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The conditional chalky grain mutant '*flo11-2*' of rice (*Oryza sativa* L.) is sensitive to high temperature and useful for studies on chalkiness

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ABSTRACT

Chalky grains of rice are increased due to high temperature (HT) during a ripening period. However, the underlying mechanisms of the chalkiness are not well known, seemingly due to the obtuse response of wild type and lack of effective mutants. In this study, we isolated and characterized the *flo11-2* mutant, which showed higher number of chalky grains than wild type under HT but quite small number of chalkiness under cool temperature as well as similar growth, development, and yield to that of the wild type. Using this high sensitivity of the *flo11-2* mutant, we identified the most critical meteorological factor and developmental stage affecting chalkiness with 5 days HT treatments over 4 consecutive years. The results demonstrated that daily maximum temperature was more causative than daily mean or minimum temperatures which have been regarded as important factors before this. Besides, the developmental stage around 20 days after flowering (DAF) was most sensitive to HT rather than the early stage up to 15 DAF. In addition, we found that the *flo11-2* mutant with a high chalky ratio was vulnerable to preharvest sprouting, which has never been reported before for chalky grains, but could cause significant yield and quality loss after extremely hot and dry summers followed by rainy cool autumns. The *flo11-2* mutant is, therefore, a useful material for chalky grain research.

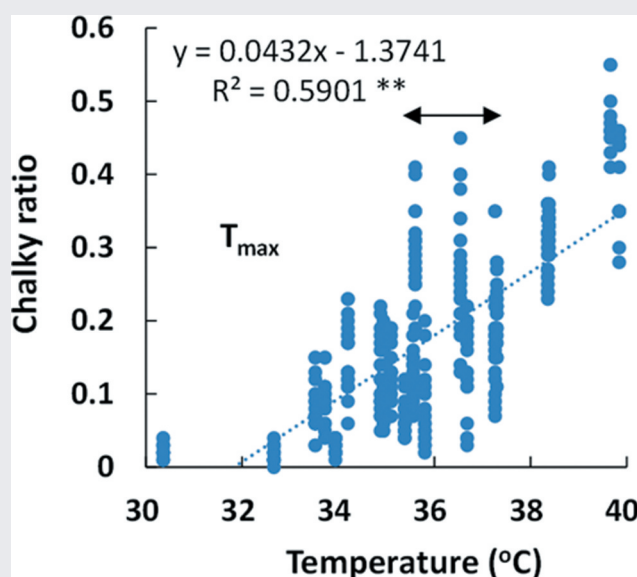
Abbreviations: T_{max} : Daily maximum temperature; T_{mean} : Daily mean temperature; T_{min} : Daily minimum temperature; DAF: Days after flowering; DAH: Days after heading; Ext_{30} : Excess temperature above 30 °C; HDT: High day temperature; HNDDT: High night and high day temperature; HNT: High night temperature; HT: High temperature; HTR: High temperature resistant; HTS: High temperature susceptible; BW: Basal white; MW: Milky white; WB: White back

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Introduction

Endosperm chalkiness is qualitative and quantitative characteristics of improper grain filling in determining grain appearance quality (Peng et al., 2014; Sun et al., 2015; Zhu et al., 2018), caused by HT (Nevame et al., 2018; Zhao & Fitzgerald, 2013). Occurrence of grain chalkiness has been a major problem in recent years; improvement in grain quality by reducing chalkiness is becoming a desired goal for rice breeders and producers. The abnormal HT during grain filling periods can change caryopses component of rice, such as starch and storage proteins causing chalky grains (Kaneko et al., 2016), making the grain vulnerable to breakage during milling (Buggenhout et al., 2014; Fitzgerald & Resurreccion, 2009). Scanning electron microscopy analysis reveals that the opaque part of heat-exposed chalky endosperm is filled with small, loosely packed starch granules, and protein bodies (Han et al., 2012; Ishimaru et al., 2009; Sreenivasulu et al., 2015; Xi et al., 2014). Grain chalkiness might be caused by common consequences, such as accelerated grain filling rate and reduced grain filling duration, poor grain weight, low amylose content, and/or reduced assimilate supply (Chen et al., 2012; Liu et al., 2013). The adverse effects of heat on grain chalkiness depend on the timing, duration, and intensity of the HT treatments (Morita et al., 2016; Zhen et al., 2019). Morita et al. (2016) reported that mean surface air temperatures greater than 26°C during the grain filling could profoundly cause chalky grains with reduced weight.

