Studies on the chalky grain mutant *floury endosperm11-2* (*flo11-2*) of rice (*Oryza sativa* L.)

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Summary

Rice (*Oryza sativa* L.) is the staple food of more than half of the world's population (Khush, 2005). The rise in temperature associated with climate change is predicted to bring adverse effects on rice production globally. High temperature (HT) during the grain filling of rice diminishes the grain quality as well as productivity. Chalky or opaque grain is one of the main visible damages of grain caused by HT, which leads to lower grade and price, lower milling efficiency and lower palatability of rice. The endosperm of chalky grains is loosely packed and has numerous air spaces between the starch granules.Chalky grains are classified into several types depending on the position of chalky part in the grain: white belly, white core, milky white, basal white or white back, which are caused from different timing and intensity of environmental stress. The molecular mechanism underlying the rice grain chalkiness is complicated and largely unknown, preventing sophisticated development of resistant cultivars and effective agronomical practices. A novel chalky rice mutant '*floury endosperm11-2* (*flo11-2*)' was isolated from heavy ion beam-irradiated rice cultivar Nipponbare of 1,116 lines. The chalkiness of *flo11-2* mutant varied from year to year in a field condition. Thus the mutant seems to be temperature-sensitive and useful for analysis of grain chalkiness under HT stress. The present study was conducted to (i) clarify the underlying molecular mechanism of chalkiness in the *flo11-2* mutant and (ii) determine the most critical meteorological and physiological factors influencing the HT-triggered chalkiness.

The HT response of the mutant was quantitatively analyzed under different temperature conditions with wild type. it was found that under HT at 28 °C of mean temperature, the mutant showed significantly higher chalky ratio (0.4-0.6), which is

defined by the ratio of chalky area to grain area, than the wild type, while showing similar chalky ratio to the wild type under low temperature, indicating that the chalkiness of the *flo11-2* mutant is highly sensitive to HT. To obtain the responsible gene of chalkiness, an exome analysis was performed and found three candidate mutated genes including *flo11-2*, which codes plastid localized 70 kDa heat shock protein 2 (cpHSP70-2). Genotyping of the preceding M_3 generation of the mutant for the three genes showed no association between the other two genes and chalkiness. The flo11-2 has an amino acid substitution on the 259^{th} aspartic acid with valine (D259V) in the ATPase domain. The intrinsic ATPase activity of recombinant cpHSP70-2 was lowered by 23% in the D259V type than in the wild type, indicating that D259V of cpHsp70-2 has important conformational and functional significances. Because Hsp70 is highly conserved in all organisms, a complementation test was carried out using DnaK (HSP70 homologue)-defective *Escherichia coli* (*E. coli*) cells which are sensitive to HT. The growth of the *E. coli* expressing DnaK with D201V mutation (equivalent to the D259V mutation) was severely reduced at 37 °C, but the growth of the *E. coli* expressing the wild type DnaK was not. Thus, the lower ATPase and chaperone activities of cpHSP70-2 are one of the causes for chalkiness in the *flo11-2* mutant. Moreover, the transgenic plants of the mutant expressing the wild type *cpHSP70-2* were generated, to investigate the effect of accumulation level of cpHSP70-2 on chalky ratio (dose effect). A negative correlation was found between the chalky ratio and the accumulation level of cpHSP70-2 in the transgenic mutant lines. The chalky ratio of the WT was lower than the regression line, suggesting that endogenous mutated cpHsp70-2 is competitive to the exogenous WT cpHsp70-2 for some co-factors. However, it was suggesting that enhancing cpHSP70-2 by breeding or agronomical practices such as ‘priming’ might be useful as countermeasure to heat stress.

