to clarify the optimum number of spikelet and to improve grain quality grown under high temperature (Experiment 1). In addition, I examined the relationship among the number of spikelet, the amount of nitrogen up-take of aboveground part, plant height, number of tillers and leaf color value at panicle formation stage to establish the rapid method for predicting the spikelet number by using growth diagnosis (Experiment 2). Furthermore, I studied a nitrogen application method for controlling the number of spikelet and improvement nitrogen nutrient condition (Experiment 3).

1.2 Materials and methods

Experiment 1

An experiment was performed at a field of Fukuoka Agricultural Research Center in Chikushino City of Fukuoka Prefecture, in 2009 and 2010. The experiment was performed at varietal soil texture and fertility conditions to investigate the relationship between the number of spikelet, grain quality and their yield (Table 1). Rice seedlings (*Oryza sativa* L. 'Hinohikari', medium maturing cultivar) were transplanted at 18-24th June in 2009 and at 18-25th June in 2010, respectively. The young seedlings with 3.0-3.5 leaf stage were transplanted with planting density of 19-22 hills/m^2. Varietal amounts of nutrient fertilizers were applied at each stage, as basal at pudding, as top-dressing at panicle formation stage and booting stage (Table 1).

The rice plants were harvested at maturity stage. Then threshing and husking treatments were carried out after air drying adjusted at 15% water content, and preparation was sieved at 1.85mm. Inspection grade was evaluated as 10 degrees, 1st grade (1, 2, 3), 2nd grade (4, 5, 6), 3rd grade (7, 8, 9) and below standard (10) according to Fukuoka Region center of Kyusyu Regional Agricultural Administration Office.

Table 1 The amount of nitrogen fertilizer application to rice plants grown at Fukuoka Agricultural Research Center Chikushino station: Experiment 1.

Year	Field No.[1]	Transplanting Time (Mon. Day)	Number of the experimental plot	Amount of nitrogen fertilizer application[3] (N kg/10a)
2009	1, 2	6.18-6.24	20	5-12.5: 3,5,7,9 + 0,2,3 + 0,1.5
2010	1, 2, 3	6.18-6.25	28	0-10.5: 0,3,5,7 + 0,2 + 0,1.5

1) Fields No. 1 and 2; Sand loam. Field No.3; Clayey soil.
2) Available nitrogen content in rice-cultivated soil in 2011. Field No. 1; 11.1mg/100g), Field No. 2; 6.9mg/100g, Field No. 3; 5.7mg/100g.
3) Amount of nitrogen fertilizer application: Total: Basal stage＋panicle formation stage＋booting stage.

Kernel quality such as a perfect kernel and white immature kernel was measured with grain quality analyzer RGQI20A (Satake Co. Ltd., Japan), White immature kernel is a total value of milky white kernel including white core kernel, white based kernel and white-back kernel including white belly kernel (Tsubone et al., 2008). The spikelet architecture in terms of panicles and grain quality was counted from 10 hills per experimental plot in 2010. Thirty panicles without damage were randomly selected among 10 hills, and were classified in every ten panicles by length (long, middle and short panicle), and four panicles were selected from each ten panicles. Then, selected total 12 panicles were classified into the primary and secondary branches, and threshing and husking treatments were carried out, and were measured 1000-grain weight and grain quality with grain quality analyzer described above.

Experiment 2

An experiment 2 was performed at a field of Fukuoka Agricultural Research Center in Chikushino City, Fukuoka, and Fukuoka Agricultural Research Center Chikugo Branch in Ooki Town, Fukuoka, in 2009 and 2010 (Table 2). Rice seedlings (*Oryza sativa* L. 'Hinohikari') were transplanted at 10-25th June in Chikushino, and 19th June-10th July in Chikugo, respectively.

Varietal amounts of nutrient fertilizers were applied at each stage, as basal dressing at puddling, as top-dressing at panicle formation stage and booting stage (Table 2). At the panicle formation stage, which is 18-20 days before heading (DBH), ten plants were measured by plant height, the number of tillers and leaf color value. Leaf color value was measured at the second leaf from top of an unfolded leaf by using chlorophyll meter SPAD-502 (Konica Minolta Co. Ltd., Japan). In addition, 3-4 hills per experimental plot were sampled at both the panicle formation stage and the full heading stage, and then the dry weight of above-ground part was measured. The dried samples were milled, and then the nitrogen content was measured by using Kjeldahl method; the amount of nitrogen uptake was evaluated by dry weight × nitrogen content. At the maturity stage, ten plants were harvested, and were measured by the number of spikelet.

Experiment 3

An experiment 3 was performed at a field of Fukuoka Agricultural Research Center in Chikushino City, Fukuoka, in 2010 and 2011. Rice seedlings (*Oryza sativa* L. 'Hinohikari') were transplanted at 18th June in 2010 and at 23th June in 2011, respectively. The young seedlings with 3.0-3.5 leaf stage were transplanted with planting density of 19-22 hills/m^2.

Varietal amounts of nutrient fertilizers were applied with the 5 levels as top-dressing at panicle formation stage and booting stage to examine a nitrogen application method for controlling the number of spikelet and for improving nitrogen nutrient condition and rice grain quality (Table 3).The rice plants were harvested at maturity stage, and grain yield and quality were measured with the same method described above. Protein content of brown rice was measured with Infratec 1241 （Foss Co. Ltd., Japan）.

Table 2 The amount of nitrogen fertilizer application to rice plants grown at Fukuoka Agricultural Research Center, Chikushino station and Chikugo station: Experiment 2.

Place	Number of the experimental fields	Soil texture	Available nitrogen in soil (mg/100g)	Experimental plot	Transplanting Time (Mon. Day)	Amount of nitrogen fertilizer application[1)] (Nkg/ 10a)
Chikusino	4	Sand loam	3-11	2009 n=23 2010 n=19	6. 10-6.25	0-12.5: 0,3,5,7,8.5,9 +0,2,3 + 0,1.5
Chikugo	4	Clayey soil	12-16	2009 n=24 2010 n=18	6. 19-7.10	0-8.5: 0,3,5,6 +0,2 + 0,1.5

1) Amount of nitrogen fertilizer application: Total: Basal stage＋panicle formation stage＋booting stage.

Table 3 The amount of nitrogen fertilizer application to rice plants grown at Fukuoka Agricultural Research Center Chikushino station: Experiment 3.

Year	Soil Texture[1)]	Transplanting Time	Amount of nitrogen fertilizer application[3)] (N kg/10a)
2010	Sand loam	June 18	5+2 (18 (standard), -7 and -1DBH[2)]),
2011		Jun 23	5+2(18DBH[2)])+1.5(11DBH[2)]), 5+0+0

1) Available nitrogen content in rice-cultivated soil in 2011, 11.1mg/100g.
2) Number in parentheses shows topdressing date of before heading stage(DBH).
3) Amount of nitrogen fertilizer application: Total: Basal stage＋panicle formation stage＋booting stage.

1.3 Results

1.3.1 Relationship between the number of spikelet, grain quality and yield

Transition of average temperature during 20 DAH in 2009 and 2010 is shown Fig. 1. These data were recorded by a weather station located close to the experimental field. Heading stage of 'Hinohikari' in 2009 and 2010 was August 26-31 and 27-29, respectively. Therefore, the average temperature during 20 DAH of 'Hinohikari' in 2009 and 2010 were 24.3-25.3°C and 27.3-27.9°C, respectively, and that in 2010 was around 2-3 °C higher than in 2009.

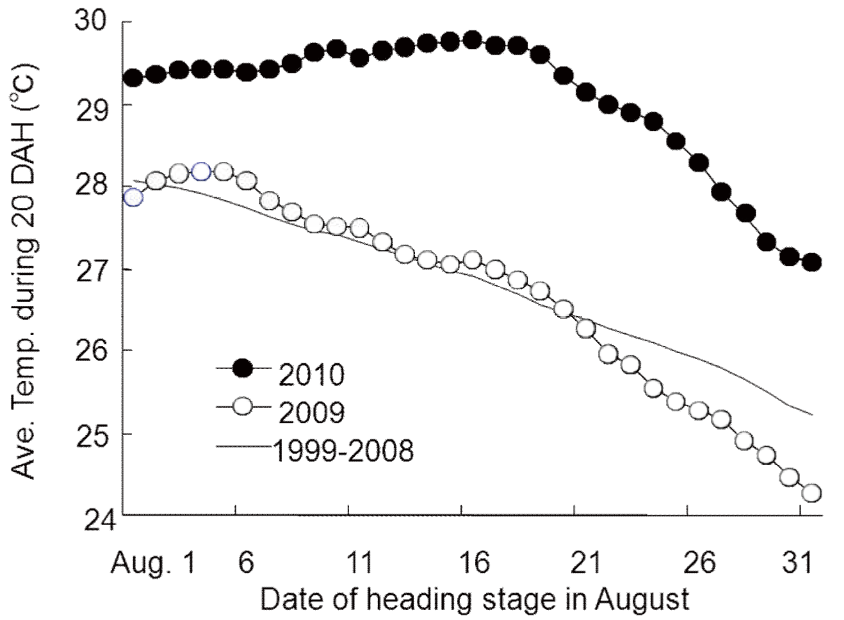

Fig. 1 Transition of average air temperature during 20 days after heading (DAH) cultivated in 2009 and 2010, respectively (Dazaifu AMeDAS 1999-2010).

1) Dazaifu is located close to Chikusino Station.

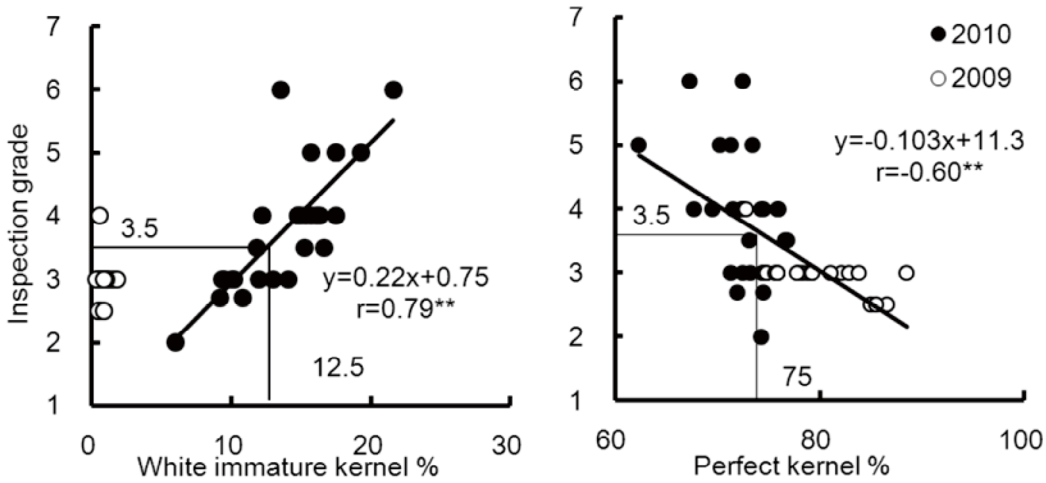

Fig. 2 Relation between inspection grade and occurrence of white immature kernel and perfect kernel.

1) Average air temperature 20 days after heading (DAH), 27.3-27.9◦C in 2010, 24.3-25.3◦C in 2009.
2) Inspection grade of kernels ranged in 10 degrees: 1st grade (1-3), 2nd grade (4-6), 3rd grade (7- 9) and below standard (10).
3) ** are significant at 1% level.

Fig. 2 shows relationship among inspection grade, white immature kernel and perfect kernel. In 2010, when was a particular hot summery, the inspection grade was highly correlated with white immature kernel at 1% levels, and it was estimated that less than 12.5% of white immature kernel rates is threshold to maintain the first inspection grade. However, kernels harvested in 2009, inspection grade was not correlated with the occurrence of white immature kernels. In contrast, the occurrence of perfect kernels was highly correlated with the inspection grade of grains harvested in both of the two years, and it was estimated that more than 75% of perfect kernel rates is threshold to the first inspection grade.

Fig. 3 shows relationship between the number of spikelet and rate of perfect kernel. The number of spikelet per square meter ranged from 21,800 to 35,200; the rate of perfect kernel decreased due to increase of the number of spikelet, indicating the significance of the correlation coefficient at 1% significance level. 30,500 of the number of spikelet per square meter is threshold to maintain more than 75% of perfect kernel rates. In addition, Fig. 4 shows relationship between the number of spikelet and grain yield. The grain yield increased due to increase of the number of spikelet, indicating a significant correlation at 1% level. When the number of spikelet per square meter was 28,000, the grain yield was 543kg/10a for the two years, whereas 531kg/10a in 2010 when was a particular hot summer.

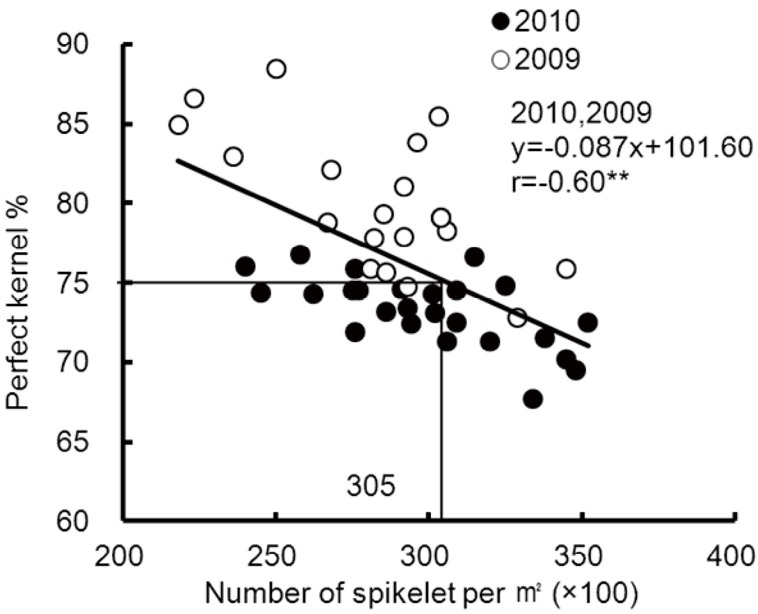

Fig. 3 Relation between the number of spikelet and occurrence of perfect kernel rating.
1) ** are significant at 1% level.

The 1000-grain weight, rates of perfect kernels and white immature kernels in the primary and the secondary branches of each panicle were examined (Table 4). In the secondary branch of panicle, 1000-grain weight was 4.3g lighter, percentage of occurrence of the perfect kernel was 30.6 points lower and that of white immature kernel was 5.8 points higher than those of the primary branch of panicle, respectively. Fig. 5 shows relationship between the number of spikelet and rate of the secondary branches. The number of spikelet was positively correlated with rate of the secondary branches at 1% significance levels. These results indicate that the perfect kernel increased due to decrease of the number of spikelet because of decrease the rate of the secondary branches.

Table 4 1000-grain weight, perfect kernel and white immature kernel in the primary and the secondary branches of each panicle.

Category	primary branches ①	secondary branches ②	①−②
1000-grain weight (g)	24.1	19.8	4.3 **
Perfect kernel (%)	77.1	46.5	30.6 **
White immature kernel (%)	12.2	17.9	-5.8 **

1) ** are significant at 1% level (n=21).

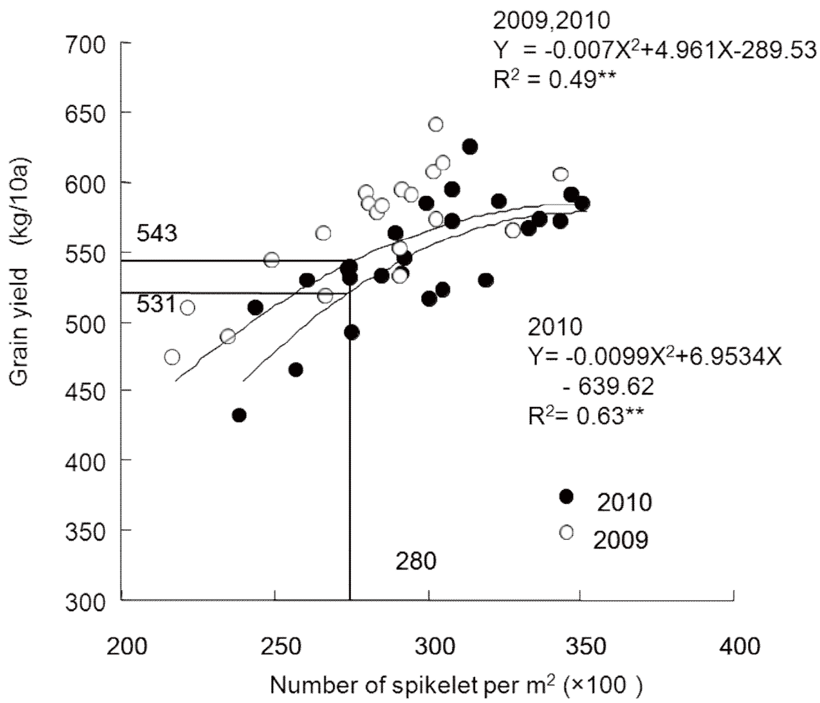

Fig. 4 Relation between the number of spikelet and grain yield.
1) ** are significant at 1% level.

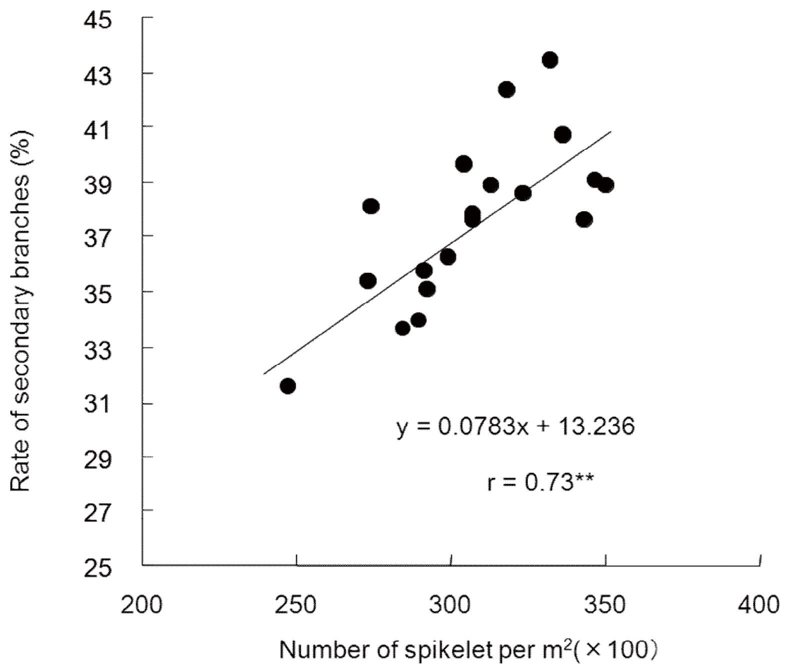

Fig. 5 Relation between the number of spikelet and rate of the secondary branches.
1) ** are significant at 1%level.

1.3.2 Relationship between the number of spikelet, amount of nitrogen up-take, plant height, the number of stems and leaf color value at panicle formation stage

The number of spikelet was a significant positive correlation with the amount of nitrogen up-take until each growth stages, from emergence of seedling to panicle formation stage and from panicle formation to full heading stage, respectively (Table 5). Therefore, I examined a multiple regression analysis using the number of spikelet as response variable, and the amount of nitrogen up-take until each growth stages, 1) from emergence of seedling to panicle formation stage and 2) from panicle formation to full heading stage as predictor variable. As a result, amount of nitrogen up-take of rice plants from emergence of seedling to panicle formation stage greatly contributed to the number of spikelet (Table 6).

Table 5 Single correlation coefficient with the number of spikelet and amount of nitrogen up-take until different growth stages.

Category	Amount of nitrogen uptake		
	from emergence of seedling to full heading stage	from emergence of seedling to panicle formation stag	from panicle formation stage to full heading stage
Number of spikelet	0.847**	0.790**	0.322**

1) ** are significant at 1%levels . ns:not significant at 5% level.

Table 6 Correlation with the amount of nitrogen uptake and the number of spikelet.

