Original Paper

Reduced rice grain production under high-temperature stress closely correlates with ATP shortage during seed development

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Abstract High-temperature stresses during seed development reduces rice grain yield and causes poor milling quality because of insufficient grain filling. We monitored the progress of seed enlargement and the fluctuation in ATP content during seed development. When the rice cultivars Nipponbare and Taichung-65 seeds were developed in a high-temperature condition, a shortage in the amount of ATP was detected in the early and middle stages of seed development. This observation suggested that high temperature caused the insufficient accumulation of storage substances leading to the formation of small seeds along with chalky or white core endosperm. The *flo2* mutant, which is thought to lack a regulatory factor for storage starch biosynthesis, produced chalky endosperm that resembled the typical phenotype of the high-temperature ripening-grain. The *flo2* mutant exhibited a shortage of ATP content in immature seeds, suggesting that the gene responsible for the *flo2* mutation is also involved in ATP production during seed development. In contrast, Kinmaze seeds maintained sufficient ATP content and their features did not differ significantly between mature grains developed in the normal- and high-temperature environments. These findings suggest that Kinmaze has some trait that is involved in the tolerance to high-temperature stress.

Key words: High-temperature ripening, seed development, rice, whole white and white core endosperm.

The activity of grain production is sensitive to abiotic stresses such as high or low temperature and submergence or desiccation. The upcoming global warming will cause high-temperature stress during the grain-filling stage, which will reduce crop yield and quality (Peng et al. 2004). The panicles are extremely sensitive in the developing stage (Tashiro and Wardlaw 1991; Morita et al. 2004). During seed development, many features of the rice grains are changed by a hightemperature environment, and the grains may display chalky texture, milky appearance, and lower grain weight (Yamakawa et al. 2007). This phenomenon is considered to be caused by the formation of large airspaces in the endosperm because of the insufficient growth of starch granules (Tashiro and Wardlaw 1991; Zakaria et al. 2002). In high-temperature ripening, expression of multiple genes, such as those for starch biosynthesis in the endosperm, is repressed, resulting in reduced amylose content and aberrant amylopectin structure (Yamakawa et al. 2007).

Many rice mutants show peculiar grain features that resemble those harmed by high-temperature stress. Five Cold stress increases the expression of the genes for mitochondrial adenosine triphosphate (ATP) synthesis and electron transport (Byun et al. 2009). Microarray data on high-temperature-ripened rice seeds (Yamakawa et al. 2007) show decreased expression of genes predicted to function in ATP synthesis. ATP is a representative energy compound that is essential for multiple cellular functions. We analyzed the relationship between the process of seed enlargement and fluctuations in ATP content in developing seeds and compared these between three rice cultivars and the *flo2* mutant, which had been ripened at high-temperature. Here, we report on

rice *floury* mutants seem to mimic the chalky endosperm seen in high-temperature-ripened grain (Satoh and Omura 1981; Kaushik and Khush 1991). Among them, the *flo2* mutant is unique because of the reduced expression of multiple genes involved in storage starch biosynthesis. This suggests that the *flo2* mutant has a defect in the activity of some regulatory network that influences multiple cellular functions and that the gene responsible for the *flo2* mutation encodes a regulatory factor (Kawasaki et al. 1996).

Abbreviations: ATP, adenosine triphosphate; DAF, days after flowering.

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the differences between strains in tolerance to the hightemperature stress during seed development, and discuss the importance and regulation of the ATP supply on the sufficient grain filling.

Materials and methods

Rice cultivars, mutants and growth condition

Oryza sativa L. japonica rice cultivars, Nipponbare, Kinmaze, and Taichung65, and *flo2* mutants were used in this study. The *flo2* mutants EM37, EM139, EM280, and EM373 are derived from Kinmaze, and EM924 is from Taichung65 (Satoh and Omura 1981). Plants were grown in pots in the open air in the summer season. For the high-temperature treatment, at 5 days after flowering (DAF), the plants were transferred into a growth chamber and grown after under a 12h/12h day/night cycle at 33°C/28°C with constant 70% humidity, according to the method of Yamakawa et al. (2007). The immature seeds were harvested at 5, 7, 10, 15, 20, and 25 DAF, and stored in a deep freezer. The mature seeds were harvested at 40 DAF. Morphological changes were determined by measuring of seed size and weight. White core and other phenotypes of the endosperm were determined by illumination using backlight.

ATP measurement

ATP content in immature seeds was measured by the luciferase assay system coupled with the reaction from luciferin to oxyluciferin, using a Lucifer250-plus kit (Kikkoman Co., Japan) (Kimmich et al. 1975) and the luminescent intensity of oxyluciferin was detected by ARVOTMLight luminometer (PerkinElmer, USA). The weight of each immature seed was measured and the seed was powdered independently in liquid nitrogen using an AutoMill TK-AM5 (Tokken Inc. Japan), then it was suspended in ATP extraction buffer of the Lucifer250-plus kit.