So far, two types of temperature treatment systems were employed for the chalkiness studies, artificial growth chamber (Chaturvedi et al., 2017; Yamakawa et al., 2007) and natural field (K. J. Lee et al., 2015; Thuy & Saitoh, 2017). The artificial growth chamber can control temperature but usually possess a narrow vertical difference in temperature and may have different meteorological factors, such as light intensity, humidity, wind speed, and so on to natural conditions, while natural experimental fields with or without heating systems are similar to actual crop production environments but a substantial diurnal variation occurs with temperature differing by 7–12°C during summer of temperate and subtropical regions (Poorter et al., 2016). Moreover, unresolved issues remain with respect to the different impacts between realistic, sinusoidal diurnal temperature profiles under field conditions, and artificially controlled square- or triangle-wave temperature profiles under chamber conditions on chalkiness.

In addition, 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 not been fully elucidated. It has been reported that HNT led to the chalky phenotype (Dhatt et al., 2019; Okada et al., 2011; Shi et al., 2013, 2017; Song et al., 2013). The studies on HNT reported that abundance of sugar and sugar alcohols (Dhatt et al., 2019), a reduction in non-structural carbohydrate pool size (Shi et al., 2013) or higher maintenance respiration which then decreases assimilate supply (Cheng et al., 2009; Mohammed & Tarpley, 2010) influence chalk formation. An increase in the HNT during the R8 stage (when at least one brown hull appears on the main stem panicle) degrades the overall appearance of milled rice and generally results in lower head rice yield (Ambardekar et al., 2011; Lanning et al., 2011). Meanwhile, Dai et al. (2009) suggested that HNT exerted similar but less effects compared with HDT. Li et al. (2011) also found that HDT had greater influence on chalkiness compared to the HNT, although grain weight, amylose content, and milled rice rate were lower in HNT. According to Xiong et al. (2017), the combined effect of HNNT on chalkiness was greater compared to each of the HDT and the HNT. Shi et al. (2017) found that chalkiness of the grains significantly increased under all temperature treatments, but with HNT mainly resulting in white-belly chalky grains due to insufficient assimilate availability while HDT and combined stress of HNNT resulted in high proportions of MW kernels under inefficient utilization of reserves.

In rice, certain stages of grain filling are suggested to be more sensitive to stress. Yamakawa et al. (2007) exposed 'Nipponbare' cultivar to HT (33°C day/28°C night) from 5 to 20 DAF and found that grain chalkiness appeared in 84% of heat-ripened grains with weight reduction by 6.5%. Some of the studies specified that average temperature above 26°C during 15 DAF (Lur et al., 2009) or 20 DAF (Wakamatsu et al., 2007) greatly influenced rice grain chalkiness. In particular, the appearance of white-belly chalky kernel was assumed to be more associated with assimilate supply during 20–30 DAF than that of initial grain filling period by shading experiments (Tashiro & Ebata, 1975). Conversely, Ishimaru et al. (2019) showed that the HT sensitivity was found at the early storage phase (5–10 DAF) or the middle phase (15–20 DAF) depending on the chalk grain types. Despite decades of agronomic research, the effects of difference in the developmental stage on the sensitivity to HT remain imperfectly solved.

In this study to comprehend the effect of HT on chalky grains in the wide range of grain filling stages, we isolated and used conditional chalky grain mutant '*flo11-2*' for the comparative quantitative analysis. The mutant has a single amino acid substitution at the conserved ATPase domain of cpHSP70-2, which is important for translocation of amyloplast-localized proteins, such

as granule bound starch synthase I, resulting in lower ATPase and chaperone activities than that of wild type (Tabassum et al., 2020). The mutant showed extreme chalkiness under averaged T_{mean} of 28°C during a grain filling in natural environment, whereas the agronomical traits were similar to wild type and chalkiness becomes less severe under cool temperature (averaged T_{mean} of 24°C). In the present paper, we exploit the high sensitivity of the mutant to HT for the better understanding of the rice chalkiness. The purpose of the present study was to determine the most critical environmental factor and developmental stage for chalkiness formation during grain filling and to evaluate the usefulness of the mutant for chalkiness studies.