Category	Predictor variable	
Multiple correlation coefficient R		0.856 ***
Standard partial regression coefficient	Amount of nitrogen uptake from emergence of seedling to panicle formation stage	0.793 ***
	Amount of nitrogen uptake from panicle formation stage to full heading stage	0.329 ***

1) Multiple regression analysis was calculated by using the number of spikelet as response variable, and the amount of nitrogen up-take until each growth stage as a predictor variable.
2) *** are significant at 0.1% level.

I investigated the relationship among the number of spikelet, plant height, the number of tillers and leaf color value measured with chlorophyll meter, at panicle formation stage (Table 7). The number of spikelet was positively correlated with the number of tillers and leaf color value at the panicle formation stage at 1% level. Therefore, multiple correlation analysis was applied using the number of spikelet as response variable and the number of tillers and leaf color value on the panicle formation stage as predictor variable, and the regression equation for predicting the number of spikelet is accepted at 0.1% level (Fig 6).

Table 7 Single correlation coefficient with the number of spikelet, plant height, the number of tillers and leaf color value.

Category	Plant height	Number of tillers	Leaf color value
Number of spikelet	0.057 ns	0.601 **	0.598 **

1) Plant height, number of tillers and leaf color value were measured at panicle formation stage.
2) **indicates significance at 1% levels.

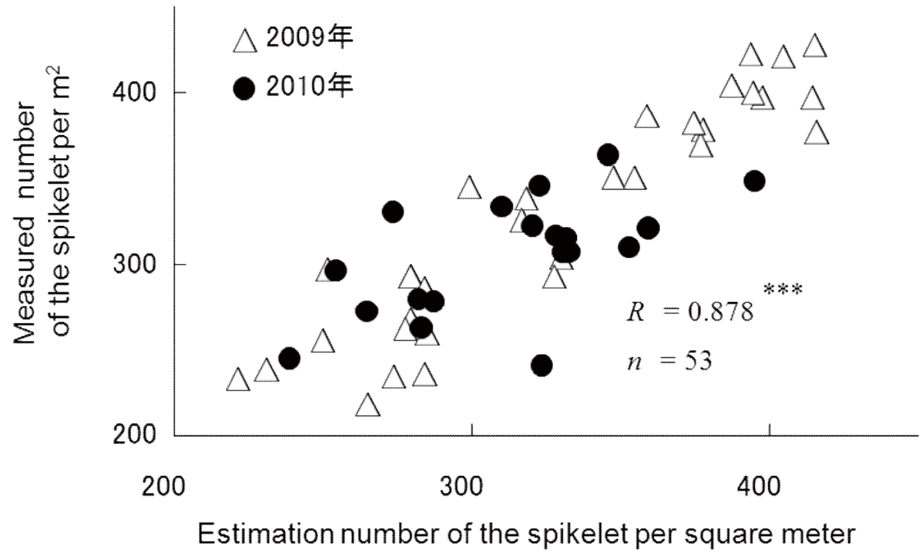

Fig. 6 Relation with estimated number and measured number of spikelet.
1) Estimating equation: $y = 0.34^{***}x_1 + 9.64^{***}x_2 - 231.53^{***}$
y :The number of spikelet per square meter (×100).
x_1: Stem number per square meter at panicle formation stage.
x_2:Leaf color value at panicle formation stage.

1.3.3 Effects of different application for top-dressing nitrogen on yield and grain quality

Table 8 and Fig. 7 indicate effects of different applications for top dressing nitrogen on yield and grain quality. When date of application for top dressing nitrogen was changed from 18 days before heading (DBH) to 1 DBH, the number of spikelet per area and occurrence of white immature kernel decreased in comparison with those at 18 DBH, which is standard application method for top-dressing adopted in Fukuoka Prefecture. When application for top-dressing by nitrogen was applied at 7 DAH, the yield and protein content of brown rice were not significantly different compared with that cultivated by standard application method, whereas the yield decreased by 5.5% compared with that cultivated by standard application method when they were applied by nitrogen 1 DAH. When top-dressing was applied twice, protein content of brown rice increased by 0.1 points, and white immature kernel increased, whereas the yield was not significantly different compared with that cultivated by standard application method.

When top dressing was omitted, the yield decreased by 4.6% and occurrence of white immature kernel tended to increase in produced under high temperature year 2010 compared with that of standard application method.

Table 8
Effects of different application for top-dressing nitrogen on yield and protein content of brown rice (2010-2011year).

Amount of nitrogen [2]	Timing of top dressing[3]	Culm length	Panicle length	Spikelets per m^2	Ripened Grains	1000-Grain Weight	Grain yield	Protein
Nkg/10a		cm	cm	×100粒	%	g	kg/a	%
	18(Standard)	82	18.5	307	85	23.5	56.1	6.7
5+2+0	7	80	18.2	291	85	23.6	55.7	6.7
	1	79	18.0	273	85	23.5	53.0	6.8
5+2+1.5	18, 11	81	18.5	318	82	23.8	57.4	6.8
5+0+0	−	80	18.1	280	85	23.4	53.5	6.4

1) Average temperature 20 days after heading (DAH) : 27.9°C (2010) and 26.8°C (2011).
2) Amount of nitrogen fertilizer application: Basal stage＋panicle formation stage＋booting stage.
3) Timing of top dressing: 18, 7 and 1 indicate top-dressing date of before heading stage.

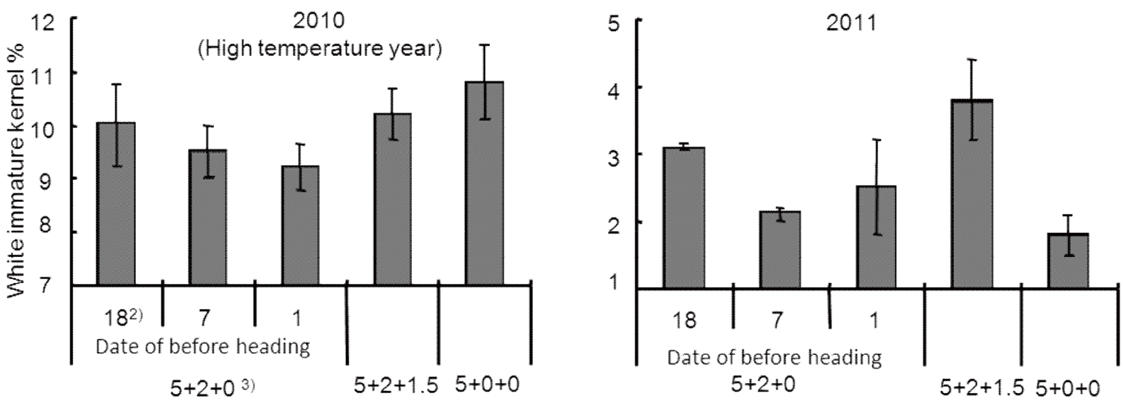

Fig. 7 Effects of different nitrogen application method on occurrence of white immature kernel of rice cultivar 'Hinohikari'.
1) Occurrence of white immature kernel was measured by using grain quality analyzer RGQI20A (Satake Co. Ltd., Japan).
2) 18, 7 and 1 indicate top-dressing date of before heading stage.
3) Amount of nitrogen fertilizer application (N kg/10a): Basal stage＋panicle formation stage＋booting stage.

1.4 Discussion

1.4.1 Optimum number of spikelet for improving grain quality of 'Hinohikari' grown under high temperature

According to the data published by Fukuoka Region center of Kyusyu Regional Agricultural Administration Office, there are two main causative factors ranking grain quality below the second inspection grade (Fukuoka Region center of Kyusyu Regional Agricultural Administration Office 2009-2010). The first factor is an occurrence of immature thin kernel, which reduces yield and lowers milling quality due to deep creases on the surface, which hinder removal of the bran grain (Yonemaru and Morita 2012), and the second factor is an occurrence of white immature kernels such as milky white kernel and white based kernel. But, the rate of those occurrences depends on weather conditions in each year. In 2009, when it was recorded as normal air temperature in the summer, main causative factor ranking kernel quality below the second inspection grade was occurrence of immature thin kernel. On the contrary in 2010 when it was recorded as an extremely hot summer, an occurrence of white immature kernel increased as a main causative factor. In addition, although it has been reported that occurrence of white immature kernel grown under high temperature, there are few reports about relations with inspection grade (Tsubone et al., 2008). Therefore, it is necessary to establish an useful diagnostic indicator for evaluation of the grain quality.

In this study, the occurrence of white immature kernel was not correlated with inspection grade in 2009. On the contrary, it was closely correlated with inspection grade at 1% level in 2010 due to high air temperature during ripening period (Figs. 1-2). In contrast, the occurrence of perfect kernel was correlated with inspection grade at 1% level in both two years, and it was estimated that more than 75% of perfect kernel rate was threshold to the first inspection grade (Fig 2). Therefore, it was considered that 75% of perfect kernel rate was an useful indicator for evaluating the grain quality as the first or second inspection grade in this examination.

It has been reported that abundance of the number of spikelet and high temperature condition during ripening period enhanced a reduction of grain quality (Terashima et al., 2001, Sato et al., 2002). Kobata et al. (2004) reported that a lack of assimilate supply to grains increased the proportion of milky white immature kernels, because high temperatures during the grain filling period could increase the grain growth rate without profoundly affecting assimilate production. In this study, the number of spikelet per unit area was significantly and negatively correlated with an occurrence of perfect kernel at 1% level. When the number of spikelet per square meter was less than 30,500, the occurrence of perfect kernel was estimated more than 75 % (Fig. 3). It has been reported that the optimum number of spikelet per square meter of 'Hinohikari' ranged from 30,000 to 32,000 per square meter in Fukuoka Prefecture (Manabe et al., 1990). However, these results suggested that it is necessary to suppress the number of spikelet against occurrence of white immature kernels grown under high temperature.

In consideration of the average yields (499kg/10a) in Fukuoka Prefecture (Ministry of Agriculture, Forestry and Fisheries, 2012), it was appropriated that target of yield was around 530 kg/10a. In this study, the number of spikelet per square meter was significantly and positively correlated with grain yield at 1% level, thus when the number of spikelet per square meter is more than over 28,000, the grain yields are estimated over more than 530kg/10a (Fig. 3). Therefore, to obtain a yields more than 530 kg/10a, and perfect kernel rate more than 75%, the number of spikelet per square meter was estimated to be 28,000-30,000 suggesting that it is the optimum number of spikelet per square meter of 'Hinohikari' against high temperature condition.

1.4.2 Prediction number of spikelet by growth diagnosis on panicle formation stage and a nitrogen application method improving rice grain quality.

It is important to control the amount of top dressing at panicle formation stage to maintain the optimum number of spikelet (Kanada et al., 1986). In this study, the number of spikelet per unit area was significantly and positively correlated with amount of nitrogen up-take at 1% level (Table 5). In addition, amount of nitrogen up-take during the growth period, from emergence of seedling to panicle formation stage contributed the number of spikelet at 0.1% level (Table 6).

It has been reported that the amount of nitrogen up-take of rice plants during emergence of seedling and panicle formation stage was closely related with the growth development at the panicle formation stage (Suenobu et al., 1994, Araki et al., 2005). Therefore, these results indicated that it is possible to predict the number of spikelet as a growth diagnosis on panicle formation stage. In fact, the number of spikelet was positively correlated with the number of stems and leaf color values at the panicle formation stage of rice (Table 7), and the regression equation for predicting the number of spikelet significantly indicated at 0.1% level (Fig. 6).

Table 9 shows the calculation chart to predict the number of spikelet based on the equation of regression. When the number of spikelet is predicted less than the optimum number according to this chart, it is necessary to increase an amount or times of top dressing at a panicle formation stage. On the contrary, when the number of spikelet is predicted over the optimum number, it is necessary to decrease an amount of topdressing at the panicle formation stage. However, it has been reported that deterioration of yield and grain quality of rice resulted from lack of nitrogen nutrient condition when the amount of nitrogen decreased at top-dressing (Tanaka et al 2010). Therefore, further research was necessary for the nitrogen application method improving rice grain quality. In this study, when application timing of top dressing nitrogen was delayed from 18 DBH, which is standard application method in Fukuoka Prefecture, to 7 DBH, the number of spikelet and occurrence of white immature kernel per unit area decreased (Table 8, Fig. 7).

Table 9
Calculation chart for predicting the number of spikelet.

Leaf color value	Number of tillers per m^2 on panicle formation stage				
	300	400	500	600	700
30	160	194	228	262	296
32	179	213	247	281	315
34	198	232	266	300	334
36	218	252	286	320	354
38	237	271	305	339	373
40	256	290	324	358	392
42	275	309	343	377	411
44	295	329	363	397	431

1) Number of spikelet: × 100 per square meter.

Although the yield and protein content of brown rice were not significantly different between the two application times describe above (Table 8). These result indicated that this method is effective to suppress the spikelet number and improving rice grain quality.

It has been reported that an occurrence of white immature kernel increased in a field cultivated soybean in the previous summer due to increase of the number of spikelet recorded over 35,000 per square meter in the field (Yoshino et al. 2011). In these cases, it is difficult to reduce the number of spikelet within a range of 28,000 to 30,000 per square meter, which was optimum number of spikelet against high temperature condition, by delaying time of top-dressing. It has been reported that the number of rice spikelet was suppressed by reduction of the amount of basal application of nitrogen (Tanaka et al 2010, Miyazaki et al 2011). For the purpose of maintaining kernel quality, it is necessary to reduce the amount of basal application of nitrogen as well as to delay time of top-dressing nitrogen application in highly soil fertility fields where the number of spikelet become abundant.

1.5 Summary

I studied the relationship among the number of spikelet, grain quality and yield of 'Hinohikari' to clarify the optimum number of spikelet for improving grain quality grown under high temperature. Occurrence of perfect kernel of 'Hinohikari' was correlated with inspection grade, and it was estimated that more than 75% of perfect kernel rates was threshold to the first inspection grade. In addition, the number of spikelet per unit area was negatively correlated with an occurrence of the perfect kernel, and it was estimated that less than 30,500 of the number of spikelet per square was threshold to 75 % of the perfect kernel rates. Furthermore, the number of spikelet per unit area was positively correlated with grain yield, and it was estimated that more than 28,000 of the number of spikelet per square to 530kg/10a. Therefore, to obtain the perfect kernel rate more than 75% and a yield more than 530 kg/10a, the number of spikelet per square meter was estimated to be 28,000-30,000 suggesting that it was the optimum number of spikelet per square meter of 'Hinohikari' against high temperature condition.

I showed the calculation chart to predict the number of spikelet based on the equation of regression, which was estimated by using the number of stems and leaf color values at the panicle formation stage. According to this chart, it is able to control amount of top-dressing to maintain the optimum number of spikelet. Especially, when application timing of top-dressing nitrogen was delayed from 18 days before heading (DBH) to 7 DBH, the earlier which is a standard application timing method in Fukuoka Prefecture, the number of spikelet per square meter and occurrence of white immature kernel decreased. Nevertheless, the yield and protein content of brown rice were not significantly different. These results indicated that this method is effective to suppress the spikelet number and improving rice grain quality.

Chapter 2

Changes in NMR relaxation times, gene expression and quality of grains: Response to different temperature treatments before and after the heading stages of rice plants

2.1 Introduction

Thermal stress during the grain ripening stages usually causes deleterious effects on the yield and quality of crop products (Chowdhury and Wardlaw, 1978). Low temperature produces defective and small endosperm of rice (Hong., et al., 1995), and that was more sensitive at the early ripening stage of rice plants (Funaba et al., 2006a). On the other hand, high temperature influences an occurrence of white immature kernels (Tashiro et al., 1991).

Grain ripening is associated with biochemical and physiological changes in tissues along with dehydration. The dynamic states of water compartments in grain tissues correlate with the organic properties of macromolecular structures related to grain development (Iwaya-Inoue et al., 2001). Nuclear magnetic resonance (NMR) is a useful technique for analyzing water conditions. Spin–lattice relaxation time (T_1) and spin–spin relaxation time (T_2) are used as indicators of dynamic states of water in biological tissues as they reflect the motion of water molecules (Farrar et al., 1971; Lenk et al., 1991; Iwaya-Inoue et al., 2004a). Funaba et al. (2006b) revealed that the change in T_1 of developing rice grains (*Oryza sativa* L. cv. Hinohikari) closely related with the quantity of water until the mid-mature stage, while T_2 was more sensitive diagnostic indicator for accumulation of dry matter and quantity of kernel during grain maturity.

It has been reported that several starch synthesis-related genes in rice seeds are down regulated by high temperature (Yamakawa et al., 2007). High temperature stress also influences the period of grain maturation by changing the process of photosynthetic rate and dehydration of rice plants (Hirotsu et al., 2005). Furthermore, it has been reported that white immature kernels occurred when an average air temperature for 20 days after heading (DAH) was above 27°C and over (Wakamatsu et al., 2007). However, the influence of temperature treatments on the quality of rice grains before the heading stage and its relationship with the water status remain unknown.

Membrane permeability for water depends on both the state of the lipid bilayer and the water channels of plant cells (Maurel et al., 1995). It has been reported that rice has 33 aquaporin genes (Sakurai et al., 2005). Particularly in grains, aquaporins, the proteins forming membrane water channels, comprise 5% to 10% of the total membrane proteins (Maurel 1997). However, effects of thermal stress on the expression of the aquaporin genes in rice before or after the heading stages has not been reported yet.

Therefore, the purpose of this study was to evaluate, in relation to the quality of the grain, changes in NMR relaxation times (T_1 and T_2) of grains and aquaporin genes expression of grains that was enhanced in response to different temperature treatments before and after the heading stage of rice plants.

2.2 Materials and methods

2.2.1 Plant materials

Rice cultivar (*Oryza sativa* L. japonica type) 'Hinohikari', medium maturing cultivar and 'Koshihikari', early maturing cultivar were used in this study in 2011 and 2012, respectively. In 2011, 'Hinohikari' were transplanted to 1/5000 Wagner pots on June 16 at a rate of three plants per pot. In 2012, 'Koshihikari' were transplanted by same method described below on April 23. Irrigation and pesticides were applied to ensure optimal plant growth. Compound fertilizer (N-P_2O_5-K_2O:4-4-4%) at 0.3 g N was added to each pot as a basal dressing. Additionally, 0.84 g N ammonium sulfate (N: 21%) was top-dressed during the panicle formation and booting stages.

Plants were allowed to grow at an experimental field of Kyushu University until 4 weeks (2011) or 2 weeks (2012) before the heading stage. Thereafter, all pots were transferred to growth chambers, and the plants were subjected to two temperature treatments, at 25°C and at 30°C. On the heading stage, half of the pots grown at 25°C and 30°C were transferred to different growth chambers at 30°C and 25°C, respectively, for a total of four temperature treatments: 25°C/25°C (before/after the heading stage, treatment temperatures), 25°C/30°C, 30°C/25°C and 30°C/30°C, whereas 30°C/30°C treatments was carried out only in 2011 (Fig. 8). Plants were grown under the same conditions until maturity.

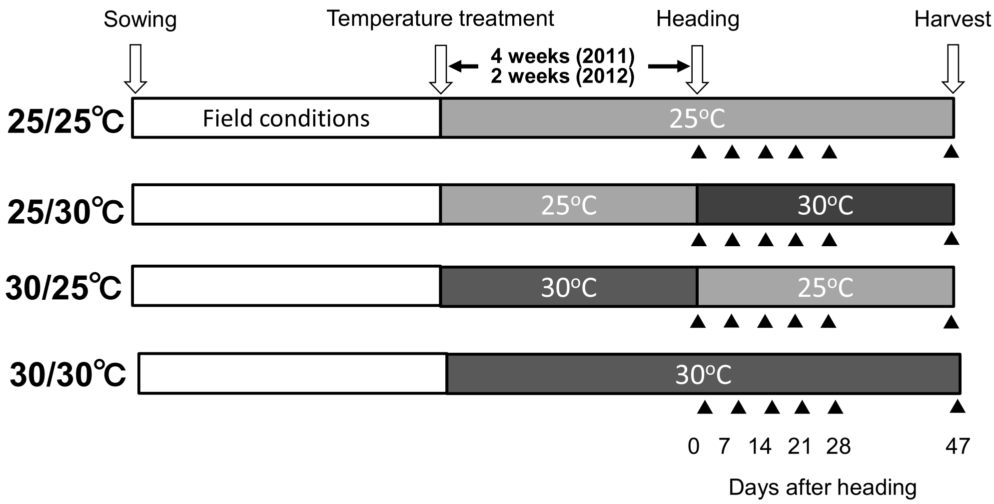

Fig. 8 Temperature treatment and cultivation method for rice plants.

