Isolation of the chalky grain mutant 13–45 in rice (*Oryza sativa L.*)

T. Katsube-Tanaka,[*1] R. Tabassum,[*1] T. Dosaka,[*1] C. Masuoka,[*1] M. Tsuyama,[*1] Y. Mukai,[*1] C. Nukigi,[*1] H. Ichida,[*2] R. Morita,[*2] and T. Abe[*2]

High temperature during the grain filling of rice diminishes the grain quality as well as productivity. Chalk values and peak head rice yields were quadratically increased and linearly decreased, respectively, against an increase in the nighttime air temperature during the R8 stage where one brown hull appeared on the main stem panicle.[1)] Chalky grains have several air spaces between the starch granules in amyloplast,[2)] resulting in lowered palatability[3)] as well as substantial yield loss due to reduced polishing efficiency.[4)] The molecular mechanism underlying the rice grain chalkiness is supposed to be complicated and largely unknown.

In this study, a novel chalky rice mutant was isolated from heavy ion beam (Carbon, 20 Gy, LET: 22.6–60.3 keV/μm)- irradiated rice (*O. sativa* ssp. *japonica* cultivar Nipponbare) of 1,116 lines. The mutant tentatively named 13–45 showed significantly higher chalky ratio (0.4–0.6), which was defined by the averaged ratio of chalky area to grain area of all brown rice grains within a panicle than the wild type (<0.1) at 28°C (Fig. 1). Meanwhile, the chalky ratio of both the wild type and the mutant became low (<0.06) and similar to each other at 24°C. These results suggested that the rice grain of the mutant 13–45 is very sensitive to high temperature and is useful for the analysis of the mechanism underlying the chalkiness caused by high temperature during grain filling.

Prolamin, the second most abundant and hydrophobic seed storage protein in rice, is composed of 13 kDa and 10 kDa polypeptides. Prolamin is often demonstrated to decrease under heat stress. When the total seed proteins were compared by western blotting with anti-10 kDa prolamin antibody, a band around 70 kDa clearly disappeared in the mutant 13–45. The 70 kDa polypeptide of the wild type was insoluble and purified by 2D-PAGE for Peptide mass fingerprinting, resulting in the identification of chloroplastic 70 kDa heat shock-related protein: cpHsp70.

Rice genome contains two *cpHsp70* genes: *cpHsp70-1* and *cpHsp70-2*. The transcription levels for the two genes were not different between the mutant 13–45 and the wild type. Thus, the coding region and promoter region (~2 kbp) of the two genes in the mutant 13–45 were cloned and sequenced, demonstrating that a single nucleotide polymorphism (SNP) was exclusively detected at the 3' region of the second exon of *cpHsp70-2*. The SNP resulted in amino acid substitution of the 259th aspartic acid with valine (D259 V) in an ATPase domain. The whole exome sequencing analysis of the mutant 13–45 confirmed that only three homozygous mutations including the SNP in *cpHsp70-2* caused non-synonymous substitutions and frameshifts in the coding sequences.

Fig. 1. External appearance of brown rice for the wild type (Nipponbare) and mutant 13–45 grown under conventional field condition (high, 28°C) and cool temperature condition (low, 24°C).

Transgenic plants of the mutant 13–45 expressing wild type *cpHsp70-2* were produced and the chalky ratio averaged by six panicles was compared with those of the mutant 13–45 and the wild type. The chalky ratio of the transgenic line 7(6/12) was significantly lower (0.08) than that of the mutant (0.15–0.23) and comparable to that of the wild type (0.07). The results indicated that *cpHsp70-2* is a causal gene for the chalkiness of the mutant 13–45.

The intrinsic ATPase activity of recombinant cpHsp70-2 was compared between the mutant 13–45 and the wild type. The results showed that the Km of the mutant 13–45 type was slightly lower (higher in affinity) than that of the wild type and Vmax of the mutant 13–45 type was significantly lower by 23% than that of the wild type. Besides, the growth of DnaK (Hsp70 homologue)-defective *Escherichia coli* (*E. coli*) cells complemented with DnaK with D201 V mutation (equivalent to the rice D259 V mutation) was significantly reduced at 37°C. The growth of DnaK-defective *E. coli* cells complemented with wild type DnaK was not different from that of the non-complemented DnaK-defective *E. coli* cells at 37°C. Taken together, these results suggest that the lowered function of *cpHsp70-2* is involved with the chalkiness of the mutant 13–45.

*1 Graduate School of Agriculture, Kyoto University
*2 RIKEN Nishina Center

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