Materials and methods

Plant materials and cultivation

Rice (*O. sativa* L. subsp. *japonica* 'Nipponbare') seeds were soaked in water in the dark for 3 days at 28°C, then irradiated with carbon-ion beams (20 Gy, linear energy transfer, LET: 22.6, 29.9, 37.4, 48.0, 60.3 keV μm^{-1}) at the RIKEN RI-beam factory (Saitama, Japan). The irradiated seeds (M_1 seeds) were germinated and the plants were grown in a paddy field. A total of 1,116 M_2 lines were grown in a paddy field with conventional agronomical practices, in Tottori University 2006 and in Kyoto University 2007 and 2008, Japan. The appearance of the matured brown rice was compared by visual inspection to isolate the *flo11-2* mutant that expressed higher chalkiness than the wild type over two locations and 3 years.

Rice plants of cultivars Nipponbare, Hatsuboshi (HTS), Aichi123 (HTR), and the *flo11-2* mutant were grown in 1/5000a Wagner pots filled with soil containing 0.36 g each of nitrogen, phosphate, and potassium. The pots were placed in a phytotron where the air temperature was set at 26°C/24°C (day/night) temperature and upland field of Kyoto Farm, Graduate School of Agriculture, Kyoto University. Only the main stems of 20 plants were allowed to grow in each pot by removing emerged tillers based on the method of Satake (1972). Some plants were subjected to short daylength treatment (10 h light/14 h dark) for 7 days at the panicle initiation stage (mid to late June) to accelerate a flowering period. Flowering of those plants was mostly observed mid to late July, which was 2–3 weeks earlier than the conventional flowering date of the cultivar Nipponbare in Kyoto and first flowering date was recorded for each panicle.

The wild type and the *flo11-2* mutant were also grown in a paddy field with the plant density of 22.2 plants m^{-2} and with conventional practices (basal fertilizers N:P:K = 6:4.7:5.6 g m^{-2}) in Kyoto University. In 2014, plants

were grown in plots (5 m x 2.5 m each cultivar) for yield and growth measurements. Sowing and heading dates were 8 May and 16 August, respectively for both the wild type and the mutant. In total, 10 plants medial in panicle number out of contiguous 22 plants were used for measurements. In 2018 and 2019, plants were grown in plots (2.5 m x 2 m each cultivar) for grain size and chalkiness measurements. Sowing and full heading dates for both the wild type and the mutant in conventional practices were 23 April and 11 August, respectively, in 2018, and 19 April and 13 August, respectively, in 2019. In 2018 and 2019, a short daylength treatment was applied for a part of the plants during 14–20 June by covering the plant canopy with a black sheet from 5:00 pm to 7:00 am.

Heat stress treatments

Eight temperature treatments were given to plants grown in pots assigned for continuous HT (H), continuous low temperature (L), six regimes of 5 days HT treatment (High1–High6) imposed at different developmental stages of a grain filling period up to 30 DAF in 2017 to 2019 (Table 1). In 2016, the High1–High3 treatments were preliminarily conducted. Around 20 plants (single stem) with different flowering date in each pot were used for one regime. The plants assigned to 5 days HT treatments were temporarily shifted from phytotron (26°C/24°C, cool temperature) to the outside field or glasshouse conditions (HT) and then moved back to the phytotron after HT treatment for 5 days was completed. The plants for L and H treatments were placed in the phytotron and the outside field (or the glasshouse), respectively, for the entire grain filling period (30 days). Harvested rice grains were air-dried and stored at room temperature before testing. Fully filled grains were used for the measurement of chalky ratio.

Heat stress treatments in a paddy field were spontaneously conducted by accelerating heading dates with short daylength treatments in 2018 and 2019.