The different impact of daytime, nighttime and whole day temperatures represented by daily maximum (T_{max}), daily minimum (T_{min}) and daily mean (T_{mean}) temperature, respectively, on grain chalkiness has received little emphasis. In this study, the advantage of the mutant’s high sensitivity was taken by exposure to 5 d short HT stress in each grain filling stage from 2016 to 2019. The 5 d HT treatment was ineffective for the evaluation of temperature response in wild type. Meanwhile, the impacts of 5 d HT treatment in the mutant were obvious and differed year to year. The combined four years quantitative data (n=394) demonstrated that T_{max} was more causative (R^2=0.59, **) for chalkiness than T_{mean} (R^2=0.35, **), while no correlation with T_{min} (R^2=0.01, ^{ns}) was observed. The analysis on the confined variable data within T_{max} of 35.5-37.5 ºC demonstrated that the developmental stage around 20 DAF was most sensitive to HT. Because starch accumulation reaches to maximum around 20 DAF, it was assumed that the more starch accumulate, the more vulnerable endosperm cells are to heat stress. It should be noted that the mean flowering date of all grains in a panicle is several days behind the first flowering date in the panicle. In this study, chalkiness was evaluated at a panicle basis (a mean value of all grains), while the maturing stage (DAF) of a panicle was counted from the first flowering date. Thus, a single grain may be most susceptible to HT at several days earlier than 20 DAF. To confirm the importance of T_{max} for chalk formation, 30 d of continuous high and cool temperature treatments were conducted under field and glasshouse conditions. It was found that the chalky ratio of the mutant in glasshouse was much lower (<0.1) than that of field condition (0.50) with lower T_{max} in the glasshouse than in the field (34.6 ºC vs. 35.9 ºC), even though T_{mean} was similar to each other. The difference in T_{max} as well as cumulative effect of HT and solar radiation between the two growth conditions may cause such large variation in chalkiness of the mutant. The present quantitative analysis indicated that alternative emphasis should be put on for causative factors of chalkiness.

The binding immunoglobulin protein (BiP), endoplasmic reticulum (ER)-localizing HSP70 is increased in the ER after an accumulation of misfolded proteins by some environmental stress. In rice seeds, BiP is associated with prolamins that are hydrophobic and are likely to be easily denatured under heat stress, causing ER stress**.** To cope with ER stress, cells activate the unfolded protein response (UPR) by increasing levels of ER protein-folding enzymes and chaperones, enhancing the degradation of misfolded proteins, and reducing protein translation (Hiramatsu *et al.*, 2015). In this study, only insoluble form of cpHSP70-2 of the wild type was detected using anti-10 kDa prolamin. On the other hand, BiP was increased with decrease of cpHSP70-2 in the mutant. Thus, it is hypothesized that severe ER stress caused by HT changes the localization of cpHSP70-2 and promotes interaction of cpHsp70-2 with10 kDa prolamin denatured by ER stress, leading to poor starch accumulation under heat stress. Future experiments will be directed toward determining whether the cpHsp70-2 involves with ER stress response or not.

The previous chalky rice studies have firstly categorized different types of chalky grain using superior caryopses at a specific panicle position with visual inspection. Meanwhile, the present study used imaging analysis to quantify chalky area on scanned grain images of all grains in a panicle at once that is high-throughput with avoiding sometimes uncertain classification of chalky types. Moreover, a large variation in chalky ratio was found within a panicle, which may be reflected by different flowering date and different sensitivity to HT over developmental stages in a panicle. Inside the grain, starch biosynthesis initiates from the central region and extends towards the periphery through discrete transportation routes of assimilates. Thereby, the classification of chalky grain is crucial to understand how and when the heat stress is exposed within a panicle. Further investigations on the caryopsis location-specific along with chalky type-specific image analysis are necessary to clarify.

The current study demonstrated the involvement of cpHSP70-2 and the impact of T_{max} rather than T_{mean} at around 20 DAF of grain filling stage for the occurrence of chalkiness in the novel chalky rice mutant ‘*flo11-2*’. Because the primary thermal sensing molecular machinery in endosperm cells seemed to be different from cpHSP70-2 but common between wild type and the mutant, the findings in this study and the *flo11-2* mutant with high sensitivity to HT would be useful for the chalky grain research of wild type as well.