Results and discussion

Comparison of features of high-temperatureripening rice grains and flo2 mutant

To estimate the tolerance of the three rice cultivars, Nipponbare, Taichung-65, and Kinmaze to the hightemperature stress during seed development, we compared the phenotypic properties of grains developed at high temperature. The mature grain average weights were 6% and 7% less for Nipponbare and Taichung-65 grown under high temperature, respectively, than for grains grown under the normal condition (Figure 1).

We measured the grain length, width, and depth of mature grains of Nipponbare and Taichung-65. The Nipponbare seeds became narrower but thicker when developed at the higher temperature (Figure 1). The mature Taichung-65 grains developed at high temperature were significantly narrower and thinner than those grown under the normal condition, although the length of the grains did not change (Figure 1). These observations indicate that, when developed at high temperature, the Taichung-65 grains were smaller than those developed at the normal temperature, and that the Nipponbare grains exhibited changes in their features. In contrast, Kinmaze seeds showed no obvious difference in feature when matured at high and normal temperatures. The weight of these grains did not differ significantly between the normal- and high-temperature conditions, although their mature seeds showed shorter, wider, and thicker features in average as compared with those developed in the normal condition (Figure 1).

We analyzed the progress of the grain-filling process in developing seeds of these cultivars and compared them between the grains developed at high and normal temperatures. Among these cultivars, Taichung-65 seeds showed a repressed grain-filling process under hightemperature stress, but no significant difference was found for the cultivars Nipponbare and Kinmaze (Figure 2).

The early (\leq 5 DAF) and middle (7–20 DAF) stages of seed development are important for grain filling because storage starch and proteins are actively produced and contribute to enlarging the size of developing seeds. In immature seeds exposed to high-temperature stress during these stages, we assumed that their viability would be reduced because of the inhibition of seed growth and the insufficient accumulation of storage substances. Seeds with insufficient grain filling show the formation of chalky and white core features because of the generation of many airspaces in the endosperm. After the seed developmental stages, grain weight decreases through desiccation. Under the normal ripening condition, the total weight decreased by about 15-35% in the mature seeds of each cultivar. However, in the high-temperature environment, the magnitude of the decrease in grain weight differed between cultivars.

The major defective feature of Nipponbare grain grown under high-temperature stress appeared as a chalky area in the dorsal and proximal side or white core endosperm, such as basal-white and white-back grains (Figure 1A, B, Table 1). In contrast, Kinmaze and Taichung-65 seeds showed no obvious changes in their features, although a high proportion of Kinmaze grain produced basal-white grains under our experimental condition (Figure 1C-F, Table 1). These results indicate that the response to the high-temperature stress differed between cultivars. Nipponbare was very sensitive to the high-temperature stress, whereas Taichung-65 was only moderately sensitive, as shown by an apparent reduction in yield because of decreased grain size. In contrast, Kinmaze appeared to have a resistant trait that may overcome the high-temperature stress because Kinmaze grains showed no significant alteration in either grain weight or visible features.



Figure 1. Properties of grains developed in the normal- and high-temperature environment. (A) Grain weight of Nipponbare, Taichung-65, Kinmaze, and EM37 (*flo2* mutant). Open and filled boxes indicate average weight of 60 mature grains developed in the normal- and high-temperature condition, respectively. Error bars represents standard errors of the mean. (B–G) Appearance of mature grains of Nipponbare (B, C), Taichung65 (D, E), and Kinmaze (F, G) developed in the normal environment (B, D, F) and high-temperature environment (C, E, G). Photographs were taken under backlight illumination. Bar=5 mm. (H–J) Seed length, width, and depth of mature grains of Nipponbare (H), Taichung-65 (I), and Kinmaze (J). Open and filled boxes indicate the average data for the grains developed in the normal environment and high-temperature environment, respectively. Error bars show standard errors of the mean.

Table 1. Percentage of damaged grains developed in the normal- and high-temperature environments.

Grain feature	Nipponbare		Taichung 65		Kinmaze		EM37	
	Normal	High	Normal	High	Normal	High	Normal	High
Perfect grain	97.4	7.1	90.8	85.5	43.4	44.6	0.0	0.0
Basal white	1.3	57.1	0.0	1.6	47.4	42.9	0.0	0.0
White back	0.0	75.7	1.5	6.5	5.3	8.9	0.0	0.0
White belly	1.3	5.7	1.5	0.0	5.3	5.4	0.0	0.0
White core	0.0	7.1	6.2	6.5	9.2	14.3	0.0	0.0
Milky white	0.0	0.0	0.0	3.2	1.3	0.0	100.0	100.0

Typical appearances of damaged grain features are indicated. Values are expressed as percentages of the total grains. The sum of the values was not 100% because several grains showed duplicated features. EM37 is a *flo2* mutant derived from Kinmaze. "Normal" and "High" indicate grains developed in the normal- and high-temperature conditions, respectively.