1) Rice cultivar (Oryza sativa L. japonica type) 'Hinohikari' and 'Koshihikari' were used in this study in 2011 and 2012, respectively.
2) □, field conditions ; ▒ , 25℃; ■ , 30℃; ▲, sampling time.
3) 30/30℃ treatment was carried out only in 2011.

After heading, five pots were randomly selected for four individual treatments every 7 days from 0 to 28 days after heading (DAH) for evaluating the parameters described below. Grains on the primary branches and on the first and second rachis branches were used for the studies. Each experiment was performed with five replications. The grain quality of the rice cultivar 'Koshihikari' harvested after maturity in 2012 was analyzed with a grain quality analyzer (RGQI20A; Satake Co. Ltd., Japan).

2.2.2. ^{1}H-NMR relaxation times, its analysis, and water content of rice grains

Spin-lattice relaxation times (T_1) and spin-spin relaxation time (T_2) of the samples were measured using a ^{1}H-NMR spectrometer with a magnet operating at 25MHz for ^{1}H (Mμ25A, JEOL Ltd. Tokyo, Japan). T_1 and T_2 of the samples were measured based on the procedure described by Funaba et al.(2006). Twenty to twenty-four grains (rough rice) were prepared for the measurements of NMR relaxation times. The sample was placed in an NMR tube (10 mm in diameter) set in the NMR spectrometer. The probe temperature was controlled at 30°C with a thermostat connected to the sample chamber of the spectrometer. For T_1 measurement, the saturation recovery method (90°–τ–90° pulse sequence) was used. T_2 was measured using the Carry–Purcell–Meiboom–Gill (CPMG) method and the solid echo method. The decay curve of the echo signal was analyzed using a non-linear least-squares method on semi-log plots of signal intensity. There were five replications of each treatment. It was measured that the fresh weight of the rice grains used in NMR determinations and then dried them for 24 h at 90°C. Water content was expressed as a percentage of fresh weight.

2.2.3. Gene expression profiling of rice grains using DNA microarray analysis

This experiment was performed in 2012. Total RNA was extracted from developing grains harvested from 25°C/25°C, 25°C/30°C and 30°C/25°C treated plants 14 DAH of the rice cultivar 'Koshihikari'. The cRNA was amplified, labeled, and hybridized to a 44K Agilent 60-mer oligo microarray according to the manufacturer's instructions. All hybridized microarray slides were scanned using an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). Raw signal intensities and flags for each probe were calculated from hybridization intensities and spot information, according to the procedures recommended by Agilent. The raw signal intensities of two samples were $\log_2$-transformed and normalized by a quantile algorithm with the "preprocessCore" library package (Bolstad et al., 2003) of Bioconductor software (Gentleman 2001).

2.3 Results

2.3.1. Dry weight and water content of ripening grains under different temperatures applied before and after the heading stage

The dry weight and water content of rice cultivar 'Hinohikari' were measured for four temperature treatments, 25°C/25°C, 25°C/30°C, 30°C/25°C and 30°C/30°C before and after the heading stage (Fig. 9). The dry weights of rice grains grown under the four treatments did not change significantly until 7 DAH. While, they under 25°C/30°C, 30°C/25°C and 30°C/30°C increased linearly compared with those under 25°C/25°C treatment at 7 to 21 DAH stages (Fig. 9A). The water content of rice grains for all the treatments increased at 7 DAH and thereafter decreased linearly until 28 DAH. However, at 14 DAH, the water contents of rice grains grown at 25°C/30°C, 30°C/25°C and 30°C/25°C were lower than those of seeds grown at 25°C/25°C (Fig. 9B).

The dry weight and water content of rice cultivar 'Koshihikari' were measured for three temperature treatments, 25°C/25°C, 25°C/30°C, and 30°C/25°C before and after the heading stage (Fig. 10). The dry weights of rice grain grown under the three treatments did not change significantly until 14 DAH. However, the dry weights of grains grown under 25°C/30°C and 30°C/25°C increased linearly compared with those grains grown under 25°C/25°C treatment at 14 to 21 DAH stages and decreased after 21 DAH (Fig 10A). The water content of rice grains for all the treatments increased at 14 DAH and thereafter decreased linearly until 28 DAH. However, at 21 DAH, the water contents of rice grains grown at 25°C/30°C and 30°C/25°C were lower than those of grains grown at 25°C/25°C (Fig. 10B).

2.3.2. NMR relaxation time (T_1,T_2) in ripening grains under different temperature treatments before and after the heading stage

NMR relaxation times (T_1 and T_2), which correspond to the water status, were measured in rice grains during the period of ripening under different temperatures before and after the heading stage. The T_1 of rice grains of 'Hinohikari' for all the treatments were prolonged at 7 DAH and thereafter shortened in a linear manner until 28 DAH (Fig. 11A). However, at 14 DAH, T_1 of rice grains grown at 25°C/30°C, 25°C/30°C and 30°C/30°C were lower than those at 25°C/25°C. This tendency was similar to the changes in water content observed in the early ripening stage (Fig. 9B). T_2 was a more sensitive diagnostic indicator for accumulation of dry matter and the quantity of grain during grain ripening (Funaba et al., 2006b).

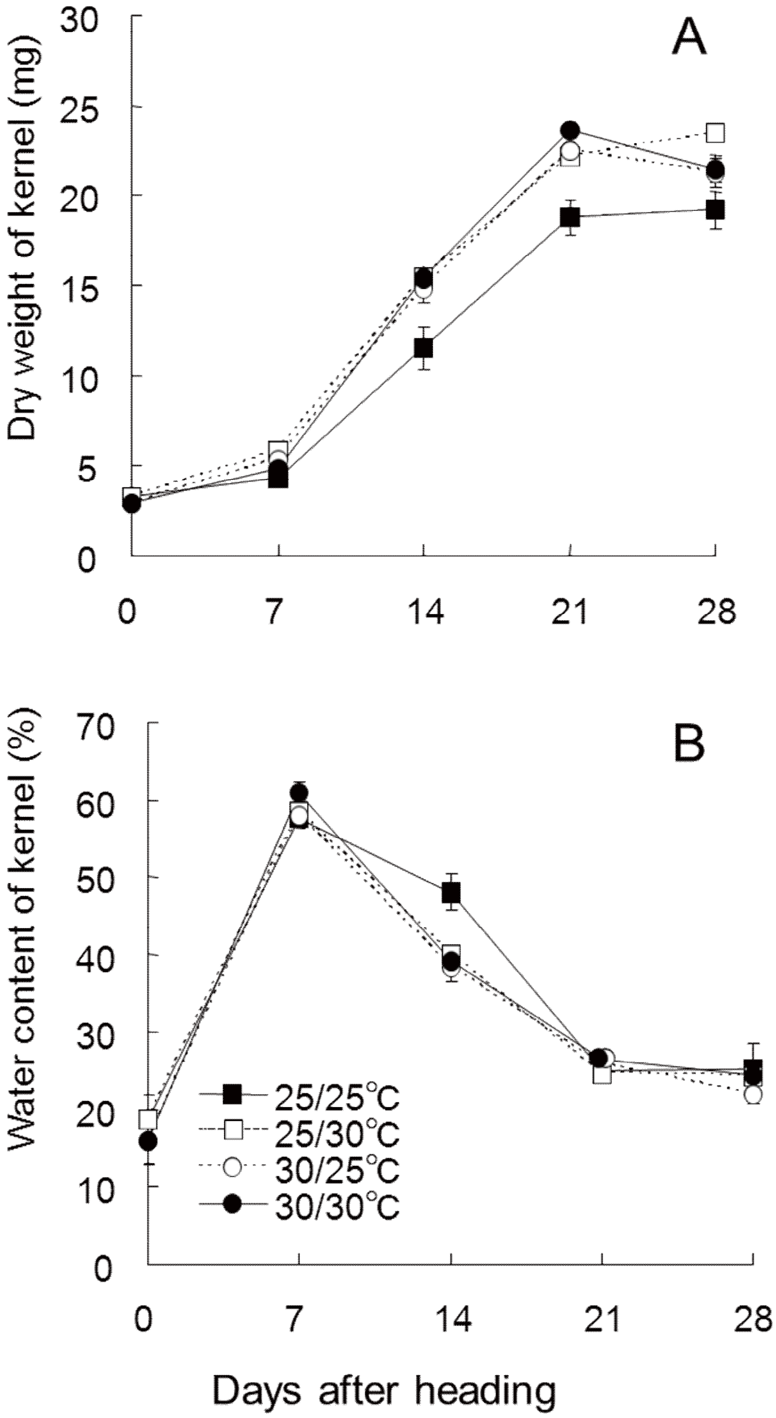

Fig. 9 Dry weight (A) and water content (B) of rice grains during grain ripening stage under the different temperature treatments before and after the heading stage of 'Hinohikari'.

1) Values are mean ± SE (n=5).

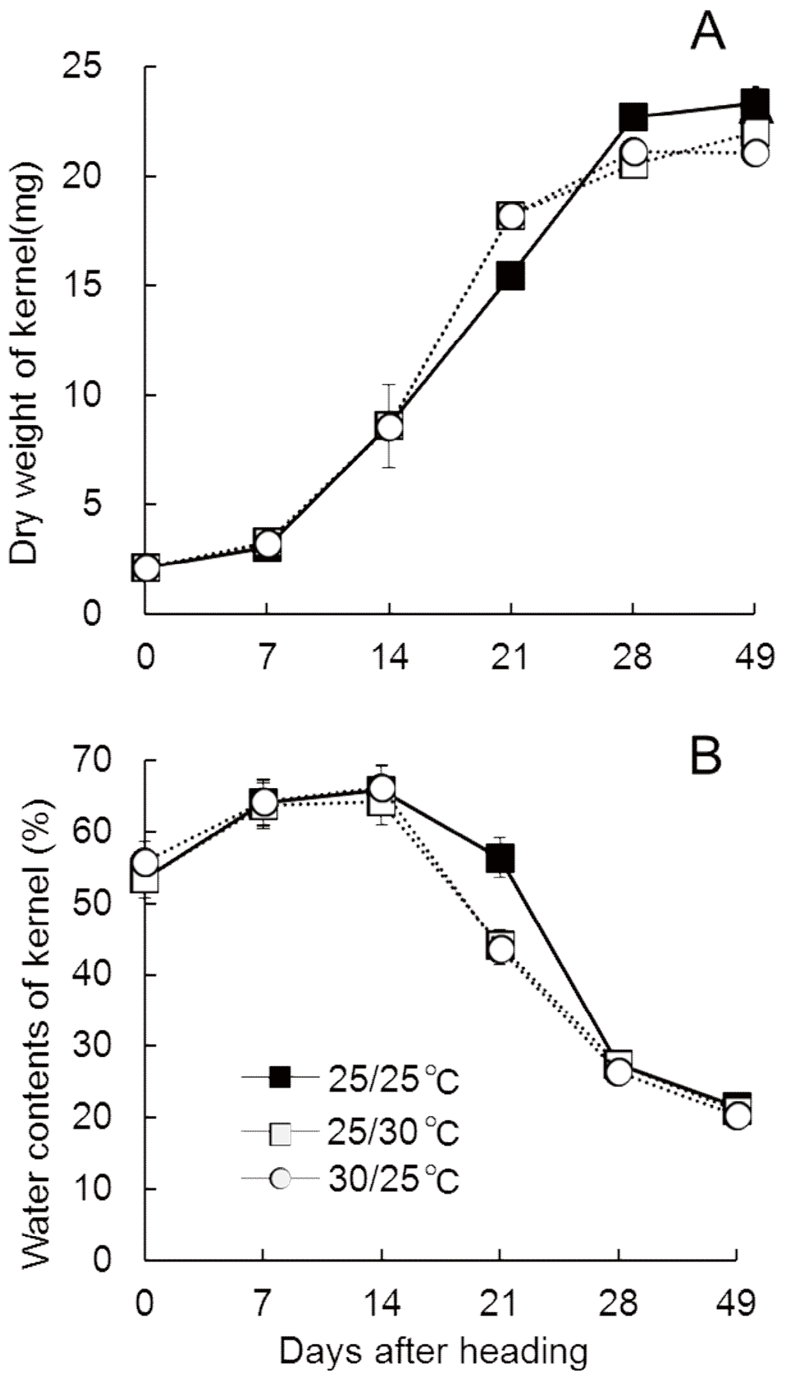

Fig. 10 Dry weight (A) and water content (B) of rice grains during grain the ripening stage under different temperature treatments before and after the heading stage of 'Koshihikari'.

1) Values are mean±SE (n=5).

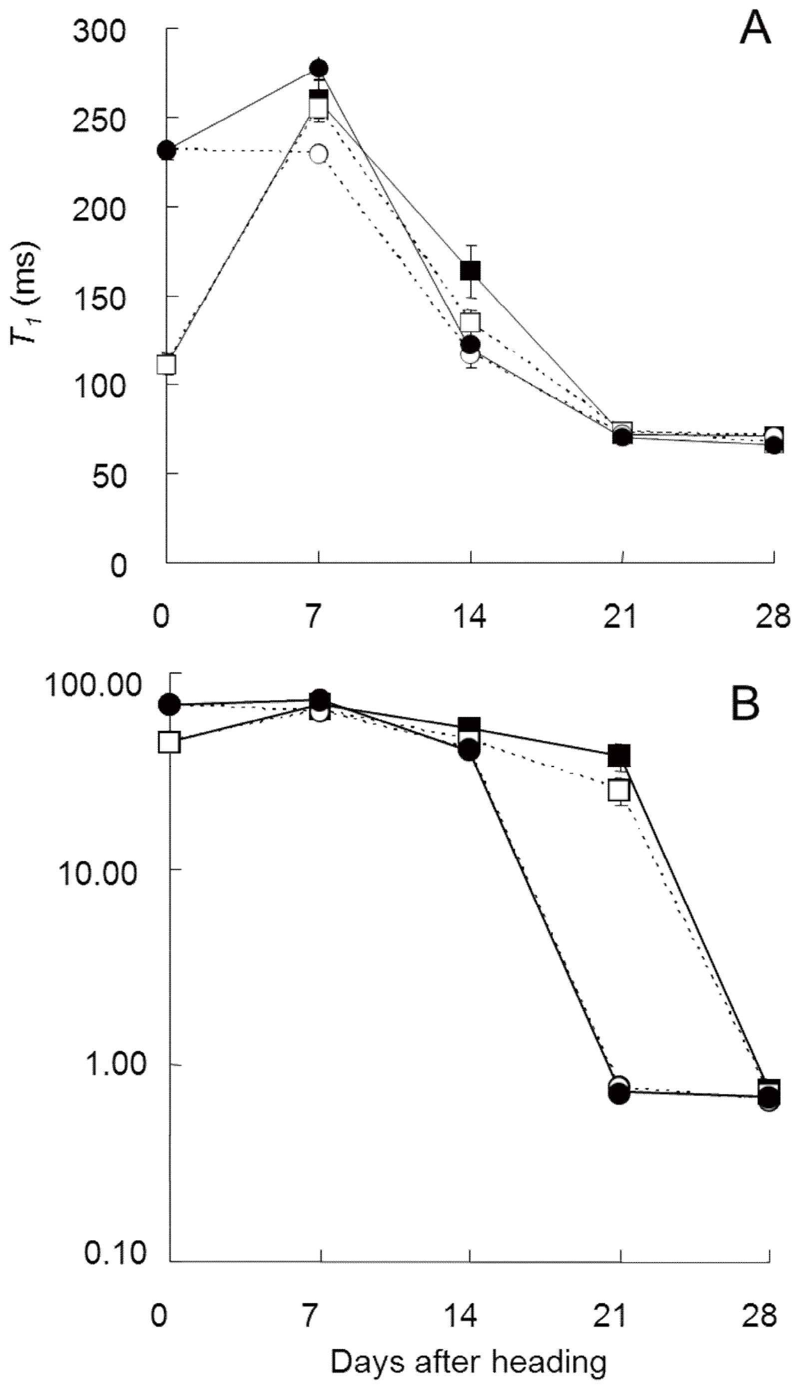

Fig. 11 NMR spin-lattice relaxation time, T_1 (A) and spin-spin relaxation time, T_2 (B) of water protons in rice grains during the ripening stage under different temperatures before and after the heading stage of ‘Hinohikari’

In this study, T_2 of rice grains grown under the four temperature treatments did not significantly change until 14 DAH. However, abrupt decreases in the T_2 values were observed in the rice grains grown at 30°C/25°C and 30°C/30°C treatments at 21 DAH (Fig. 11B) , whereas T_2 of the grains grown at 25°C/25°C and 25/30°C treatments were still over 20 ms at the stage.

The T_1 of rice grains of 'Koshihikari' for all the treatments were prolonged at 7 DAH and thereafter shortened in a linear manner until 28 DAH (Fig. 12A). However, at 21 DAH, T_1 of rice grains grown at 25°C/30°C and 25°C/30°C were lower than those at 25°C/25°C. This tendency was similar to the changes in water content observed in the early ripening stage (Fig. 10B). T_2 of rice grains grown under the three temperature treatments did not significantly change until 21 DAH. However, abrupt decreases in the T_2 values were observed in the rice grains grown at 25°C/30°C and 30°C/25°C treatments at 28 DAH (Fig. 12B), whereas T_2 of the grains grown at 25°C/25°C treatment was still over 40 ms at this stage.

2.3.3. The expression of aquaporin genes in rice grains at 14 DAH and quality of the rice grain under different temperatures before and after the heading stage of 'Koshihikari'

Aquaporins are channel proteins with six transmembrane domains, and most of them can facilitate water passage through bio membrane water systems (Preston et al., 1992). Therefore, to investigate aquaporin genes responding to different temperatures before and after the heading stage, genes whose transcript levels were induced by high temperature stress were identified. The gene expression analysis was carried out with the developing grains harvested at 14 DAH using the Agilent rice 44-K oligo DNA microarrays.

Compared to levels under 25°C/25°C, among 32,000 rice genes on the microarray, 513 and 1,182 genes were upregulated more than two folds under 25°C/30°C and 30°C/25°C, respectively. Seventy-seven genes were commonly upregulated in both 25°C/30°C and 30°C/25°C treatments (Fig. 13). In addition, 320 genes were commonly downregulated by less than half a fold in both treatments (data not shown). In the 77 commonly upregulated genes, I was focused on the *PIP1;1* aquaporin gene. *PIP1;1* was induced by thermal stress, with the 25°C/30°C and 30°C/25°C treatments increasing its expression by 3.09 and 3.42 fold, respectively (Table 10).

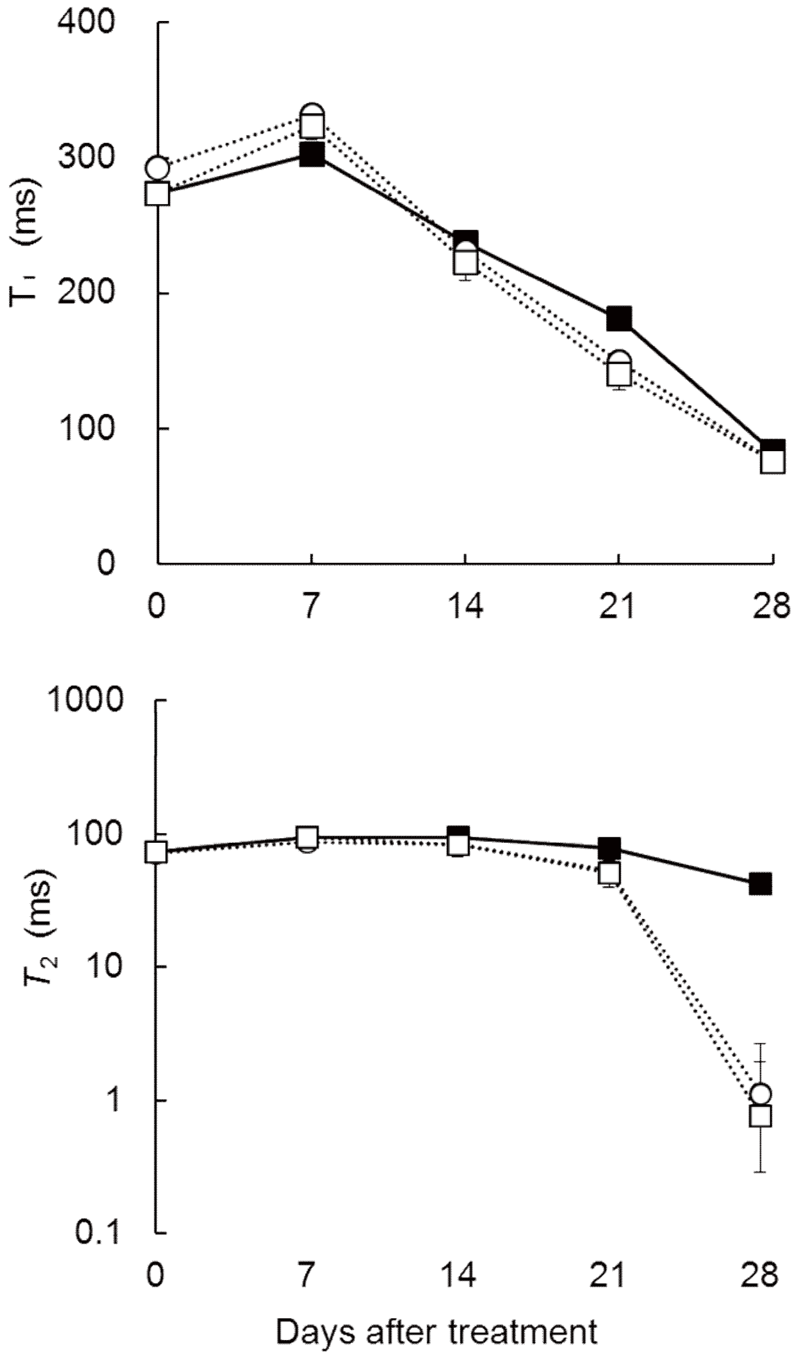

Fig. 12 NMR spin-lattice relaxation time, T_1 (A) and spin-spin relaxation time, T_2 (B) of water protons in rice grains during the ripening stage under different temperatures before and after the heading stage of 'Koshihikari' .