Daily mean, daily maximum, and daily minimum temperatures were calculated with air temperature data (T) recorded at 10 min intervals. Excess temperature above 30°C [$\text{Ext}_{30}(T)$] was defined as incremental temperature from 30°C ($T - 30$) when $T > 30^\circ\text{C}$ and zero when $T \leq 30^\circ\text{C}$. Temperature data were also obtained from Tottori and Kyoto local meteorological observatories nearby to experimental fields (<https://www.data.jma.go.jp/obd/stats/etrn/index.php>).

Chalky ratio, grain size, and preharvest sprouting measurements

Lateral images of all brown rice grains in each panicle grown in pot experiments were collectively scanned by

Table 1. Regimes of 5 days HT treatment.

Year	Regime	Duration	Location	T_{mean} (°C)	T_{max} (°C)	T_{min} (°C)
2016	High1	29 July–2 August	Outside	28.69	38.38	23.28
	High2	3 August–7 August	Outside	30.28	39.66	23.96
	High3	8 August–12 August	Outside	29.18	39.84	21.90
2017	High1	30 July–3 August	Outside	28.50	36.68	24.12
	High2	4 August–8 August	Outside	28.30	35.10	24.82
	High3	9 August–13 August	Outside	28.28	37.26	22.76
	High4	14 August–18 August	Outside	26.71	33.94	22.90
	High5	19 August–23 August	Outside	28.50	36.54	23.66
	High6	24 August–28 August	Outside	28.09	35.60	22.28
2018	High1	3 August–7 August	Glasshouse	28.92	35.56	24.86
	High2	8 August–12 August	Glasshouse	28.98	34.20	24.74
	High3	13 August–17 August	Glasshouse	28.63	34.94	24.84
	High4	18 August–22 August	Glasshouse	27.76	35.38	22.90
	High5	23 August–27 August	Glasshouse	28.87	33.52	25.00
	High6	28 August–1 September	Glasshouse	28.36	33.72	24.88
2019	High1	2 August–6 August	Outside	30.28	37.30	25.06
	High2	7 August–11 August	Outside	30.41	38.36	24.46
	High3	12 August–16 August	Outside	29.05	34.88	25.58
	High4	17 August–21 August	Outside	28.19	35.80	24.00
	High5	22 August–26 August	Outside	25.52	32.66	21.12
	High6	27 August–31 August	Outside	24.58	30.36	21.46

Duration and location indicate the period and place exposed with 5 days HT treatment.

T_{mean} , T_{max} , and T_{min} indicate averaged values within 5 days for daily mean, daily maximum, and daily minimum temperature, respectively.

Grain Scanner RSQ110A (Satake, Hiroshima, Japan). The chalky ratio (the proportion of chalky area to the projected grain area for each grain) was measured and a mean value of all grains in a panicle was calculated. Chalky ratio and grain size (length, width, and area) were also measured with the Grain Scanner for bulked and randomly chosen grains, which were grown under conventional practices in paddy fields in 2018 and 2019 ($n = 278$) and under short daylength treatments in 2018 ($n = 75$). Preharvest sprouting was measured as

germinating grain number to total grain number (percentage) in each panicle with 10 randomly selected panicles.

Statistical analysis

The results were presented as mean $\pm$ SE (standard error) or mean $\pm$ SD (standard deviation). Statistical analysis was performed using BellCurve for Excel software (Social Survey Research Information Co., Ltd. Tokyo, Japan).

Results and discussion

Isolation and grain appearance of the *flo11-2* mutant

The *flo11-2* mutant was isolated as a mutant that produced a higher number of chalky grains than the wild type rice 'Nipponbare' under field conditions in Tottori 2006 and Kyoto 2007 and 2008. The LET condition used for the isolation of the *flo11-2* mutant was C ions at 29.9 keV μm^{-1} , which has shown the highest mutation frequency (Kazama et al., 2008) and has caused more single-base substitution and small indels in Arabidopsis than Ar ions (Kazama et al., 2017). Therefore, it was considered that these irradiation conditions would likely also be effective for rice. Although the mutated gene *flo11-2* was not homozygous yet at M_3 generation (Tabassum et al., 2020), the number of chalky grains observed in the *flo11-2* mutant seemed to fluctuate depending on temperature (data not shown). The averaged T_{mean} during 30 DAH at nearby local meteorological observatories considerably varied in the two locations and the 3 years (2006–2008), to 25.8°C, 27.7°C, and 26.3°C, respectively. The descendent line of the *flo11-2* mutant (homozygous in the causal gene) and the wild type Nipponbare were, therefore, grown and compared in a phytotron and in a field or a glasshouse as described below.