Figure 2. Time course of seed enlargement during seed development of Nipponbare (A), Taichung-65 (B), and Kinmaze (C). Values are expressed as the average weights of six immature seeds harvested at 5, 7, 10, 15, 20, and 25 days after flowering (DAF), and mature seeds (40 DAF). Open and filled boxes show those developed in the normal- and high-temperature environments, respectively. Error bars represent standard errors.

Fluctuation in ATP content in developing seeds during the grain-filling process under hightemperature stress

These cultivars were developed in the normal and the high-temperature conditions, and the ATP content of the developing seeds was measured at 5, 7, 10, 15, 20, and 25 DAF. As shown in Figure 3, the ATP content fluctuated depending on the developing stages. In the middle stage of seed development (7–20 DAF), higher ATP content was maintained in the immature seeds that developed under the normal conditions. These stages coincided with those when storage starch was actively produced, although the timing of the high ATP concentration shifted slightly, depending on the cultivar.



Figure 3. Time course of fluctuation in ATP content during seed development in the normal- and high-temperature environments. ATP contents were measured in 6 immature seeds of Nipponbare (A), Taichung-65 (B), and Kinmaze (C) at 5, 7, 10, 15, 20, and 25 DAF. Open and filled boxes indicate those developed in the normal- and high-temperature environments. Error bars represent standard errors.

In immature Kinmaze seeds, the ATP content was elevated after 10 DAF, whereas seeds of the other cultivars showed increased ATP content at 5 DAF. In addition, Kinmaze seeds showed a peculiar pattern of fluctuating ATP content, as shown by another peak of ATP concentration at 20 DAF.

When immature seeds were developed at high temperature, Nipponbare and Taichung-65 showed low ATP content through the seed development stages (Figure 3A, B); a significant decrease in ATP content was observed at 7 DAF and 15 DAF. In contrast, the immature Kinmaze seeds had a higher ATP content under these conditions in the early stage, similar to the level developed under the normal conditions, and lower ATP content in the immature seeds in the late-middle stage (20 DAF) (Figure 3C). These results suggest that Kinmaze and the other cultivars have different patterns of fluctuation in the ATP content during seed development.

Nipponbare was very sensitive to the high-temperature stress, as shown by the decrease in the size and weight of the seeds and the visible changes in their features. The immature seeds of these cultivars also had reduced ATP content, suggesting that ATP production was impaired by the high-temperature stress. Nipponbare seeds developed in the high-temperature condition showed an adequate increase in size, but an inadequate amount of ATP was supplied during seed development, suggesting that they accumulated an insufficient amount of storage substances. Similarly, reduced ATP content was also observed in the flo2 mutants, which produced small grains with a milky white phenotype. ATP is required for multiple cell functions including plant growth and storage substance accumulation. We conclude that the occurrence of the phenotypic changes such as small grains or white core endosperm were strongly linked to the decrease of ATP content in the developing seeds.

In contrast, Taichung-65 produced small-sized grains, which significantly reduced the grain yield when they were developed at the higher temperature. Grains with normal features were produced from Taichung-65, and the inefficient enlargement of seed growth was the typical phenotype of the high-temperature-ripened Taichung-65 seeds. These results suggest that Taichung-65 is moderately sensitive to high-temperature stress, which reduces productivity without any visible changes.

In Kinmaze, the weight and appearance of the mature grain did not differ between seeds developed in the high and normal temperature environments. The high-temperature-ripening immature Kinmaze seeds continuously maintained a relatively high ATP content throughout the seed development stages, and the ATP shortage seemed to be rescued in the immature Kinmaze seeds. Although low ATP content was detected in the late-middle stage of seed development, the ATP shortage at this stage had little effect on seed development. Enlargement of the grain size and production of storage substances would be almost completed before the late seed-development stage. This observation suggests that Kinmaze has some trait that is involved in tolerance to high-temperature stress. We assume that this trait gene enhanced or rescued the ATP production in immature Kinmaze seeds, which were able to grow adequately and accumulate sufficient storage substance.

Comparison of flo2 mutant seeds with hightemperature-ripening wild-type seeds

The *flo2* mutant produced milky white grains with chalky endosperm, which resembled the typical phenotype of the high-temperature-ripened Nipponbare seeds (Figure 4A, B). The grain weight was 27–37% smaller in the mature *flo2* seeds than in seeds of their own background cultivars, Kinmaze and Taichung-65

(Figure 1).