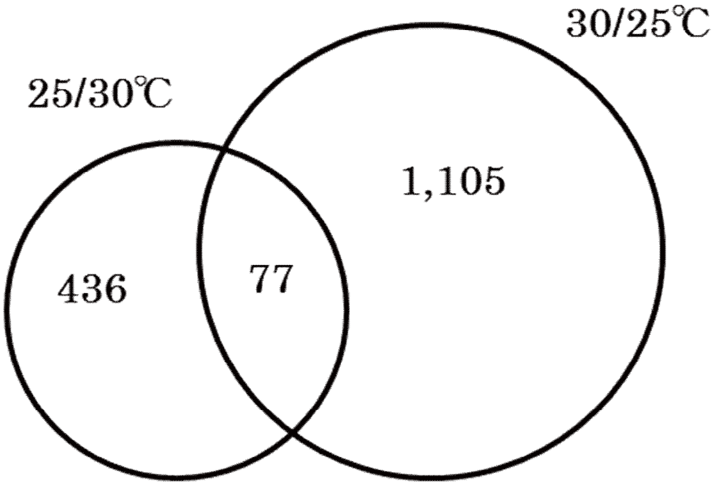

Fig. 13 Venn diagrams of gene expression of rice grains induced by high temperature stresses before and after the heading stage of 'Koshihikari'.

Tabel 10 Expression level of *PIP1;1* by high temperature stress before and after the heading stage of 'Koshihikari'.

	Microarray			Foldchange	
	25℃/ 25℃	25℃/ 30℃	30℃/ 25℃	25℃/ 30℃	30℃/ 25℃
PIP1;1	4,271	13,222	14,619	3.09	3.42

Rice grains quality under different temperature before and after the heading stage of 'Koshihikari' is shown in Fig. 14. Percentages of perfect kernels of rice grains grown at 25°C/30°C and 30°C/25°C treatments were 77.5 and 77.1%, respectively, owing to the occurrence of milky white kernel, white-back kernel, white belly kernel and immature kernel. In contrast, the percentage of perfect kernels under the 25°C/25°C treatment was 84.3%.

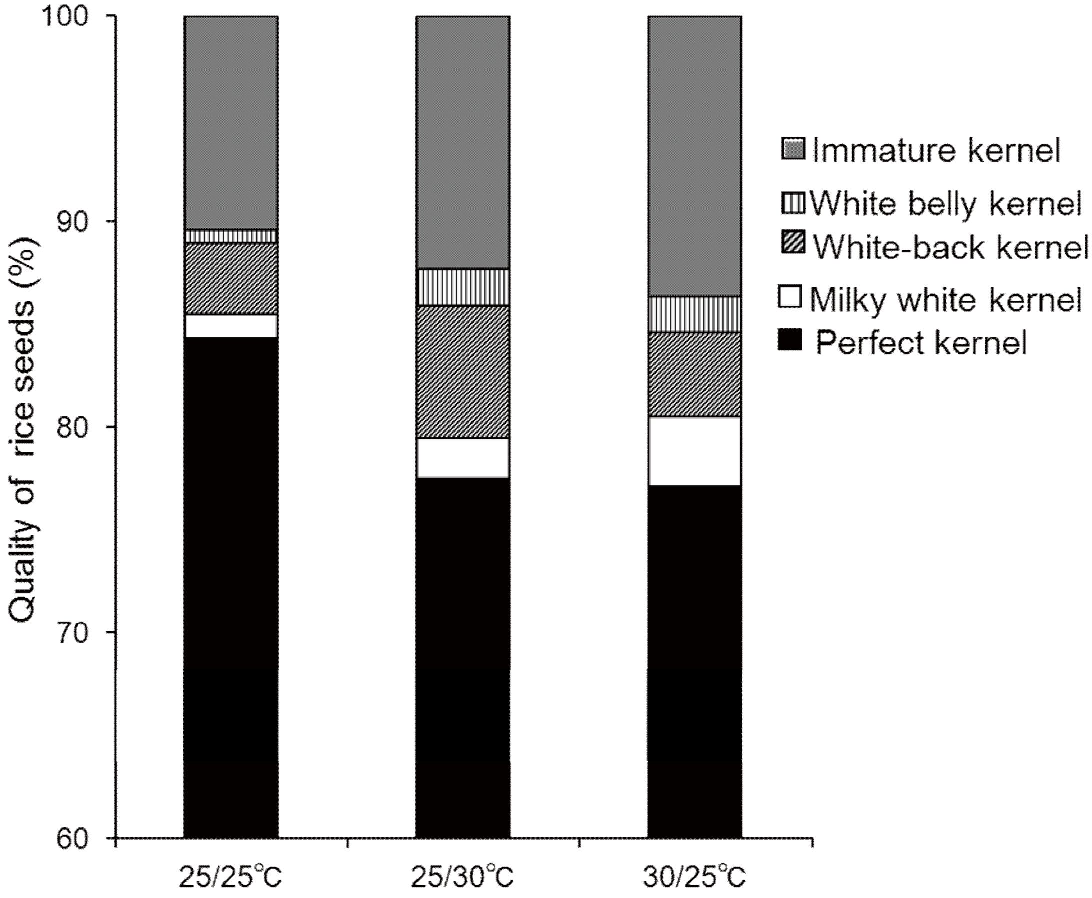

Fig. 14 Rice grains quality under different temperature before and after the heading stage of 'Koshihikari'.

2.4. Discussion

2.4.1. Effect of temperature treatments on dry weight, water content and NMR relaxation time (T_1, T_2) in rice grains during maturation.

High temperature accelerated the rice grains development during the early ripening stages, but reduced the duration of grain growth and ultimately resulted in a decrease in final grain weight (Wilhelim et al., 1999; Morita et al., 2005a). Funaba et al (2006b) determined an influence of low/high temperature during the ripening stages on water status in ripening grains of rice cv. Hinohikari, and they revealed that 20°C treatment suppressed a grain growth rate by delaying dehydration process of the grains.

In this study, it was showed that high temperature treatments both before and after the heading stage accelerated an increase in dry weight and dehydration of rice grains between 7 and 21 DAH stages (Figs. 9, 10). These results indicate that the changes in rice grains exposed to 30°C before or after heading stage showed a different response to 25°C treatment through the stages, and that high temperature stress, either before or after the heading stage accelerated increase in dry weight and dehydration of rice grains during ripening stage.

The NMR relaxation times (T_1 and T_2) of water protons in biological systems provide important clinical information that the relaxation mechanism depends on the intrinsic state of water in cells and tissues(Köckenberger, 2001). Therefore, it was focused on T_1 and T_2 values of the ripening grains grown under different temperatures before and after heading stage.

The T_1 values exposed to 30°C before or after the heading stage were lower than those of 25°C treatment at 14 or 21 DAH stage (Figs. 11A, 12A), and the changes in T_1 values at 30°C before or after the heading stage were similar to the changes in the degree of dehydration (Figs. 9B, 10B). Thus, this fact suggested that T_1 values closely related with the quantity of water in rice grains (Funaba et al., 2006b).

T_1 and T_2 values between 100 ms and 3 s show the existence of free water, and T_1 and T_2 values between 100 μs and 100 ms show the presence of loosely bound water (Hills and Remigereau, 1997; Iwaya-Inoue and Nonami, 2003; Iwaya-Inoue et al., 2004a, b). As mentioned above, the T_1 values were significantly differences at 14 or 21 DAH stage between different temperature treatments, however those values were over 100 ms (Figs. 11, 12). In contrast, T_2 values in rice seeds were less than 100 ms, while that tendency of changes in T_1 and T_2 values showed similarity at each stage (Figs. 11B, 12B). These results indicated that the changes in T_1 and T_2 values of developing rice grains reflected dynamic states of water such as free water and bound water, suggesting that they can be used as a diagnostic indicator for characteristics of water status in ripening grains treated by different temperature treatments. Especially, abrupt decrease in the T_2 values was observed in the rice grains grown at 30/30°C and 30/25°C treatments at 21 DAH, while that of under 25/25°C and 25/30°C treatment was still over 20 ms (Fig. 11B). In addition, abrupt decrease in the T_2 values was observed in the rice grains grown at 25/30°C and 30/25°C treatments at 28 DAH, while that of under 25°C/25°C treatment was still over 10 ms (Fig. 12B). It has been reported that relaxation times detected using NMR and MRI of the cereals' embryo and endosperm are specific parameters associated with the metabolic and physiological information such as germinative energy, α-amylase activity, and starch synthesis (Gruwel et al., 2002; Ishimaru et al., 2009; Rolletsheck et al., 2011). Therefore, these results indicate that T_2 values were more sensitive diagnostic indicators of dry matter accumulation in response to temperature treatments before and after the heading stage.

2.4.2. Effect of temperature treatments on the expression of aquaporin genes in rice seeds at 14 DAH and quality of the rice seed in 'Koshihikari'

Aquaporins are channel-like transporter proteins with six transmembrane domains, and most of them can facilitate water passage through biomembrane systems (Preston et al., 1992). In this study, a high level of expression of aquaporin gene *PIP1;1* in grains at 25°C/30°C and 30°C/25°C was detected using DNA microarray analysis at 14 DAH (Fig. 13, Table 10). Aquaporin *PIP1;1* , one of the most abundant aquaporins in leaves and roots of rice plants, functions as an active water channel and plays important physiological roles. In addition, the germination rate and α-amylase activity of PIP1;1 overexpressed grain were significant higher than the control (Liu et al., 2013). It has been reported that the expression of α-amylase genes is up regulated by thermal stress during ripening (Yamakawa et al., 2007) and that the ectopic overexpression of α-amylase genes results in grains with white immature kernels even under ripening at ambient temperature (Asatsuma et al., 2005). These reports suggest that these alterations in gene expression might contribute to the low quality of grains by formation of white immature kernels.

It has been reported that the sink-source balance of carbohydrates is disrupted, and white immature kernels are produced when rice plants are exposed to high temperature stress during the ripening period (Morita et al., 2008). These reports also state that and that white immature kernels appear when the average air temperature for the first 20 DAH is above 27°C and that the cultivars differ in the percentage of such kernels under high temperature stress (Wakamatsu et al., 2007; Tanaka et al., 2009).

In this study, grain quality grown under 25°C/30°C and 30°C/25°C treatments was inferior to that of grains under 25°C/25°C treatment because of the occurrence of white immature kernels. These results suggested that high temperature stress either before or after the heading stage led to a reduction in the quality of rice grains as well as changes in NMR relaxation times (T_1 and T_2) along with an expression of aquaporin gene, *PIP1;1* .

2.5. Summary

I performed a biophysical approach by using ^{1}H-nuclear magnetic resonance (NMR) relaxation times (T_1 and T_2) to investigate the effects of different temperature treatments before and after the heading stage of rice cultivars 'Hinohikari' and 'Koshihikari', which has the most largest cultivation area in Japan. Dehydration of rice grains was accelerated by heat stress at 30°C before and after the heading stage, and the changes in T_1 values of grains exposed to 30°C before and after the heading stage showed parallel relationship to the changes in the degree of dehydration. In addition, the T_1 values of grains exposed to 30°C before and after the heading stage were lower than those of 25°C treatments at 14 or 21 DAH stage. Furthermore, the T_2 values of grains exposed to 30°C before and after the heading stage treatments (except for 30°C after the heading stage treatment of 'Hinohikari') were abruptly shortened at 21 or 28 DAH, while those at 25°C treatments were hardly shortened. These results indicated that T_1 and T_2 are useful diagnostic indicators for the water status during grain ripening of rice plants. Furthermore, I focused on the water channels called aquaporins, which control the dynamic state of water, especially, aquaporin genes expression of grains that was enhanced in response to different temperature treatments before and after the heading stage of rice cultivar 'Koshihikari'. A high level of expression of aquaporin gene *PIP1;1* in grains at 30°C before and after the heading stage was detected using DNA microarray analysis at 14 DAH. Furthermore, grain quality exposed to 30°C before and after the heading stage was inferior to that of grains under 25°C/25°C treatment because of the occurrence of white immature kernels. These results suggested that high temperature stress either before or after the heading stages led to a reduction in the quality of rice grains as well as changes in NMR relaxation times and expression of aquaporin genes.

Chapter 3

Effects of high air temperature in the summer of 2010 on the grain quality of heat-tolerant rice cultivar ‘Genkitsukushi’

3.1 Introduction

High temperature reduces grain quality and yields of rice according to global warming (IPCC 2007) in Japan (Morita, 2008). In particular, it was reported that the occurrence of white immature kernels is one of the most important factors for reduction of the grain quality of rice cultivar ‘Hinohikari’ as a leading cultivar in the Kyushu district region (Sakai et al., 2007). The first inspection grade of rice was greatly less than 50% in Fukuoka Prefecture since 2002 (Hamachi 2010). It is predicted that these harmful effects will cause greater problem in the near future.

Recently, heat tolerant rice cultivar ‘Genkitsukushi’ was developed at the Fukuoka Agricultural Research Center (Wada et al., 2010) using evaluation facility for high‐temperature tolerance, which evaluates treated by warm water at 35°C during grain ripening period (Tsubone et al., 2008). The objective of this breeding program was to develop a rice cultivar with high palatability and excellent grain appearance even under the high temperature conditions.

High air temperatures in the summer of 2010 caused a lower level of quality of rice kernels in a large area of Japan. Nevertheless, the first inspection grade of rice kernels of ‘Genkitsukushi’ cultivated in Fukuoka Prefecture was more than 90% even when an average of the first inspection grade of kernels of rice cultivars in Japan was 63% (Ministry of Agriculture, Forestry and Fisheries, 2011). Therefore, it is expected that ‘Genkitsukushi’ will spread out as a leading heat-tolerant cultivar in the cultivation area.

It has been reported that grain filling status and quality are varied depending on the spikelet positions in the panicle. Milky white kernels occurred at inferior spikelets, such as lower part and secondary branch in panicles (Kido and Yanatori, 1968; Takahashi, 2006), white based kernels occurred at upper branches in panicle (Kido and Yanatori, 1968), and white-back kernels occurred at inferior spikelets under high temperature conditions during ripening periods (Nagato and Ebata, 1965).

Therefore, it is important to investigated a relationship between spikelet architecture and extent of white immature kernel from both of heat-tolerant and heat-sensitive rice cultivar to evaluate heat-tolerance.

In this study, the rice grain quality was analyzed using ‘Genkitsukushi’, ‘Tsukushiroman’ and ‘Hinohikari’ cultivated under the extreme high temperature hot summer in 2010 to clarify relationship between spikelet architecture and occurrence of white immature kernels.

3.2 Materials and methods

3.2.1 Plant materials

Experiments for three rice cultivars were performed at a field of Fukuoka Agricultural Research Center in Chikushino City, Fukuoka, in 2010. The experimental soil condition was composed of coarse grained gray lowland soil and low-medium soil fertility, which was cultivated only paddy rice every year. For the experiments, three kinds of variety of *Oryza sativa* L. 'Genkitsukushi' and 'Tsukushiroman', early maturing cultivars, and 'Hinohikari', a medium maturing cultivar were used. The grain appearance of 'Genkitsukushi'was superior to that of 'Tsukushiroman' and 'Hinohikari', and its grain quality was classified to be of the first grade, even the former grew under a high temperature conditions (Wada et al., 2010).

Seedlings with 3.0 leaf stages of 'Genkitsukushi' and 'Tsukushiroman' were transplanted at 13th May, while those of 'Hinohikari' were transplanted at 17th May, respectively. They were transplanted with planting density of 21.2 hills/ m^2. 5.0kg N/10a was applied as basal, additionally 2.0kg N/10a as topdressing at about days 18 before heading, and 2.0 or 1.5kg N/10a at about 8-11 days before heading. Each area of experimental field was 5.7 m^2. Two replicated experiments were carried out.

Furthermore, to compare to the grain quality with different field conditions, the experiments were performed at fields of Fukuoka Agricultural Research Center Chikugo Branch in Ooki Town, Fukuoka, in 2010. Experimental soil condition was composed of fine grained gray lowland soil and highly soil fertility. Experimental fields were two preceding crops, wheat cropping (Chikugo 1), wheat cropping and soybean product for alternation (Chikugo 2). The rice varieties of 'Genkitsukushi', 'Tsukushiroman' and 'Hinohikari' (*Oryza sativa* L.) were transplanted at 4th June. The young seedlings with about 2.0 leaf stages were transplanted with planting density of 21.8 hills/ m^2. 3.0kg N/10a was applied as basal, additionally topdressing at same stage with Chikushino City. Each area of experimental field was 10.5 m^2. Two replicated experiments were carried out.

3.2.2. Analyses

The rice plants, 60 and 100 hills per experimental plot both in Chikusino City and Chikugo City were harvested at maturity stage, respectively. Then threshing and husking treatments were carried out after air drying adjusted at 15% water content, and preparation was sieved at 1.85mm. Inspection grade was evaluated as 10 degrees, 1st grade (1, 2, 3), 2nd grade (4, 5, 6), 3rd grade (7, 8, 9) and below standard (10) according to Fukuoka Region center of Kyusyu Regional

Agricultural Administration Office. Contents of perfect kernels and white immature kernels were measured with grain quality analyzer RGQI20A (Satake Co. Ltd., Japan), White immature kernel is a total value of milky white kernel (including white core kernel), White based kernel and white-back kernel (including white belly kernel) (Tsubone et al., 2008). The spikelet architecture in terms of panicles and grain quality were measured on two cultivars 'Genkitsukushi' and 'Tsukushiroman', 5 and 10 hills per experimental plot both in Chikusino City and Chikugo City were harvested at full ripened stage, respectively. Each rachis-branch was separated into the three positions (upper positions, central positions and lower positions), and classified into the primary and secondary spikelet, respectively (Fig. 15). Then threshing and husking treatments were carried out. Perfect kernels and white immature kernels were also measured with grain quality analyzer RGQI20A. Air temperature was measured at the position of panicles with thermograph (TR-71U, T and D Co. Japan).