Agronomical traits, such as growth parameters and yield components, were not significantly different between the wild type and the mutant under a conventional field environment in 2014 (Table 2), where averages of T_{mean} (T_{max}) during 30 DAH were 25.9°C (30.9°C). Under conventional practices in paddy fields where averages of T_{mean} (T_{max}) were 26.4°C (33.5°C) in 2018 and 27.0°C (35.0°C) in 2019 for 30 DAH and 27.1°C (34.7°C) in 2018 and 26.6°C (33.7°C) in

Table 2. Yield components and growth parameters at maturity.

	Panicle number (number plant ⁻¹)	Plant height (cm)	Grain number (number plant ⁻¹)	Panicle weight (g plant ⁻¹)	Total dry weight (g plant ⁻¹)
Nipponbare	8.20 $\pm$ 0.33	96.1 $\pm$ 0.53	714.1 $\pm$ 34.5	22.7 $\pm$ 0.77	41.7 $\pm$ 0.81
<i>flo11-2</i>	8.20 $\pm$ 0.20	97.1 $\pm$ 0.97	698.7 $\pm$ 25.7	21.8 $\pm$ 0.56	43.5 $\pm$ 0.88
t-test	n.s.	n.s.	n.s.	n.s.	n.s.

Plants were grown in paddy field plots with conventional practices in Kyoto University in 2014. Mean value $\pm$ SE. Statistical analysis was conducted with Student's *t*-test. n.s.: not significant at $p < 0.05$ ($n = 10$).

Table 3. Size and chalkiness of grains grown under conventional practices in paddy field.

		Length (mm)		Width (mm)		Area (mm ²)		Chalky ratio	
		mean	SD	mean	SD	mean	SD	mean	SD
2018	Nipponbare	5.13	0.22	2.62	0.16	10.69	1.00	0.08	0.11
	<i>flo11-2</i>	5.18	0.23	2.61	0.16	10.71	0.96	0.45	0.19
	<i>t</i> -test	**		ns		ns		**	
2019	Nipponbare	5.01	0.20	2.56	0.16	10.12	0.88	0.03	0.06
	<i>flo11-2</i>	4.99	0.19	2.60	0.14	10.21	0.82	0.28	0.19
	<i>t</i> -test	ns		**		ns		**	

Plants were grown in paddy field plots with conventional practices in Kyoto University in 2018 and 2019. Statistical analysis was conducted with Welch's *t*-test.