We analyzed two different types of the *flo2* mutants, EM924, and others derived from Taichung-65 and Kinmaze, respectively. EM924 showed highly repressed grain enlargement, which differed from grains of high-temperature-ripened Taichung-65 (Figure 4C). In contrast, the other *flo2* lines derived from Kinmaze showed a similar pattern of grain growth as those of Kinmaze (Figure 4D). These results indicate that the small size of grains of the *flo2* mutants from Taichung-65 and Kinmaze were caused by insufficient seed growth and strong desiccation in the seed-maturation process, respectively. These results also suggest that there were



Figure 4. Features of the *flo2* mutants. (A, B) Appearance of the mature grains of a *flo2* mutant EM37 developed in the normal- (A) and high-temperature environments (B). (C) Time course of seed enlargement of a *flo2* mutant EM924. The weights of six immature EM924 seeds (shown by circles) harvested at 5, 7, 10, 15, 20, and 25 DAF, and mature seeds (40 DAF) were measured during seed development. Those of Taichung-65 (background cultivar) developed in the normal condition (open boxes) and high-temperature condition (filled boxes) are also shown. Error bars represent standard errors. (D) Time course of seed enlargement of *flo2* mutants EM37, EM373, EM139, and EM280, and Kinmaze (background cultivar). Data are expressed as the average values of six seeds. Error bars represent standard errors.

two diverging patterns of the weight-increasing process of the *flo2* mutants, which differed according to the background cultivars.

Lower average ATP content was detected in the immature seeds of all *flo2* mutants (Figure 5). In EM924, the pattern of ATP content was similar to that of the high-temperature-ripening Taichung-65 seeds (Figure 5A). The *flo2* mutants derived from Kinmaze showed peculiar patterns of fluctuating ATP content. These immature seeds had high ATP concentration at 7 DAF, and this concentration decreased rapidly as seed development progressed, although two alleles, EM139 and EM280, also showed a slight increase in ATP content at 15 DAF (Figure 5C). These patterns of changes in ATP content differed markedly from those of the wild-type Kinmaze. These results suggest that the



Figure 5. Alteration in ATP contents in *flo2* mutants during seed development. ATP content was measured in six seeds of each immature *flo2* mutants harvested at 5, 7, 10, 15, 20, and 25 DAF in the normal environmental condition. (A) Data on EM924 (A) and Taichung-65 (normal- and high-temperature conditions), (B) data on EM37 and EM373 with Kinmaze, and (C) data on EM139 and EM280 with Kinmaze are shown. Error bars represent standard errors.

process of seed development of the *flo2* mutant differed according to the background cultivars and that the *flo2* mutation decreased ATP production during seed development. In the early stage of seed development (5–7 DAF), the *flo2* mutants derived from Kinmaze showed high levels of ATP content (Figure 5B, C). This observation suggests fluctuation of ATP content in the early stage may not be so effective in enlarging the seed size, because each of the wild type and the *flo2* mutants showed similar growth curves of seed weights (Figure 4D).

Analysis of the flo2 mutant developed at high temperature

We observed the time course of seed enlargement and the fluctuation in ATP content in the *flo2* mutant EM37 derived from Kinmaze during seed development under the high-temperature condition. The mature grains were 13% smaller on average than those developed under the normal condition, and grain chalkiness was more severe than for those developed under the normal condition. This contrasted with the observation that the mature grain of the wild-type Kinmaze showed little alteration in the size and grain features when developed under high-temperature stress (Figures 1 and 6A). The smaller effect on *flo2* seed enlargement when developed at high temperature suggests that the phenotype of this mutant can be ascribed to the strong diminution of seed size in the last process of seed maturation. The ATP content of



Figure 6. Time course of seed enlargement (A) and changes in ATP content (B) in immature EM37 seeds during seed development at high temperature. The weight and ATP content were measured in six immature EM37 seeds. Error bars represent standard errors.

EM37 grains was lower at 7 DAF when developed in a high-temperature environment, whereas ATP content was greater in the normal condition. After 10 DAF, grains developed in both environmental conditions showed similar patterns of fluctuation of ATP content (Figure 6B). These results suggest that the gene responsible for the *flo2* mutation is involved in both the formation of seed growth and ATP production in immature seeds.

Conclusion

We propose a model accounting for the regulation of ATP synthesis in immature seeds. ATP concentration increases, depending on seed enlargement and storage substance accumulation in immature seeds. The responsible gene for the *flo2* mutation is considered to be a regulatory factor because the multiple starch synthetic enzymes are reduced in mutants (Kawasaki et al. 1996). ATP production is increased by the gene responsible for the flo2 mutation, the defect of which causes marked reduction in ATP production in the middle stage of seed development. The shortage in ATP content reduces grain productivity. ATP production is also inhibited by hightemperature stress in the early and middle stages of seed development. This inhibition is rescued in immature Kinmaze seeds, suggesting that they possess a trait that also positively regulates ATP synthesis.

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