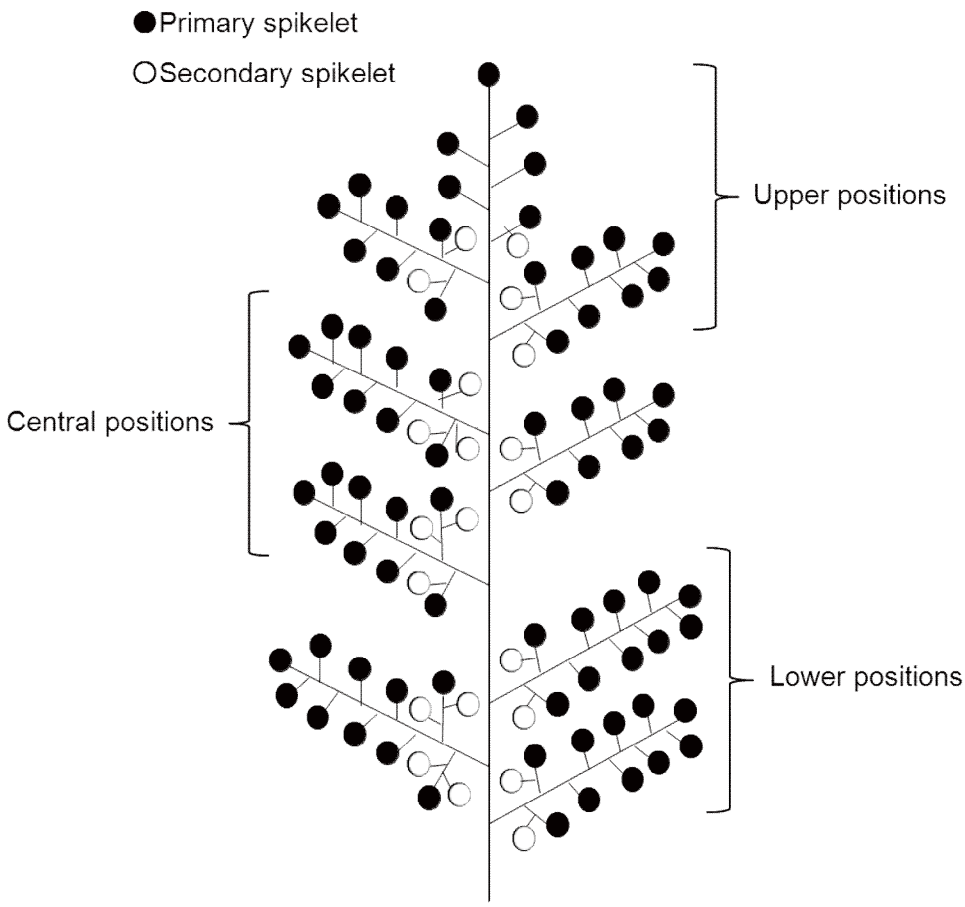

Fig. 15 Spikelet architecture in a rice panicle
1) Each rachis-branch was separated into the three positions (upper position, central position and lower position), and was classified into the primary and secondary spikelet, respectively.

3.3 Results

3.3.1. Crop situation index and grain quality in 2010.

Transition of mean air temperature and duration of sunshine during growth period of rice in 2010 are shown in Fig. 16. These data were recorded by a weather station closed to the experimental field. The mean air temperature and duration of sunshine during the transplanting stage and the middle of July were about 1°C higher and about 20% shorter than an average year, respectively. After the rainy season (around July 17), the mean air temperature was 2-3 °C higher than average year until the fourth pentad of August. Thereafter, the mean air temperature maintained at 1-3 °C higher than average year.

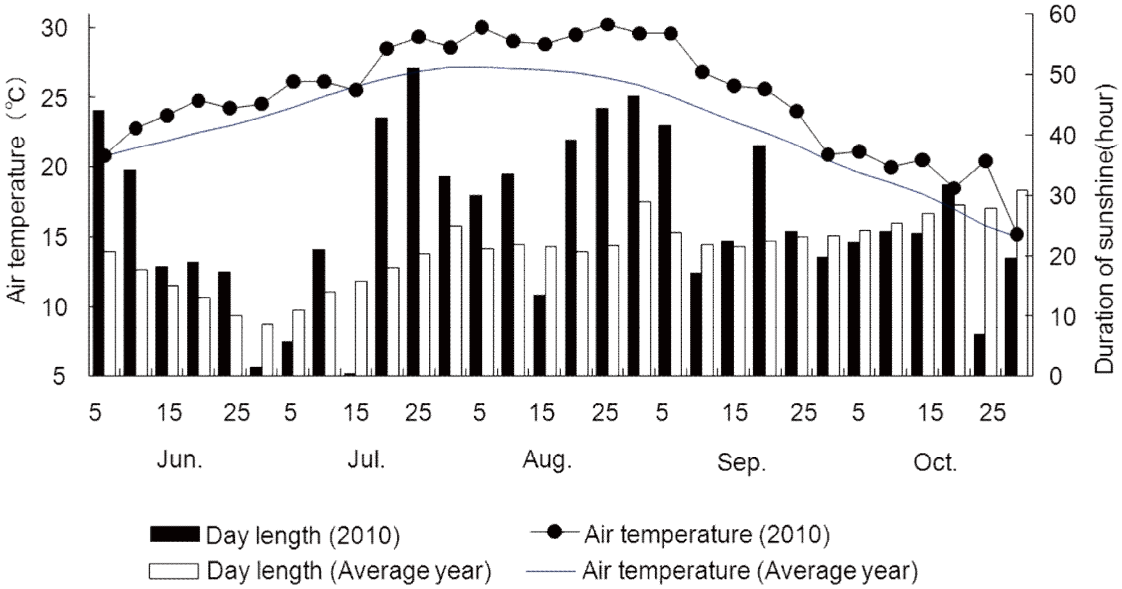

Fig.16 Transition of mean air temperature and duration of sunshine in 2010(Dazaifu AMeDAS).

1) Dazaifu is located close to Chikusino station.
2) Average: Air temperature was shown as an average of those recorded from 1979 to 2000. Duration of sunshine is shown as an average of those recorded from 1986 to 2008.

In this year, the crop situation index in Fukuoka Prefecture 2010 indicated 97, which was categorized as 'slightly low yield', because, both the panicle and spikelet numbers decreased compared with those of the average year, because of short duration of sunshine during the vegetative stage. Furthermore, the 1000-grain weight decreased because of high temperature during the ripening stage. Therefore, However, the grain quality of rice kernel in Fukuoka Prefecture remarkably decreased, and the first inspection grade was 16.6%, while that in 2009 was 36.0 % (Ministry of Agriculture, Forestry and Fisheries, 2011).

3.3.2. Influence of high temperature on the grain quality of 'Genkitsukushi', 'Tsukushiroman' and 'Hinohikari'.

Heading stage of 'Genkitsukushi' grown at Chikusino station was seven days earlier than that grown at Chikugo station. Therefore, the average temperature during 20 DAH(days after heading) of 'Genkitsukushi' grown both at Chikusino station and Chikugo station were 28.6°C and 28.3°C, respectively. In addition, heading stage of 'Genkitsukushi' was one day earlier than that of 'Tsukushiroman', and six to nine days earlier than that of 'Hinohikari'. Therefore, the average temperature 20 DAH for 'Tsukushiroman' was about the same as that of 'Genkitsukushi', and that for 'Hinohikari' was about 1°C lower than that for 'Genkitsukushi' (Table11).

Table 11 Heading stage, maturity stage and average temperature during 20 days after heading (DAH) of rice cultivars.

Experimental Station	Cultivar	Heading stage Mon. Day	Maturity stage Mon. Day	20days after heading Ave. Temp. ℃
Chikushino	Genkitsukushi	8.15	9.16	28.6
	Tsukushiroman	8.16	9.17	28.6
	Hinohikari	8.24	9.28	27.7
Chikugo 1	Genkitsukushi	8.22	9.29	28.3
	Tsukushiroman	8.23	9.30	28.2
	Hinohikari	8.28	10. 8	27.2
Chikugo 2	Genkitsukushi	8.22	9.29	28.3
	Tsukushiroman	8.23	9.30	28.2
	Hinohikari	8.28	10. 8	27.2

1) Chikugo 2: soybean was cultivated at this area in the previous summer. At the other experimental fields soybeans were not cultivated in the previous summer.

Table 12. Effects of high temperature on physicochemical properties of grains and the proportion of various types of white immature kernel of rice cultivars.

Experimental places	Cultivar	Panicle number per m^2	Spikelets per m^2	1000-grain weight (g)	Grain yield (kg/a)	rice screenings (%)	Inspection grade	Perfect kernel (%)	White immature kernel: Milky white kernel (%)	White based kernel (%)	White-back kernel (%)	Total (%)
Chikushi	Genkitsukushi	271	267	23.0	54.6	2.0	2.0a	79.2	1.6a	3.1	0.4	5.1
	Tsukushiroman	305	283	22.5	52.8	2.1	5.0b	54.9	8.1b	11.1	3.5	22.7
	Hinohikari	332	293	23.2	56.2	4.5	5.5b	64.2	9.2b	10.0	1.6	20.8
Chikugo 1	Genkitsukushi	443	296	21.6	51.2	4.9	3.0a	74.6	4.9a	1.7	1.3	7.8
	Tsukushiroman	491	294	22.2	50.8	3.6	5.5b	58.9	9.2b	13.3	3.3	25.7
	Hinohikari	416	308	22.1	54.2	5.4	6.0b	67.5	6.4ab	7.5	1.1	14.9
chikugo 2	Genkitsukushi	437	302	22.0	50.5	7.0	3.0a	74.7	5.8a	2.3	1.5	9.6
	Tsukushiroman	469	303	22.1	49.0	7.2	7.0b	52.8	11.6b	11.1	3.1	25.8
	Hinohikari	441	347	22.1	53.7	7.4	4.0a	62.9	6.5a	5.9	1.1	13.5
Average	Genkitsukushi	384	288a	22.2	52.1ab	4.6a	2.7	76.2c	4.1	2.4a	1.1a	7.5a
	Tsukushiroman	422	293a	22.3	50.9a	4.3a	5.8	55.5a	9.6	11.8c	3.3b	24.7c
	Hinohikari	396	316b	22.5	54.7b	5.8b	5.2	64.9b	7.4	7.8b	1.3a	16.4b
Chikushi		303a	281a	22.9b	54.5	2.9a	4.2	66.1	6.3a	8.1	1.8	16.2
Chikugo 1		450b	299b	22.0a	52.1	4.6b	4.8	67.0	6.8a	7.5	1.9	16.1
Chikugo2		449b	317c	22.1a	51.1	7.2c	4.7	63.5	8.0b	6.4	1.9	16.3
Cultivar (A)		ns	**	ns	*	*	***	***	***	***	***	***
Place (B)		***	***	***	ns	***	ns	ns	*	ns	ns	ns
A×B		ns	ns	ns	ns	ns	*	ns	*	ns	ns	ns

1) Inspection grade of kernels in 10 degrees: 1st grade (1-3), 2nd grade (4-6), 3rd grade (7-9) and below standard (10).
2) **and * are significant at 1% and 5% levels, respectively. ns: not significant at 5% level. Values followed by the same letter within a column are not significantly different at 5% level, based on Tukey's test.
3) Perfect kernel and white immature kernels were measured with grain quality analyzer RGQI20A (Satake Co. Ltd., Japan).

In Chikushino station the number of spikelet of the three cultivars was less than that grown at Chikugo station. Therefore, the 1000-grain weight was higher and rice screenings rate was lower than that grown at Chikugo station. In Chikugo station, the number of spikelet grown at Chikugo 2, where soybean plants were cultivated in the previous summer, increased than that grown at Chikugo 1. However, the grain yields did not significantly between two fields, because of increase in rice screening rate in Chikugo 2 (Table 12).

Percentage of perfect kernels, white immature kernels and inspection grade were not significantly different among the experimental fields, whereas ratio of milky white kernels grown at Chikugo 2 significantly increased than that grown at the other area. Occurrence of white-back kernels was low in comparison with those of milky white kernels and white based kernels. The grain quality of 'Genkitsukushi' is maintained at the first inspection grade, 2.0-3.0 (Table 12). In contrast, the grain quality of 'Tsukushiroman' and 'Hinohikari' ranged from the second to third inspection grade, 5.0-7.0 and 4.0-6.0, respectively, due to remarkable occurrences of milky white kernel, white based kernels and white-back kernels (Table 12).

It has been reported that occurrence in 10% of white immature kernels is the border line of the first inspection grade (Tsubone et al., 2008). In this study, the occurrence of white immature kernel of ‘Genkitsukushi’ was less than 10%, those grown at Chikushino, Chikugo1 and Chikugo2 were 5.1%, 7.8% and 9.6%, respectively (Table 12).

3.3.3. Occurrence of white immature kernels among the different spikelet position in the panicle.

Parameters of grain yield and quality were analyzed to examine the relationship between spikelet architecture and the occurrence of white immature kernels using two cultivars ‘Genkitsukushi’ and ‘Tsukushiroman’ (Fig. 17). In each spikelet position of the panicle, the number of spikelet, grain yields and 1000-grain weight were not significantly different between the two cultivars. Both of the two cultivars, 1000-grain weight of the primary branch of panicles were superior to that of the secondary branch of panicles. 1000-grain weight of upper position of the panicles grown at Chikushi station, was superior to that of low position in each cultivar, while that grown at Chikugo station was not significantly different between two positions. In addition, the proportion of milky white kernel was higher at the secondary branch of panicle than that the primary branch grown at Chikugo 2, and proportion of white-back kernel was higher at the secondary branch of panicle than that the primary branch grown at each experimental field. Both of the two cultivars, white based kernel of the primary branch of panicle occurred at upper position compared to those at the middle and lower positions, while that of the secondary branch of the panicle was not significantly different between three positions.

Both of the primary and secondary branches, proportions of milky white kernel, white based kernel and white-back kernel of ‘Genkitsukushi’ were lower than those of ‘Tsukushiroman’, except for proportion of milky white kernel grown at Chikugo 1, which was not significantly different between two cultivars. In ‘Genkitsukushi’, the proportion of milky white kernel was the highest at the secondary branch of the panicle grown at Chikugo 2.

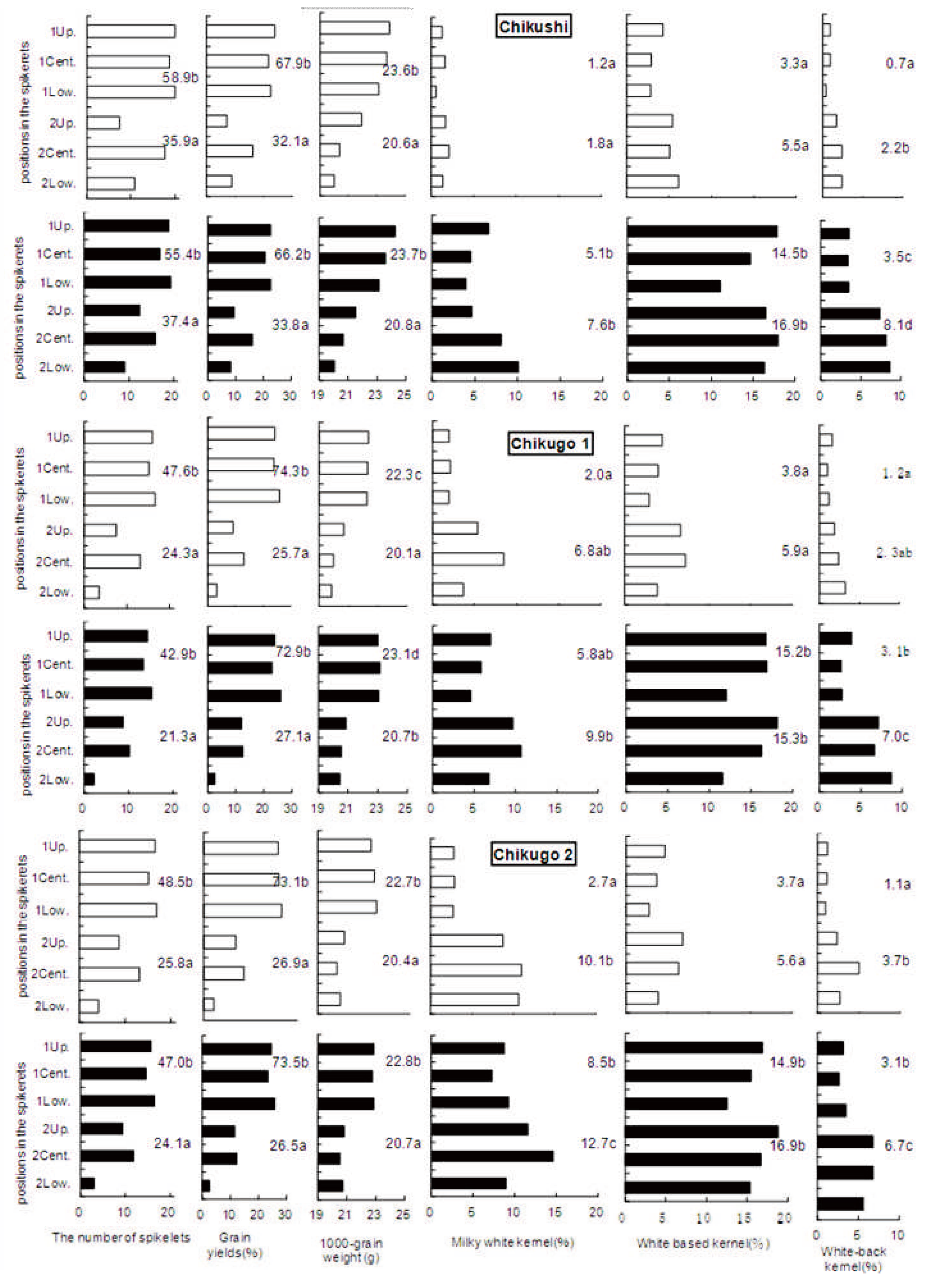

Fig. 17 Number of spikelet in a panicle, grain yields, 1000-grain weight and the proportion of various types of white immature kernels among different positions in the panicles of rice cultivar 'Genkitsukushi' and 'Tsukushiroman'.

1) White bars indicate 'Genkitsukushi' and black ones indicate 'Tsukushiroman'. A vertical axis, 1 and 2: the primary branch of a panicle and the secondary branch of a panicle, respectively. Up., Cent., and Low. : Upper, Central and lower positions in a panicle respectively.
2) Values followed by the same letter are not significantly different at 5% level, based on Tukey's test.

3.4. Discussion

Global warming is one of the most important factors for deterioration of grain quality and yield of rice in Japan, especially Kyusyu District (Morita, 2008). Recently, heat tolerant rice cultivar ‘Genkitsukushi’ was developed at the Fukuoka Agricultural Research Center (Wada et al., 2010).

Therefore, it is necessary to spread out a heat tolerant rice cultivar as a way to deal with the problem against low quality of rice kernels caused by high temperature.

It has been reported that white-back kernel and white based kernel remarkably occurred when the average temperature for the first 20 DAH was more than 27°C (Wakamatsu et al., 2007), and the first inspection grade of rice decreased (Terashima et al., 2001). During the extreme hot summer of 2010, the mean air temperature of late in August and early in September, when the periods were equivalent during 20 DAH of ‘Genkitsukushi’ was 29.9°C (26.2°C of an average year) and 28.2°C (24.7°C of an average year), respectively (Dazaifu AMeDAS 2010). Nevertheless, the percentage of the first grade kernels of ‘Genkitsukushi’ cultivated in Fukuoka Prefecture was 91.8%, whereas those of ‘Tsukushiroman’ and ‘Hinohikari’ were 12.2% and 11.1%, respectively (Ministry of Agriculture, Forestry and Fisheries, 2011).

It has been reported that grain quality was influenced by soil fertility in the fields because nitrogen nutrient condition of rice from panicle formation stage to full heading stage was affected by them (Tanaka et al., 2010). Yoshino et al. (2011) reported that occurrence of white immature kernel increased in the field cultivated soybean in the previous summer, according to increase of the number of spikelet in the field. Therefore, a field experiment was carried out to examin the influence of high temperature on the quality of grains at maturity of ‘Genkitsukushi’, ‘Tsukushiroman’ and ‘Hinohikari’ at the experimental fields with different soil fertility and different crops were cultivated in the previous summer. The average temperature during 20 DAH of ‘Genkitsukushi’ and‘Tsukushiroman’ was over 28°C, which was 1°C higher than that of ‘Hinohikari’ (Table 11). Under the high temperature condition, the number of spikelet was significantly different between experimental stations. On the other hand, the grain quality of ‘Genkitsukushi’ maintained at the first inspection grade in all experimental fields, while those of ‘Tsukushiroman’ and ‘Hinohikari’ ranged from the second to the third inspection grade because of remarkable occurrence of white immature kernel (Table 12). These results indicated that the occurrence of white immature kernels of ‘Genkitsukushi’ was significantly lower and that the rice kernel of ‘Genkitsukushi’ was superior in quality to those of other cultivars such as ‘Tsukushiroman’ and ‘Hinohikari’ under both the high temperature and the various soil fertility conditions contributing the ripening stability of ‘Genkitsukushi’.