**;*P* < 0.01; ns: not significant (*n* = 278), SD: standard deviation.

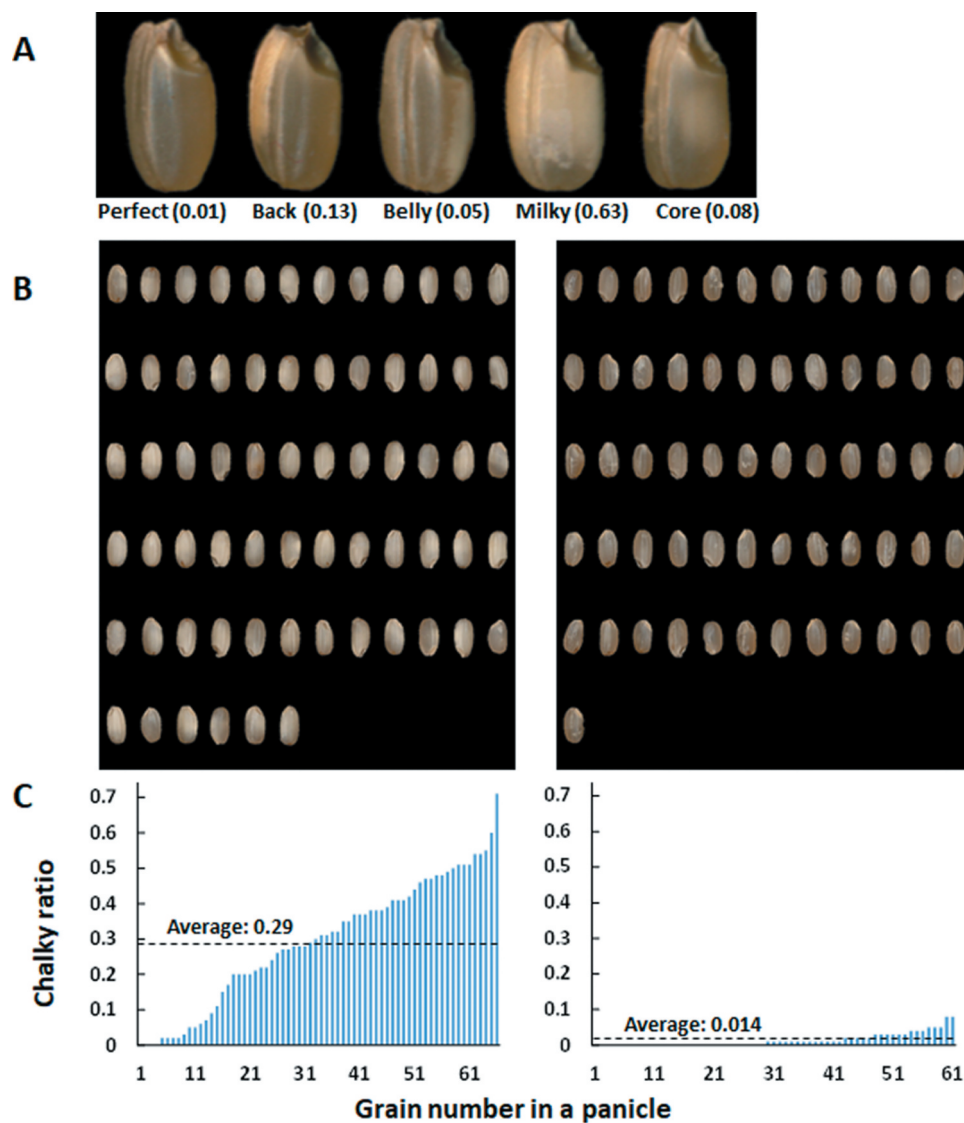


Figure 1. Variation in the chalky ratio of the wild type and the *flo11-2* mutant. (a) Representative images and chalky ratio of perfect grain (Perfect), white back (Back), white belly (Belly), milky white (Milky), and white core (Core) grains of wild type, Nipponbare harvested in Kyoto, 2018. Chalky ratio was shown in parenthesis. Images and chalky ratio measurements were taken from a same lateral side. (b) Images and (c) chalky ratio of all grains within single panicles exposed to different temperature were compared. Because the chalky ratio distributes linearly in a panicle, a mean value within each panicle was compared as a representative for the panicle. Photo and graph were examples of 5 days HT treatment in 2019. Averaged T_{mean} of the 5 days was 30.4°C (left, High2) and 25.5°C (right, High5).