Previously, it has been reported that milky white kernels occurred at inferior positions in panicle, such as the secondary branch of panicle (Kido and Yanatori, 1968; Takahashi 2006), and the occurrence of milky white kernel was promoted due to increase the number of spikelet because of a lack of assimilate supply to grains (Kobata et al., 2004; Takahashi 2006), whereas white based kernel and white-back kernel were little influenced by an increase in the number of spikelet (Wakamatsu et al., 2008). In this study, the number of spikelet and occurrence in milky white kernels of 'Genkitsukushi' were the highest grown at the Chikugo 2. In addition, proportion of milky white kernels was higher at the secondary branch in panicle than that at the primary branch (Fig. 17). Therefore, it is necessary to hold optimum number of spikelet of 'Genkitsukushi', especially in high soil fertility conditions, for maintaining the highly grain quality (Miyazaki et al., 2011).

Occurrence of white immature kernels was greatly influenced by the spikelet position of the panicle (Nagato and Ebata, 1965; Kido and Yanatori, 1968; Takahashi 2006). Therefore, parameters of grain yield and quality were analyzed to examine the relationship between spikelet architecture and occurrence of the white immature kernels among the spikelet positions of the panicle using two cultivars 'Genkitsukushi' and 'Tsukushiroman' (Fig. 16). The proportion of white immature kernel of 'Genkitsukushi' at any position of the panicle was lower than that of the heat-sensitive cultivar 'Tsukushiroman', nevertheless the number of spikelet and 1000-grain weight were not significantly different in each spikelet position of panicle between two cultivars. It has been reported that heat tolerant rice cultivar 'Nikomaru' was the high amount of the nonstructural carbohydrates (NSC) in the stem at full heading stage, which contributes to the ripening stability under high temperature condition (Morita and Nakano 2011). Tanaka et al. (2009) revealed that high temperature stress did not interrupt development of rice grains, especially of a nucellar epidermis as a morphological property for sugar transport, in heat-tolerant cultivar 'Chikushi 64' (later named 'Genkitsukushi') and 'Nikomaru' at 14 DAH while heat-sensitive cultivar 'Hinohikari' indicated a cessation of the function of the nucellar epidermis. These results suggest that the heat tolerance of 'Genkitsukushi' after heading stage was not related to panicle architecture. It has been reported that tolerance to the high temperature stress after heading stage was genetically controlled and they were relative cultivars of 'Koshihikari' (Nishimura et al., 2000). Yamakawa et al. (2007) reported that the gene expression for the syntheses of starch and storage proteins was decreased by high temperature, while that for starch consumption and heat stress response increased. Therefore, further studies are needed to elucidate the physiological, molecular and genetic mechanism for heat tolerant to accelerate rice breeding.

3.5. Summary

High air temperatures in summer of 2010 severely damaged an apparent quality of rice kernels in a large area of Japan. Nevertheless, the percentage of the first inspection grade of heat-tolerant rice cultivar ‘Genkitsukushi’ was more than 90% even when the average of air temperature during the 20 DAH was over 28°C, while the average of the first inspection grade of ‘Tsukushiroman’ and ‘Hinohikari’ were less than 20 %. In fact, in the different field conditions, such as different soil fertility and different crops cultivated in the previous summer, the occurrence of white immature kernel was significantly lower and the rice kernel of ‘Genkitsukushi’ was superior in quality to other cultivars such as ‘Tsukushiroman’ and ‘Hinohikari’. In addition, the proportion of white immature kernel of ‘Genkitsukushi’ at any position within the panicle was lower than those of ‘Tsukushiroman’. Furthermore, the number of spikelet and 1000-grain weight were not significantly different in each spikelet position within the panicle between two cultivars. These results indicated that the tolerance of ‘Genkitsukushi’ to high air temperatures was stably excellent independent of panicle architecture, suggesting that it was not related to a panicle architecture, but others contributing heat tolerance.

Chapter 4

Growth characteristics during the ripening period of assimilate translocation and gene expression of sucrose transporter, *SUT1* under heat stress in the heat-tolerant rice cultivar 'Genkitsukushi'

4.1 Introduction

High temperature is one of the major environmental stresses that limit the agricultural productivity of plants. Recently, the reduction in grain yield and quality of rice cultivated under heat stress condition is the problem of rice cultivation in Japan (Funaba et al., 2006b; Tanaka et al., 2009; Morita and Nakano, 2011). In particular, the occurrence of white immature kernels under high-temperature conditions reduces the grain quality of rice. When day/night temperature from 7 days after heading (DAH) to harvest maturity stage was 27/22°C, only a few white-core kernels occurred; when the temperatures were above 30/25°C, kernel damage (i.e, an increased percentage of milky white kernels and white-back kernels) increased (Tashiro and Wardlaw, 1991). In addition, it has been reported that white immature kernels occurred when the average air temperature for the first 20 DAH was higher than 27°C and that cultivars differed in the percentage of white immature kernels, for example, the japonica varieties such as 'Hinohikari', 'Mineasahi' and 'Hatsuboshi' were sensitive to high temperature (Morita et al., 2005a; Wakamatsu et al., 2007).

High temperature accelerated rice kernel development during the early ripening period, while it interferes with the kernel development during the late ripening period, thereby decreasing grain weight somewhat (Yamakawa et al., 2007; Ishimaru et al., 2009; Tanaka et al., 2009). It has been reported that high temperature accelerated the rate of grain dry weight (Morita et al., 2005b; Chen et al., 2006). It is conceivable that grain filling under high temperature requires more nutrition during the early development stages, which might then trigger the rapid remobilization of stored carbon. The carbohydrate supplied for rice ripening consists of two components: the nonstructural carbohydrates (NSC) in the stem at full heading and the newly assimilated carbohydrate after heading (Matsushima 1957; Weng et al., 1982). It has been reported that, in terms of carbohydrate supply, insufficient solar radiation during ripening period affects grain growth (Tsukaguchi and Iida, 2008). When rice plants were exposed to high temperature during the ripening period, the sink-source balance of carbohydrates was disrupted, and white immature kernels were produced (Morita, 2008).In fact, alteration of the carbohydrate supply affects the percentage of white immature kernels (Morita et al., 2005a; Nakagawa et al., 2006; Tsukaguchi et al., 2011). Recently, it was suggested that the level of high-temperature tolerance of rice was related to the NSC content in the stem at the full-heading stage (Morita and Nakano, 2011).

In rice plants, the sucrose transporter gene *SUT1* is highly expressed in leaf sheaths, stems, grains after heading, and also in germinating seedlings, but very low levels in roots (Aoki et al., 2003; Scofield et al., 2007). These findings suggest that SUT1 plays an important role in maintaining the supply of photoassimilates to the filling grains (Scofield et al., 2002). Furthermore, the cumulative expression level of *SUT1* in grain between 8 and 30 days after flowering was reduced under high-temperature conditions, suggesting that this alteration in gene expression is involved in grain weight reduction and/or occurrence of grain chalkiness (Yamakawa et al., 2007). Recently, our group reported that high-temperature-induced repression of the expression of *SUT1* and starch-synthesis-related genes in sink and source organs at the milky ripening stage is involved in occurrence of chalky grains (Phan et al., 2013).

High air temperatures in summer of 2010 severely damaged an apparent quality of rice kernels in a large area of Japan (Ministry of Agriculture, Forestry and Fisheries, 2011). Nevertheless, the percentage of the first inspection grade of 'Genkitsukushi' bred and cultivated in Fukuoka Prefecture was more than 90% even when the average of air temperature during the 20 DAH was over 28^{o}C, while the average of first inspection grade of 'Hinohikari' was only 11.1% (Hamachi et al., 2012). Furthermore, 'Genkitsukushi' was highly evaluated at palatability ranking of the rice production as special A indicating the highest award among 129 candidates (Japan Grain Inspection Association 2011). Therefore, it is very important to reveal growth characteristics and the mechanism of heat tolerance in 'Genkitsukushi' as a leading heat-tolerant cultivar for the purpose of extending a cultivation area in the world.

Based on these findings, the objective of this study is to gain insights into heat tolerance through the physiological differences between heat-tolerant cultivar 'Genkitsukushi' and heat-sensitive cultivar 'Tsukushiroman'. In this chapter, dynamics of carbohydrate translocation during the ripening period in both cultivars with different levels of heat tolerance was investigated by analyzing the dry weight of stem and grain, NSC content in stem and *SUT1* expression in stem and grain during the ripening period in both cultivars with different levels of heat tolerance.

4.2 Materials and methods

4.2.1 Plant materials, heat stress and leaf clipping treatments

Heat-tolerant rice (*Oryza sativa* L., japonica type) cultivar 'Genkitsukushi' and heat-sensitive rice cultivar 'Tsukushiroman' indicating the same heading date were used in this study (Miyazaki et al., 2011). 'Genkitsukushi' (former named 'Chikushi 64') was developed from a cross between ‘Tsukushiroman’ (former named ‘Chikushi 46’) and ‘Tsukushiwase’ at Fukuoka Agricultural Research Center in 2008 (Wada et al., 2010).

Growth chamber experiment was performed at Fukuoka Agricultural Research Center in 2010. Plants were transplanted into 1/5000 Wagner pots on 10 June in 2010 at the rate of three plants per pots. Irrigation and pesticide were applied to ensure optimal plant growth. Compound fertilizer (N-P_2O_5-K_2O:4-4-4%) at 0.2 N g was supplied to each pot, as basal dressing. Additionally, 0.1Ng ammonium sulfate (N:21%) was topdressed at the panicle formation and booting stages. Plants were allowed to grow to the heading stage in an experimental field. At heading, which occurred for both of the cultivars on 16 August, the pots were transferred to glasshouses and the plants were grown under individual two temperature treatments, control (26/21°C day/night) or high-temperature stress (31/26°C day/night), until harvest. The 24-hour average temperatures in the control and high temperature treatments were 23.5°C, and 27.3°C, respectively.

A field experiment was performed at the experimental field of Fukuoka Agricultural Research Center in Chikushino City, Fukuoka, Japan in 2009. Plants were transplanted in paddy field on 25 May 2009. The young seedlings with 3.0-3.5 leaf stage were transplanted with planting density of plant/ (30 × 15 cm^2). Fertilizer of 5.0kg N/10a was supplied as a basal nutrient. At the heading stage on 10 August sink-source manipulation by cutting every flag leaf blade was imposed on the five plants each cultivar. the average temperature during 20 days after heading was 27.5 ^oC.

The rice plants were harvested at the maturity stage. Then threshing and husking treatments were carried out after air drying adjusted at 15% water content in brown rice, and preparation was sieved at 1.8mm. Inspection grade was evaluated as 10 degrees, 1st grade (1, 2, 3), 2nd grade (4, 5, 6), 3rd grade (7, 8, 9) and below standard (10) according to Japan Grain Inspection Association, and perfect kernels were measured with grain discriminate RGQI20A (Satake Co. Ltd., Japan). 100 kernels of the husked grains of each temperature treatment were used for their quality and they were visually evaluated and scored according to Hoshikawa (1993).

4.2.2. Analyses of dry-matter production and nonstructural carbohydrate (NSC)

Plants were sampled at 5-30days every 5 day interval after heading (DAH). The heading stage is the date when about a half of the panicles in an area emerged. In phytotron, three pots in each treatment were sampled as a pot-experiment in 2010, and as a field experiment, five plants were sampled at each stage of the two cultivars. Plants were separated into panicles, leaf blades, and culms plus leaf sheaths (stems), oven dried an 80^{o}C for 48 hours and dry weight was measured. Dried samples of stems were ground and used for measurement of NSC. The NSC concentration in the stem was estimated according to the method of Ohnishi and Horie (1999). Distilled water (20 mL) was added to a 100 mL Erlenmeyer flask containing about 0.5g samples. The flask were boiled on a hot plate for 30 min at 100°C and then for 20 min at 180°C. After cooling, 20mL of sodium phosphate-phosphoric acid buffer containing 1.5mg of α-amylase, 0.5mg of amyloglucosidase, and 0.5mg of sodium azide was added. Each sample was incubated with continuous shaking for 24h at 40°C and then filtered through filter paper (No.5A; Advantec, Tokyo, Japan). The residues were dried at 135oC for 4 h to determine their DW. The NSC concentration (%) was estimated as;

NSC concentration = (dried stem weight – residual weight) / dried stem weight ×100,

4.2.3. RNA extraction and real-time PCR analysis

For RNA extraction, stems and grains were harvested from plants grown in three pots of each temperature treatment and cultivar at 4, 9, 14, and 21 DAH. Immediately after harvest, the stem and grain samples were frozen in liquid nitrogen and then stored at – 80°C. Total RNA was isolated from the frozen materials by an SDS/phenol/LiCl method (Chirgwin et al., 1979). cDNA was synthesized from the extracted RNA by using the ReverTra Ace reverse transcriptase kit (TOYOBO, Osaka, Japan). To synthesize single-stranded cDNA, a total 20μL of reverse transcriptase reaction mixture containing 1μg of total RNA, 2.5 M oligo-dT17, 0.5 mM of each dNTP, and 100 U of ReverTra Ace in the manufacturer's standard buffer conditions was annealed at 65°C for 5 min and then incubated at 42°C for 1 h.

Real-time PCR was performed using the MiniOpticon Real-Time PCR System (Bio-Rad Lab., Hercules, CA, USA), with SYBR Green (TOYOBO, Osaka, Japan) as the fluorescent dye, according to the manufacturer's instructions. The primer sequence of *SUT1* was according to Phan et al. (2013). PCR was performed in a 20-μL reaction mixture containing 1 μL of cDNA, 500 nM each of the 5'- and 3'-primers, 10 μL of SYBR Green buffer, and 2 μL of Plus solution containing Taq DNA polymerase under the following thermal cycling conditions: 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 30 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 (Phan et al., 2013). The specificity of the individual PCR amplifications was confirmed by using a heat-dissociation curve protocol following the final cycle of the PCR.

4.3 Results

4.3.1. Characteristics of yield and quality in heat tolerant/sensitive cultivars

The proportion of ripened grains, 1000-grain weight, physiochemical characteristics, and grain quality are shown in Table 14 and Fig. 18. High-temperature stress did not significantly affect the grain yield per pot, spikelet per pot, proportion of ripened grains, or 1000-grain weight of either cultivar (Table 13). The grain quality of ‘Genkitsukushi’ grown under high-temperature conditions during the ripening period was not significantly different from the control (Fig. 18). In contrast, the grain quality of heat-stressed ‘Tsukushiroman’ significantly decreased. The milky white kernels and white-back kernels in ‘Genkitsukushi’ were 3.2% and 2.7%, respectively for the control, while 5.8% and 12.4%, respectively for the high-temperature treatment.

Table 13
Effect of high temperature treatment on rice grain ripening, kernel quality and protein and amylose contents of 'Genkitsukushi' and 'Tsukushiroman'.

Cultivar	Temp. day/night (℃)	Grains per pot (g)	Spikelets per pot	Ripened grains (%)	1000-grain weight (g)	Inspection grade	perfect kernel (%)	Protein (%)	Amylose (%)
Genkitsukushi	26/21	18.1 b	884	94.8	21.3	2.0 a	87.0 bc	6.3 ab	17.6 d
	31/26	17.0 ab	852	95.9	21.1	2.6 a	82.8 bc	6.6 c	15.6 b
Tsukushiroman	26/21	16.1 a	798	93.4	21.4	2.0 a	89.5 c	6.4 bc	16.2 c
	31/26	16.0 a	812	94.5	20.9	9.6 b	67.5 a	6.1 a	14.4 a

1) Inspection grade of kernels in 10 degrees: 1st grade (1-3), 2nd grade (4-6), 3rd grade (7-9) and below standard (10). Different letters indicate significant differences at the 5% level (Tukey's test).

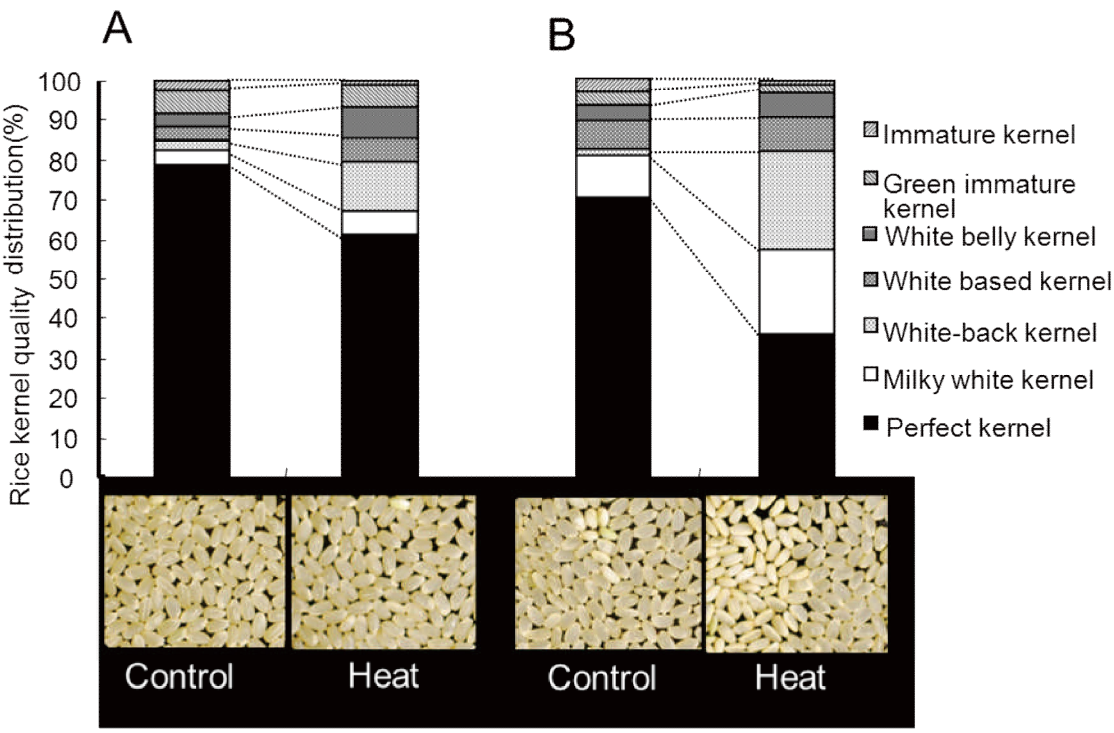

Fig. 18
Effect of high temperature treatment on the quality of grains of ‘Genkitsukushi (A)’ and ‘Tsukushiroman (B)’.

In contrast for 'Tsukushiroman' the occurrence of milky white kernels and white-back kernels of the control were 10.5% and 1.6%, respectively, while 21.3% and 25.3%, respectively for the high-temperature treatment.

The high-temperature treatment caused a significant decrease in the grain amylose content of both cultivars (Table 13). There was no significant difference in grain protein content between the two cultivars grown under the control conditions. The protein content of the grains of 'Genkitsukushi' grown under high temperature during the ripening period increased compared to the control, whereas that of 'Tsukushiroman' decreased.

In addition, effects of decreased photoassimilate on grain quality were examined byclipping treatment of flag leaf blade of rice plants at an experimental field where average air temperature was 27.5°C during 20 days after heading. Table 14 and Fig. 19 show effects of sink-source manipulation by the clipping treatment on yield and grain quality of the two cultivars. There were no significant difference of total numbers of spikelets, 1000-grain weight and ripened grain yield of the two cultivars between control and the clipping treatment. Ratios of the ripened grains and grain quality of 'Genkitsukushi' without the clipping treatment as a control indicated superiority to that of 'Tsukushiroman'. The ratios of the ripened grains and perfect kernels of 'Genkitsukushi' of the control was 93.2 and 93.4%, respectively while that of 'Tsukushiroman' was 90.8 and 76.3%, respectively (Table 14). Furthermore, percentages of milky white kernels and white backed kernels of 'Genkitsukushi' of the control were 0.5 and 8.8%, respectively while those of 'Tsukushiroman' were 7.7 and 12.0%, respectively (Fig. 19). Therefore, it was shown that sink-source manipulation by the clipping treatments of the flag leaf blade resulted in decrease in number of ripened grains, 1000-grain weight, ripened grain yield and perfect kernels of both two cultivars. However, decrease in number of ripened grains, 1000-grain weight, ripened grain yield, while in increase in milky white kernel caused by the sink-source manipulation treatment were more severely enhanced in 'Tsukushiroman' than those in 'Genkitsukushi'.

Table 14 Effect of clipping treatments of flag leaf blade on the proportion of ripened rice grains, perfect kernel of 'Genkitsukushi' and 'Tsukushiroman' grown in the field.

Cultivar	Clipping treatments	Ripened grain yield (g/m²)	Total panicles (m²)	Total spikelets (×100/m²)	Ripened grains (%)	1000-grain weight (g)	Inspection grade	Perfect kernel (%)
Genkitsu kushi	Control	459 b	336	217	93.2 b	22.6 b	2.0 a	93.4 b
	treatment	427 ab	334	212	90.3 b	22.4 b	2.6 a	91.8 b
Tsukushi roman	Control	434 ab	333	211	90.8 b	22.7 b	4.3 b	76.3 a
	treatment	388 a	348	212	81.9 a	22.0 a	4.4 b	73.5 a

1) At the heading stage on 10 August, every flag leaf blade was clipped on each five plant of the two cultivars as a sink-source manipulation. Inspection grade of kernel was evaluated of 10 degrees. ;1st grade (1, 2, 3), 2nd grade (4, 5, 6), 3rd grade (7, 8, 9) and below standard (10). The different letters indicate significant differences in the means at 5% level (Tukey's test).

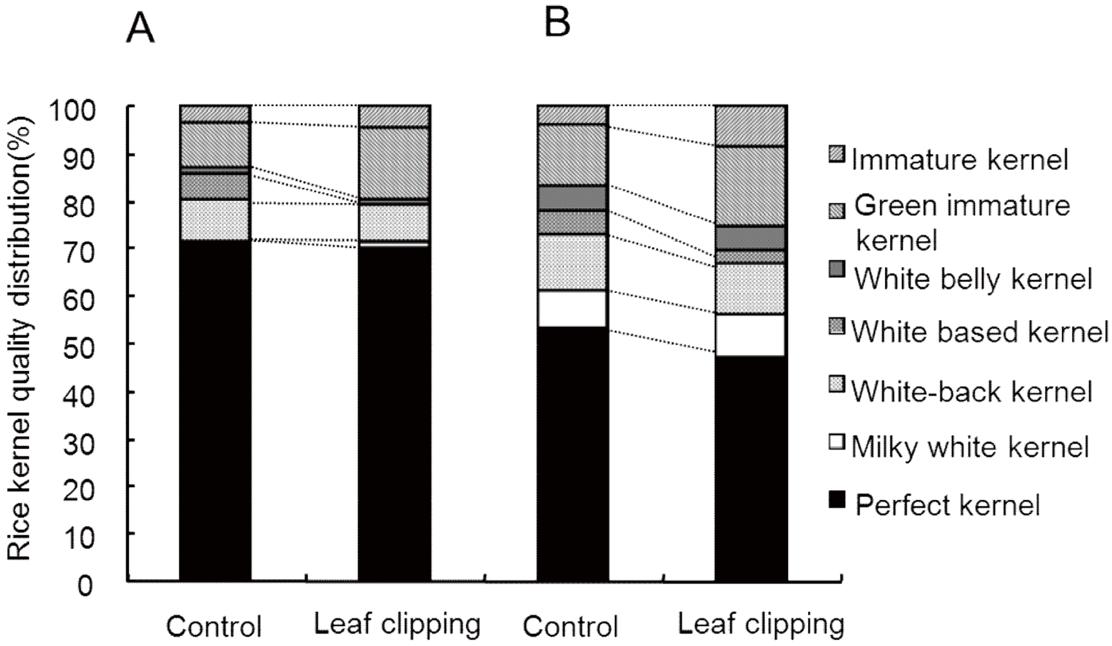

Fig. 19 Effect of clipping treatments of flag leaf blade on the quality of grain kernel of 'Genkitsukushi (A)' and 'Tsukushiroman (B)'.

4.3.2 Analysis of dry-matter production and NSC of heat tolerant/sensitive cultivars

Dry-matter production of 'Genkitsukushi' and 'Tsukushiroman' grown in different temperature chambers from 5 to 30 DAH was determined. There was no significant influence of high temperature on the kernel dry weight in 'Genkitsukushi' while that of 'Tsukushiroman' was significantly enhanced at 10 DAH by the high temperature stress (Fig. 20A B). The dry weights of whole plant per panicle both at 5 and 30 DAH of 'Genkitsukushi' in the control were 2.2 and 2.9g, respectively while that of 'Tsukushiroman' was 1.8 and 2.8g, respectively (Fig. 20C D). The increasing amount of dry-matter content in whole plant per panicle of 'Genkitsukushi' in the control from 5 to 30 DAH was lower than that of 'Tsukushiroman'. On the contrary, the decreasing amount of dry-matter both of the stem and leaf blade of 'Genkitsukushi' in the control from 5 to 30 DAH was 0.4g higher than that of 'Tsukushiroman', and the tendency was remarkably enhanced by high temperature stress. In the same way, the increase in dry weight of aboveground part of 'Genkitsukushi' from 5 to 30 DAH in the field experiment carried out in 2009 was 0.7g lower than that of 'Tsukushiroman', and the decreasing amount of dry-matter content in the stem and leaf blade of 'Genkitsukushi' from 5 to 30 DAH was 0.6g higher than that of 'Tsukushiroman' (Fig. 20E F).

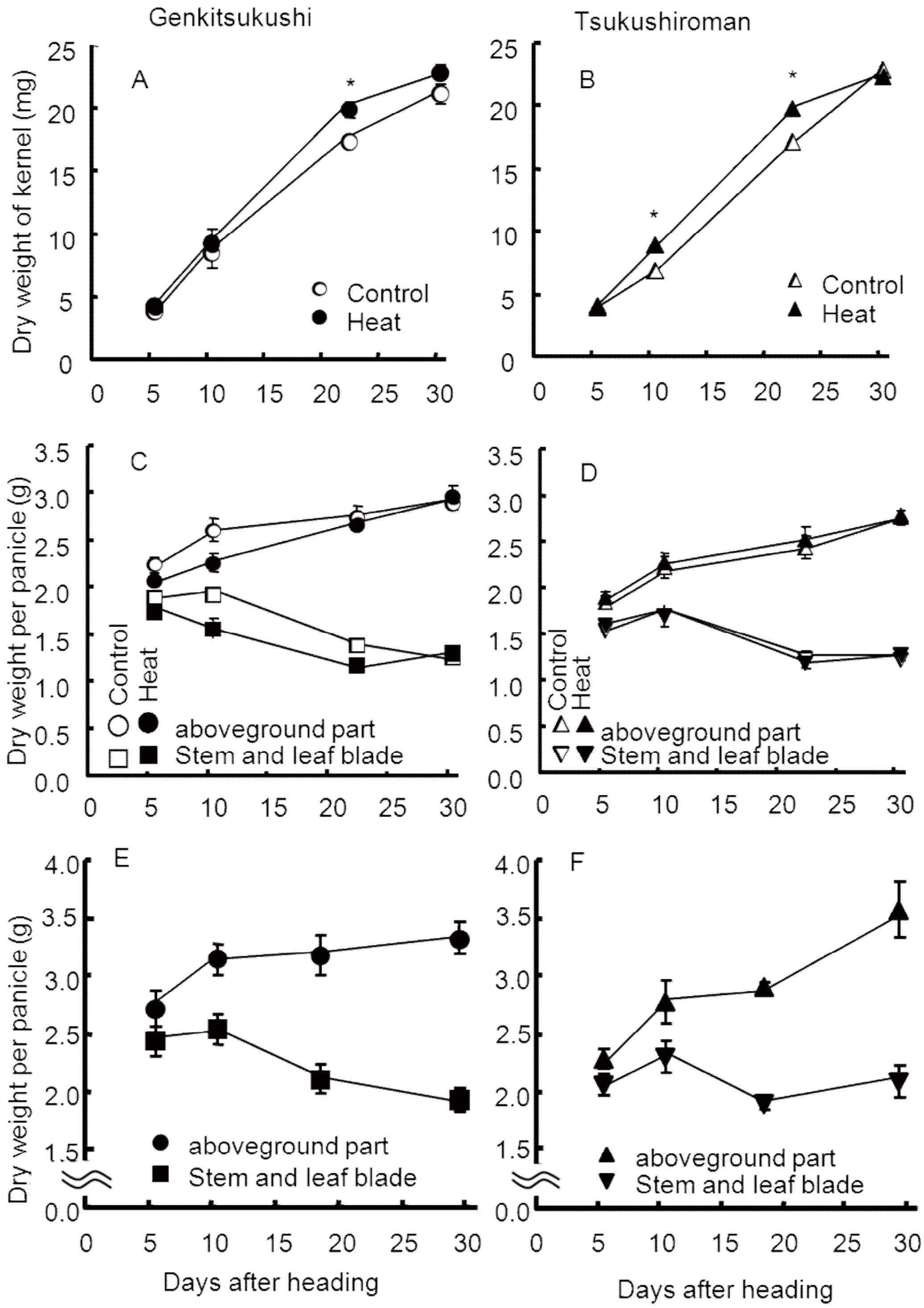

Fig. 20 Dry weight of kernels, stems, leaf brades and aboveground part in 'Genkitsukushi' (A,C,E) and 'Tsukushiroman' (B,D,F) grown under high temperature. A, B, C, D, Growth chamber experiments tested in 2010; E F, Field experiments tested in 2009. Values are means ± SE (n=5). * indicates significance at the 5% level (Tukey's test).

NSC content in the stem was also shown in Fig. 21. Especially, NSC content of the control of 'Genkitsukushi' at 5 DAH was 102mg (+26%) higher than that of 'Tsukushiroman'. NSC concentration of stem and the content per panicle of 'Genkitsukushi' remarkably decreased under high temperature stress than those of 'Tsukushiroman' from 5 to 22 DAH. Those of 'Genkitsukushi' from 5 to 22 DAH was 92mg (+50%) higher than that of 'Tsukushiroman'.
These results suggest that the dry matter translocated from stem to panicle in 'Genkitsukushi' was greater han that of Tsukushiroman', and the tendency was significantly enhanced by high temperature stress during ripening period.

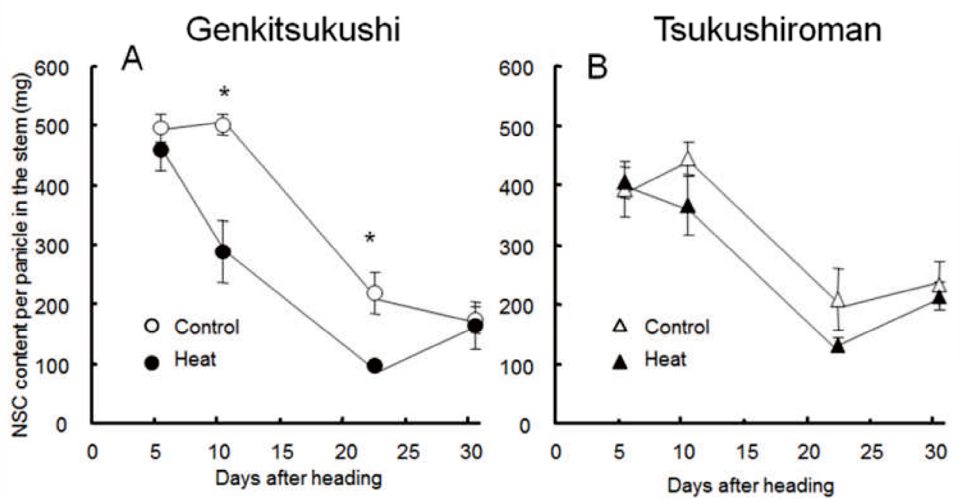

Fig. 21 Nonstructural carbohydrate (NSC) content in stems of 'Genkitsukushi' (A) and 'Tsukushiroman' (B) grown under high temperature. * indicates significance at the 5% level (Tukey's test).

4.3.3 RNA extraction and sucrose transporter gene (*SUT1*) analysis of heat tolerant/sensitive cultivars

SUT1 plays an important role in maintaining the supply of photoassimilates to the filling grains (Scofield et al., 2002). Therefore, real time PCR analysis was carried out to assess the changes in *SUT1* transcript levels in the stems and grains of cultivars 'Genkitsukushi' and 'Tsukushiroman' under high temperature (Fig. 22). In stems of 'Genkitsukushi', there was no significant difference in the expression of *SUT1* between the two temperature treatments from 4 to 9 DAH, but the expression level in that treated by the high temperature markedly increased from 9 to 21 DAH (Fig. 22A). In contrast, in stems of 'Tsukushiroman', the expression of *SUT1* under the high temperature increased at 9 DAH compared with the control, then it decreased by 14 DAH to a level significantly below the control (Fig. 22B). The patterns and levels of *SUT1* expression in the grains were noticeably different between the two cultivars. Under the control temperature, the expression of *SUT1* in the grains of 'Genkitsukushi' decreased from 4 to 14 DAH, but it increased between 14 and 21 DAH' while the gene expression of the cultivar exposed to heat stress maintained higher level compared to that in the control (Fig. 22C). In contrast, the expression of *SUT1* in grains of 'Tsukushiroman' decreased from 9 to 21 DAH in both temperature treatments, but it was significantly higher at 9 DAH under high-temperature stress than in the control (Fig. 22D). In addition, the expression level of *SUT1* during grain filling stage was markedly higher in 'Genkitsukushi' grains than in that of 'Tsukushiroman' grains, regardless of temperature.

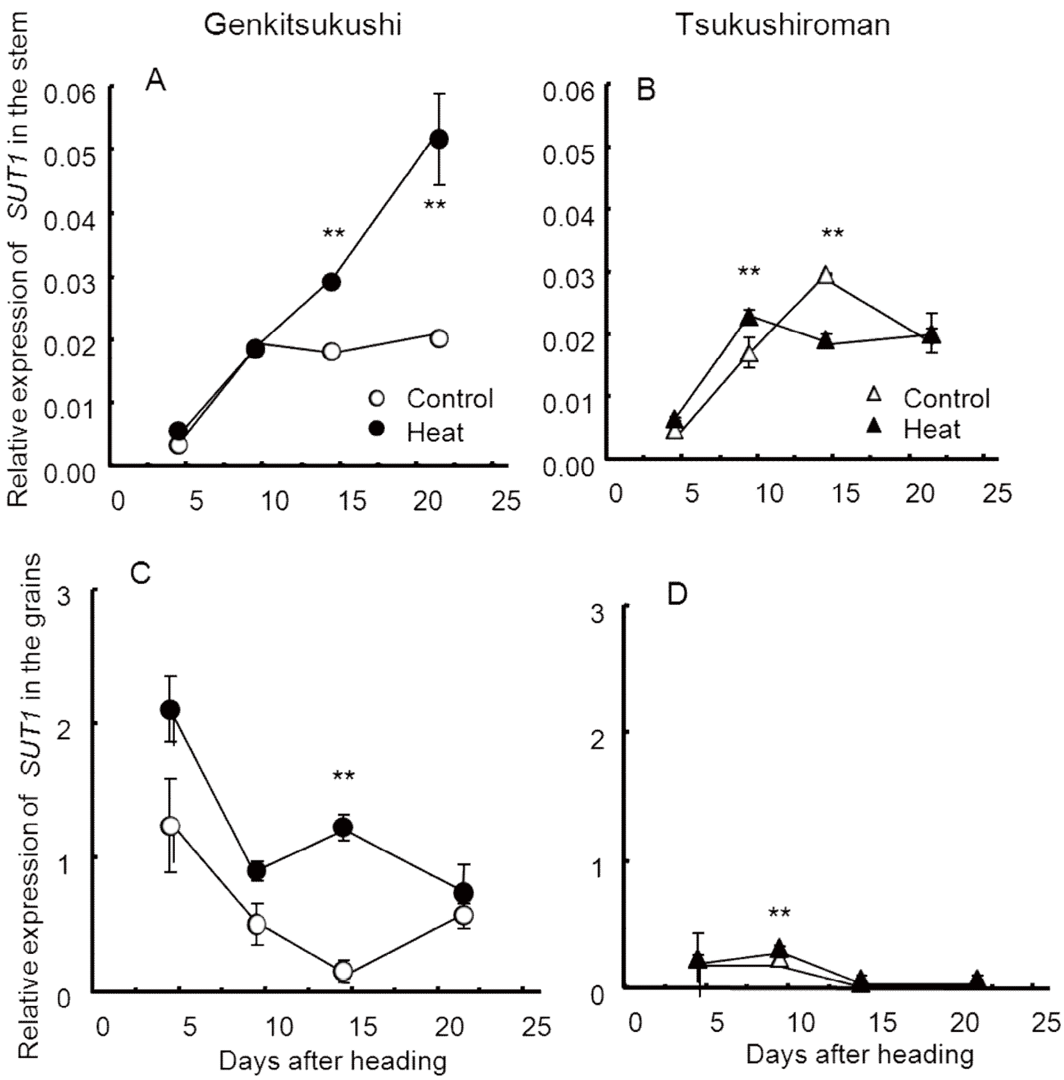

Fig. 22 Gene Expression profiles of *SUT1* in stems (A,B) and grains (C,D) of 'Genkitsukushi' and 'Tsukushiroman' grown under control (open symbols) and high-temperature (closed symbols) treatments.

1) The expression levels were determined by quantitative real-time PCR analysis and normalized to the expression of *OsActin*. Values are means ± SE (n=3). ** indicates significance at the 1% level (Tukey's test).

4.4. Discussion

4.4.1 Grain filling in 'Genkitsukushi' during the ripening period under high temperature stress

High-temperature stress did not significantly affect grain yield, spikelets per pot, percent ripened grains and 1000-grain weight (Table 13). However, the grain quality in 'Genkitsukushi' was remarkably superior to that of 'Tsukushiroman' (Fig. 18), even though the heading date of these cultivars was the same and the genetic background of the two cultivars are closely related because 'Tsukushiroman' is a mother plant of 'Genkitsukushi'. The grain of 'Genkitsukushi' grown under high temperature throughout the ripening period was maintained at the first inspection grade because only a few white immature kernels was produced (Table 13). In the recent study, Hamachi et al. (2012) reported that the proportion of white immature kernels of 'Genkitsukushi' at any position within the panicle was lower than those of the heat-sensitive cultivar 'Tsukushiroman' grown under the high air temperature of 2010 summer, which has a similar panicle architecture, suggesting that the difference of heat tolerance between the two cultivars is due to other factors rather than the panicle architecture. Microscopic observation by Zakaria et al. (2002) revealed that the chalky part of the rice endosperm contains immature starch granules, suggesting that the accumulation of starch is impaired at high temperature. Under high temperature, a lack of assimilate supply to grains caused a reduction in grain weight and increase in the proportion of milky white kernels, possibly because high temperature during the grain-filling period enhances grain growth rate without profoundly affecting assimilate production (Morita et al., 2005a). At 10 DAH, which is the most heat-sensitive phase, rapid growth led to the production of milky white kernels (Tashiro and Wardlaw, 1991). The kernel dry weight in 'Genkitsukushi' grown under high temperature was not significantly different compared with that of the control (Fig 20A). In contrast, heat stress had a significant effect on the kernel dry weight at 10 DAH in 'Tsukushiroman' (Fig. 20B). In the present study, these results indicate that the unaltered dry weight of grain at 10 DAH in 'Genkitsukushi' under the heat stress condition might contribute to maintain its higher level of grain quality.