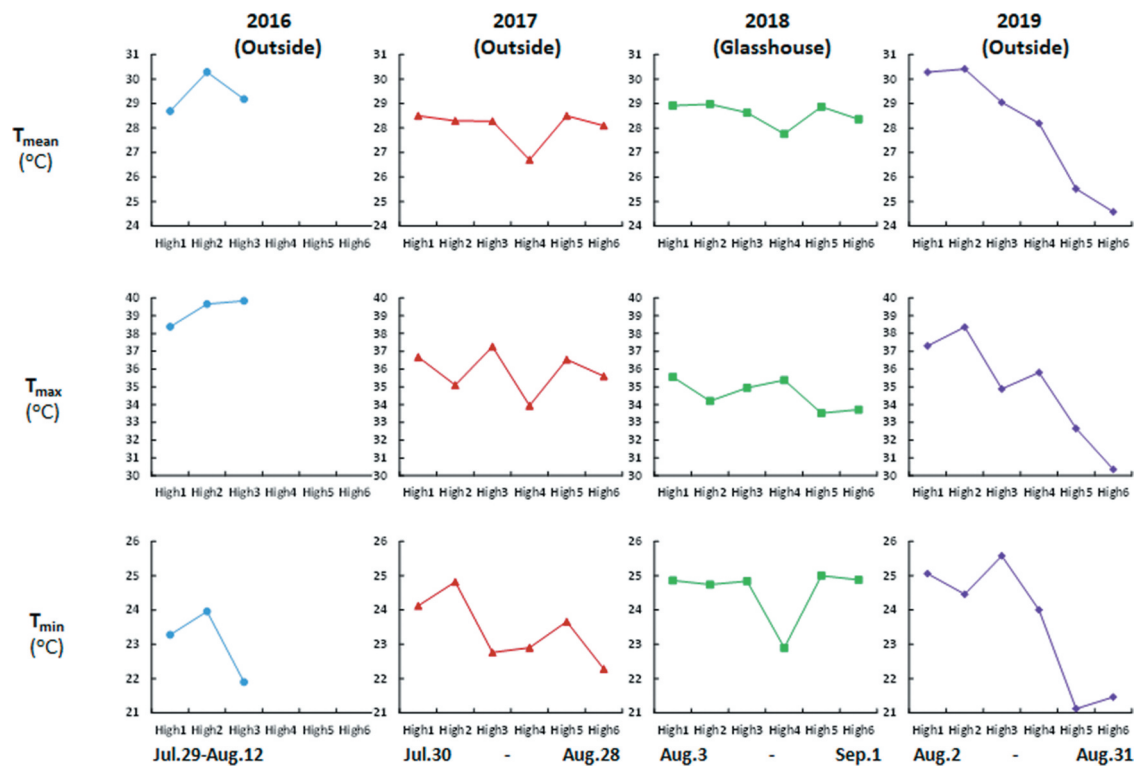


Figure 2. Temperatures of six regimes in grain filling periods from 2016 to 2019. Averaged values of daily mean temperature (T_{mean}), daily maximum temperature (T_{max}), and daily minimum temperature (T_{min}) within 5 days of HT treatments at different grain filling periods (High1 to High6 regimes) were compared in four consecutive years. In 2016, 2017, and 2019, temperature was measured outside. In 2018, temperature was for an air-conditioned glasshouse. Note that only three regimes were examined in 2016 due to a preliminary trial. Starting and ending dates of the regimes were shown under the graphs.

2019 for 20 DAH, chalky ratio was 5.6- and 9.3- fold higher in the mutant than the wild type in 2018 and 2019, respectively (Table 3). The grain length in 2018 and the width in 2019 were also significantly higher in the mutant but the differences were within 1.6% (Table 3). These observations led us to hypothesize that the rice grain appearance of the *flo11-2* mutant is fluctuating and sensitive to HT conditions during grain filling.

Mutant exhibited variegated chalky ratio within single panicle

Chalky ratio (chalky area divided by grain area) was measured at a single grain basis for quantitative imaging analysis of chalkiness (Figure 1). Chalky ratio was highly correlated with L^* , which is an indicator of whiteness, in CIE-LAB Color Space ($R^2 = 0.97$ in 2016 samples, Figure S1). Representatives of perfect, WB, white belly, MW, and white core grains of wild type from field samples grown in 2018 were selected and compared. The chalky ratio varied from 0.01 to 0.63 and well corresponded to the whiteness of grains (Figure 1(a)), suggesting the methodology for chalkiness quantification is reasonable. Similar methodology was employed by Ambardekar et al. (2011). In the meantime,

examples of the mutant from 5 days HT treatment showed the large difference in chalky ratio ranging from 0 to 0.71 at High2 treatment (30.4°C in averaged T_{mean} for 5 days) among the grains in a panicle and the little difference ranging from 0 to 0.08 at High5 treatment (25.5°C in averaged T_{mean} for 5 days). Because the chalky ratio distributes linearly in a panicle, the mean value within each panicle was compared as a representative of that panicle. There was a distinct difference between the values of the two panicles (0.29 and 0.014, for the left and right panels in Figure 1(c), respectively). Note that the chalky type of the mutant grains under HT was mostly MW, probably due to evenly high sensitivity of endosperm cells in the mutant to HT.