4.4.2 Change in sink-source balance of the stem in 'Genkitsukushi' during the ripening period under high temperature stress

It has been previously reported that a reduced carbohydrate supply to the panicle increased the percentage of milky white kernels (Morita et al., 2005a; Nakagawa et al., 2006; Tsukaguchi et al., 2011). Dry weights per panicle of the stem and leaf blade in 'Genkitsukushi' significantly decreased compared to those of 'Tsukushiroman' during the ripening period both in the growth chamber and the field experiments (Fig. 20C-F), and the decrease in dry weight of the stem and leaf blade in ‘Genkitsukushi’ was enhanced by high temperature (Fig. 20C, D). Most notably, per-panicle NSC content in the stem of ‘Genkitsukushi’ grown under high temperature was higher than that in ‘Tsukushiroman’ at 5 DAH and it decreased markedly by 24 DAH (Fig. 21). These results indicate that assimilate transport in ‘Genkitsukushi’ functioned more effectively than that in ‘Tsukushiroman’ under the high temperature condition. It has been reported that clipping treatment reduced the amount of carbohydrates supplied through photosynthesis (Nakagawa et al., 2006; Tsukaguchi et al., 2011). In the present study, I examined whether sink-source manipulation by leaf clipping influenced on the number of ripened grains, 1000-grain weight, grain yield and the percentage of milky white kernels in both cultivars (Table 14, Fig. 19). The decrease in the number of ripened grains, 1000-grain weight and ripened grain yield was statistically significant that in ‘Tsukushiroman’, whereas no effect of high temperature on that of ‘Genkitsukushi’ was significantly observed. Moreover, white immature kernels increased in ‘Tsukushiroman’ but not in ‘Genkitsukushi’ treated by leaf clipping. These results also indicated that content or translocation of assimilates in the stem of ‘Genkitsukushi’ was superior to ‘Tsukushiroman’, and these data were consistent with the results of the heat-stress treatments.

These data showed the occurrence of white-back kernel of ‘Genkitsukushi’ grown under high temperature was only 9.7%, while that of ‘Tsukushiroman’ was 23.7% (Fig.18). In addition, high temperature enhanced protein content of grains in ‘Genkitsukushi’ by 0.3% compare to the control while it suppressed that of ‘Tsukushiroman’ by 0.3% (Table 13). Wakamatsu et al.(2008) reported that white-backed kernels occurred frequently in cultivars such as ‘Hatusuboshi’ and ‘Hinohikari’ whose ripening were sensitive to high temperatures, and negative correlation was found between the protein content of grain and the occurrence of white-back kernels. In addition, Nakagawa et al. (2006) reported that white-back kernels decreased by applying nitrogen top-dressing, indicating to strengthen the transportation system of assimilates increased by applying an increment of nitrogen application.

Based on these findings and their reports, it was suggested that ‘Genkitsukushi’ promoted nitrogen accelerated under high temperature, resulting in high ratio of perfect grains and low ratio of white-back by higher transportation system of assimilates. Yamakawa et al. (2007) reported that 13-kD prolamin, one of the major storage proteins of rice grains, specifically decreased in response to high temperature, therefore, further studies are needed to clarify the function of the expression such as the 13-kD prolamin on heat tolerant/ sensitive cultivars.

4.4.3 Noticeable characteristics of sucrose transporter gene (*SUT1*) of ‘Genkitsukushi’ in the stem and grains as a heat tolerant cultivar

In developing rice caryopses, *SUT1* mRNA and protein were found at high levels both in the aleurone and maternal cell layers (Furbank et al., 2001; Scofield et al., 2002). The high level of *SUT1 e*xpression indicates its primary role in post-phloem active transport of sucrose into the endosperm. Scofield et al. (2002) reported that antisense-mediated suppression of *SUT1* led to impaired grain filling, which confirms the crucial role of *SUT1* in grain filling. In the present study, the expression of *SUT1* in the stem of 'Genkitsukushi' did not differ significantly between the control and heat-treated plants from 4 to 9 DAH; thereafter, the expression of *SUT1* in the stem of 'Genkitsukushi' exposed to high temperature linearly increased until 21 DAH, whereas that in the control slightly changed (Fig. 22A). In contrast, the expression of *SUT1* in the stem of 'Tsukushiroman' grown under heat stress increased enhanced from 4 to 9 DAH and then it decreased, reaching a level significantly below that in the control by 14 DAH (Fig. 22B). Recently, our group has reported that expression of *SUT1* in the grain of 'Hinohikari', a heat-sensitive cultivar, was decreased by heat stress (Phan et al., 2013). In the present study, *SUT1* expression in the grains of 'Genkitsukushi' was noticeably higher (2 to 30 times) than that of 'Tsukushiroman', and it increased under the high-temperature condition (Fig. 22CD). In addition, the expression of *SUT1* in the grains of 'Genkitsukushi' was significantly higher than that of 'Tsukushiroman' during the ripening period, regardless of temperature. These results indicated that sugar transport from the stem to grains functions more effectively in 'Genkitsukushi' than that in 'Tsukushiroman' under high-temperature condition during ripening period.

Kobata et al. (2011) reported that rice cultivars with a higher percentage of milky white kernels showed a higher rate of grain dry-matter increase under elevated temperatures, resulting in a lack of assimilate supply to the grains. In addition, antisense suppression of *SUT1* results in poor grain-filling accompanied by a decreased rate of sucrose uptake into seed tissues (Scofield et al., 2002). In the present study, it was shown that the heat tolerant cultivar 'Genkitsukushi' has a high NSC content in the stems at heading stage and displays a high expression of *SUT1* in stems and grains which would contribute to maintain its higher level of quality even under high temperature condition.

4.5. Summary

I carried out a physiological and molecular biological approaches such as assimilate translocation and gene expression of *SUT1* during the ripening period under heat stress for the purpose of revealing the mechanism of heat tolerance in 'Genkitsukushi'. When day/night temperatures were 31/26°C from heading until maturity, the grain quality of 'Genkitsukushi' was rated the first inspection grade (highest quality). In contrast, that of 'Tsukushiroman' showed a remarkable increase in the percentage of white immature kernels (lower quality). Non-structural carbohydrate content in the stem of 'Genkitsukushi' at the early maturation was significantly higher than that of ‘Tsukushiroman’ and it greatly decreased under high temperature. From 14 to 21 DAH, the expression of the sucrose transporter gene, *SUT1*, was higher in the stem of ’Genkitsukushi’ grown under high temperature than that of ‘Tsukushiroman’. In addition, the gene expression of *SUT1* in the grains of ’Genkitsukushi’ was significantly higher than that in ‘Tsukushiroman’ during the ripening period. These results indicated that sugar transport functions more effectively in ‘Genkitsukushi’ than in ‘Tsukushiroman’, and that the effectiveness of sugar transport contributes to maintain high grain quality in ‘Genkitsukushi’ even under high-temperature conditions.

‘Genkitsukushi’ was named to be expected that ‘Genki’ means energetic. I could revealed the noticeable characteristics on the view point of sink-source balance as a promising highest palatable rice cultivar even under high temperature through the ripening period. My study findings should be valuable for controlling assimilate supply by using molecular genetic approaches, and for breeding of heat-tolerant rice cultivar that produce good quality under high temperature condition.

ABSTRACT

Global warming reduces grain quality of rice in Japan. In particular, the occurrence of white immature kernel has become a serious problem. White immature kernel occurs when average temperature during 20 days after heading (DAH) was 27°C and over and cultivars varied great deal in the percentage of white immature kernels. Extremely hot summer 2010, it caused severely deterioration grain quality of rice all over Japan. Nevertheless, the rate of the first inspection grade of *Oryza sativa* L. 'Genkitsukushi' was more than 90%, while that of 'Hinohikari' and 'Koshihikari' that are major cultivars in Japan, and 'Tsukushiroman' was less than 20%, respectively. 'Genkitsukushi' was bred and cultivated in Fukuoka Prefecture is tolerant to high temperature, and 'Tsukushiroman' is a pollen parent of 'Genkitsukushi'. Therefore, I studied on heat-tolerant mechanism of 'Genkitsukushi' as well as improvement method for yield and quality of heat sensitive cultivars under high temperature conditions.

The number of spikelet per square meter of 'Hinohikari' was necessary to be suppressed 28,000-30,000 to obtain the perfect kernel rate more than 75% and a yield more than 530 kg/10a under high temperature conditions. Especially, it was effective method to be delayed application timing of top-dressing nitrogen from 18 days before heading (DBH) to 7 DBH to suppress the number of spikelet per unit area and occurrence of white immature kernel. Grain quality exposed to 30°C before and after the heading stage was inferior to those of 25°C treatments due to the occurrence of white immature kernels of 'Koshihikari'.

At first, a biophysical approach was carried out to investigate the effects of high temperature stress on dynamic states of water in rice grains before and after the heading stage of 'Hinohikari' and 'Koshihikari' by monitoring ^{1}H-nuclear magnetic resonance (NMR) relaxation times (T_1, T_2). The T_1 of grains showed parallel relationship to the changes in the degree of dehydration, and that exposed to 30°C before and after the heading stage was lower than those of 25°C treatments at 14 or 21 DAH. The T_2 of grains exposed to 30°C before and after the heading stage treatments were also abruptly shortened at 21 or 28 DAH. Furthermore, the next approach was focused on the water channels called aquaporins regulating the dynamic state of water molecules. The expression of aquaporin gene, *PIP1;1* , was higher in the grains of 'Koshihikari' exposed to 30°C before and after the heading stage at 14 DAH. These results suggested that the changes on dynamic states of water such as NMR relaxation times and expression of aquaporin genes in rice grain exposed to high temperature before or after the heading stage would reflect a reduction in the quality of rice.

During the extremely hot summer 2010, especially late August and early September that is an early ripening period of the cultivars used in this study, the average air temperature was over 29°C. Therefore, it was carried out physiological and molecular approaches such as sugar

translocation and expression of sucrose transporter gene, *SUT1,* during the ripening period under high temperature stress after the heading stage. Non-structural carbohydrate content in the stem of heat tolerant ‘Genkitsukushi’ at the early maturation was significantly higher than that of heat sensitive ‘Tsukushiroman’ and it greatly decreased under high temperature. From 14 to 21 DAH, the expression of *SUT1* was markedly higher in the stem of ’Genkitsukushi’ grown under high temperature than that of ‘Tsukushiroman’. In addition, the gene expression of *SUT1* in the grains of ’Genkitsukushi’ was significantly higher than that in ‘Tsukushiroman’ during the ripening period. In conclusion, it was revealed that sugar transport functions more effectively in ‘Genkitsukushi’ than in ‘Tsukushiroman’, and that the effectiveness of sugar transport contributes to maintain high grain quality in ‘Genkitsukushi’ even under high-temperature conditions.

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和文摘要

水稲品種「元気つくし」の高温耐性機構解明と品質改善技術に関する研究

地球温暖化の進行に伴い，水稲では登熟期の高温による収量および品質の低下が問題となっている．九州の主要品種である「ヒノヒカリ」は，高温感受性が高いことが指摘されており，福岡県においても 2002 年以降，1 等米比率が 50%を下回る状況が続いている．このような中，高温耐性品種として福岡県で開発された「元気つくし」は，猛暑年の 2010 年において，1 等米比率が他の品種は 20%未満であったにも関わらず 90%以上を確保し，さらに米の食味ランキングでは，2011 年産以降，3 年連続で最高ランクの「特 A」に格付され，その評価は高まっている．本研究では，「元気つくし」の高温耐性機構解明を主目的とし，「ヒノヒカリ」の高温対策技術および出穂前の高温影響評価についても検討を行った．

第１章　温暖化に対応した「ヒノヒカリ」の高品質安定栽培技術

著しい高温となった 2010 年産および高温の影響が少なかった 2009 年産の「ヒノヒカリ」を供試して，籾数と収量，検査等級および外観品質との関係を検討した．その結果，検査等級は整粒割合と相関が高く，検査等級 1～2 等の分岐点は整粒割合 75％で，これを維持するためには㎡当たり籾数を 30,500 粒以下に抑える必要があり，㎡当たり籾数が 28,000 粒以上あれば，収量 530kg/10a 以上を確保できることが明らかとなった．

以上のことから，外観品質の低下を抑え，かつ収量性を確保するという観点から，県の平年収量（499kg/10a）を考慮し，目標収量を 530～540 kg/10a とした場合，㎡当たり籾数 28,000～30,000 粒が温暖化に対応した「ヒノヒカリ」の適正籾数と考えられた．

穂肥時期（幼穂形成期）の窒素吸収量と㎡当たり籾数には高い相関が認められ，幼穂形成期までの窒素吸収量がわかれば籾数を予測できることや，幼穂形成期までの窒素吸収量は，草丈と茎数と葉色（SPAD 値）との積との相関が高いこと．㎡当たり籾数は，茎数と葉色（SPAD 値）に高い相関があることから，籾数予測のための重回帰式を作成し、それをもとに籾数予測表早見表を作成した．これにより，葉色（SPAD 値）と茎数を測定すれば，早見表により穂肥時に籾数を予測でき，穂肥診断が可能となった．さらに，籾数を抑え白未熟粒を低減するためには，穂肥時期を従来（出穂前 18 日）より遅らせ，出穂前 7 日頃に実施することが有効であることが明らかとなった。

第２章　出穂前後の温度処理による登熟中のイネ種子における水分動態、関連遺伝子発現および玄米品質の変動

「ヒノヒカリ」および「コシヒカリ」を供試して，出穂前後の高温処理が登熟中のイ

ネ種子の水分動態、遺伝子発現および玄米品質に及ぼす影響について検討を行った．登熟期間中の水分含有率と NMR スピン-格子緩和時間（T_1）は同様な推移を示し，出穂前後の 30 ºC 処理（Heat 処理）では 25ºC 処理（Control 処理）と比べて出穂後 14 日もしくは 21 日目の水分含有率および T_1 の値は有意に低下し，出穂後 21 日もしくは 28 日目では NMR スピン-スピン緩和時間（T_2）の著しい低下が認められ，自由水の急激な消失が観察された．以上のことから，NMR 緩和時間 T_1 および T_2 は登熟中のイネ種子の水分動態を示す有効な指標であると考えられた．

アクアポリンは，生体膜に存在し，主に水を選択的に輸送するチャンネルで，細菌から植物まで普遍的に存在しており，イネでは 33 種類のアクアポリン遺伝子が確認されている．そこで，出穂前後の温度処理によるイネ種子中のアクアポリン遺伝子の発現に注目して実験を行った．この結果，「コシヒカリ」の出穂前後 30 ºC 処理では，出穂後 14 日目において，アクアポリンをコードする遺伝子の 1 つである *OsPIP1;1* 遺伝子の発現量が上昇し，さらに，白未熟粒の増加により外観品質が低下した。

以上の結果から，高温ストレスは，出穂前後に関わらず登熟期間中の水分動態や *OsPIP1;1* 遺伝子発現量に影響し，外観品質は低下することが明らかとなった。

第３章　2010 年の夏期高温条件下における高温耐性水稲品種「元気つくし」の玄米品質

2010 年の夏は全国的に猛暑で，水稲の登熟期間における気温が記録的な高温となり，多くの地域で玄米品質が著しく低下した．しかし，この登熟温度 (出穂期後 20 日間の平均気温) が 28℃を超える高温年においても，福岡県内の「元気つくし」は 1 等米比率が 90%以上であり，しかも土壌の肥沃度や前年の作付けが異なる圃場においても白未熟粒の発生が少なく，玄米品質が優れていた．また，「元気つくし」は，高温により品質が低下しやすい「つくしろまん」と比較して，登熟温度がほぼ同じで，1 穂内の着粒構造が似通っているにもかかわらず，いずれの着粒位置においても白未熟粒の発生が少ない傾向にあった．

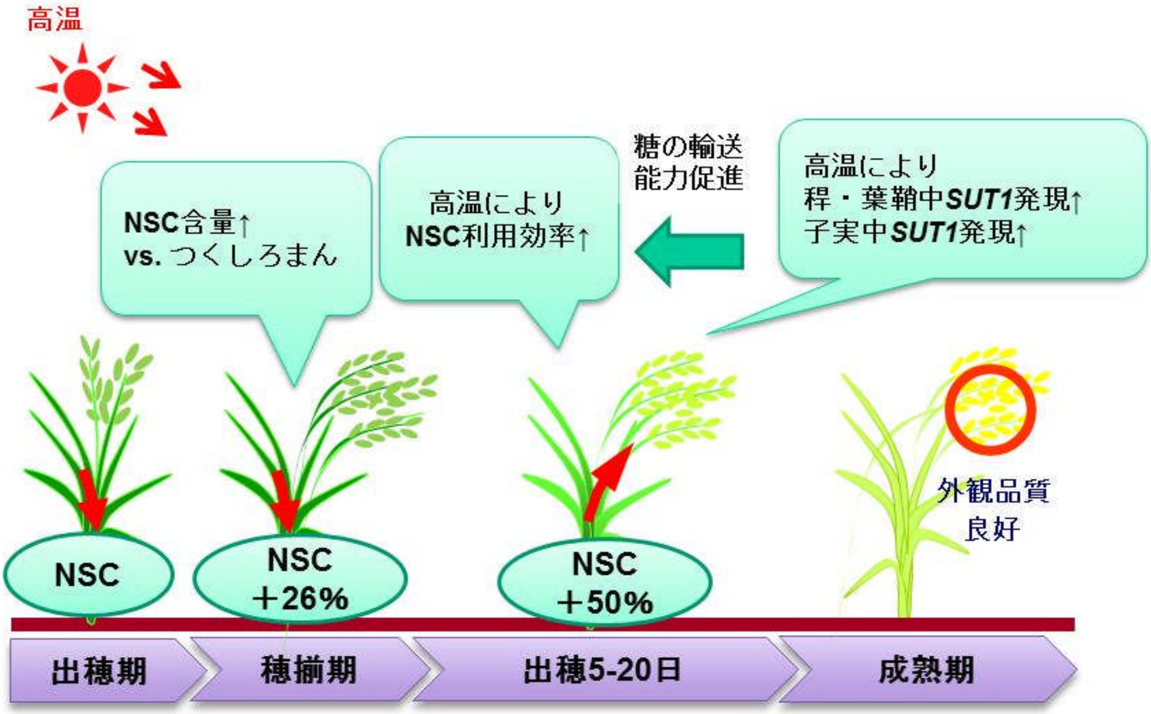
以上の結果から，「元気つくし」が有する高温耐性は安定して優れており，そのことには穂の着粒構造以外の生理的要因が関与していると考えられた．

第４章　高温耐性品種「元気つくし」の登熟期間中の高温条件下における炭水化物供給能とショ糖トランスポーター（*SUT*1）遺伝子発現の特性

高温耐性品種「元気つくし」と「つくしろまん」との比較により，とりわけ，出穂後の高温処理が炭水化物供給能とショ糖トランスポーター（以下，*SUT*1）遺伝子発現の誘導に及ぼす影響と品種間差異を検討した．「元気つくし」では，すべての処理区において，検査等級は 1 等に格付されたのに対し，「つくしろまん」の昼/夜：31/26 ºC 処理区（Heat 区）では，26/21 ºC 処理区（Control 区）と比べて白未熟粒の多発により，整粒割

合が 20%程度低下し，検査等級は 3 等～規格外に格付された．籾数，千粒重および収量は処理区による明らかな差は認められなかった．「元気つくし」では，出穂後 5-10 日間の稈・葉鞘＋葉の乾物重の減少程度が大きく，これが子実重の増大に寄与していることが示唆され，Heat 区ではこの傾向がさらに顕著であった．Control 区においても，「元気つくし」は、「つくしろまん」と比べて出穂後 5 日目の稈・葉鞘中の NSC 含量が 26%高く，出穂後 5-22 日間の稈・葉鞘中の NSC 減少量は 50%高く，Heat 区ではさらに NSC 減少量が多くなった．Heat 区の稈・葉鞘中の *SUT*1 遺伝子の発現量は，「つくしろまん」で抑制されたのに対し「元気つくし」では逆に増加した．さらに，子実中の *SUT*1 遺伝子の発現量は，いずれの処理区とも「元気つくし」の方が「つくしろまん」と比べて有意に高かった．

以上の結果から，「元気つくし」では，穂揃期までに稈・葉鞘中に蓄積された NSC が効率的に利用され，高温条件下では，より活発に利用されていると考えられた．また，その要因の一つとして *SUT*1 遺伝子の発現誘導の影響も考えられ，これらの生育特性が，良好な外観品質の維持に貢献しているものと考えられた．



SUT1:炭水化物の輸送には「糖トランスポーター」と呼ばれる遺伝子群が関与し，中でも，ショ糖トランスポーター遺伝子（*SUT1*）は米の登熟に深く関与している．近年の研究により，「ヒノヒカリ」では登熟期間中の高温により *SUT1* 遺伝子の発現量が減少し，これが白未熟や粒重低下につながる要因の一つであることが明らかとなっている(Phan et al. 2013, Ishibashi et al. 2014)．つまり，*SUT1* 遺伝子の発現量が高いほど種子への糖の輸送が容易になり，結果的に米の登熟，収量および品質に好影響を与えると考えられる．