Varied chalky ratio and temperature of short-term heat stress

Averaged temperatures of 5 days HT treatment throughout the grain filling over 4 years differed (Figure 2). T_{mean} of 2016–2018 was relatively constant around 28–29°C at all developmental stages except High2 regime of 2016 and High4 regimes of 2017 and 2018. Meanwhile, T_{mean} of 2019 was high above 30°C in the first two regimes and

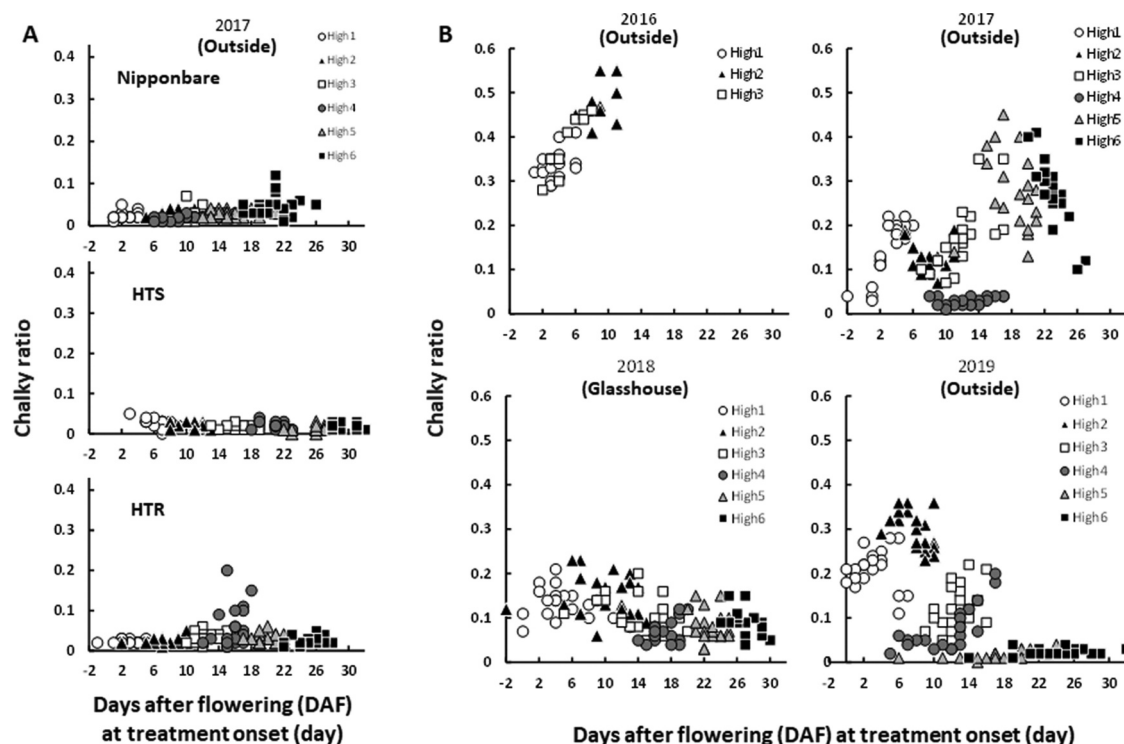


Figure 3. Chalky ratio of 5 days HT treatments. (a) Plants of wild type (Nipponbare) and HT-susceptible (HTS, Hatsuboshi) and -resistant (HTR, Aichi 123) cultivars were exposed to 5 days HT treatments at different grain filling periods (High1 to High6 regimes) in 2017. Plants were shifted from phytotron (averaged T_{mean} , 24°C) to outside (HT) only for 5 days. Around 20 plants (single stem) with different flowering date were used for one regime. After maturation, chalky ratio was measured for all grains and mean value within a panicle was plotted against days after flowering at treatment onset of the panicle. (b) Plants of the *flo11-2* mutant were similarly exposed to 5 days HT treatments in four consecutive years. In 2016, 2017, and 2019, plants were shifted from phytotron (averaged T_{mean} , 24°C) to outside (HT) only for 5 days. In 2018, plants were shifted to an air-conditioned glasshouse (HT). Note that only three regimes were examined in 2016 due to a preliminary trial.

then gradually decreased to 24°C until High6 regime. Basically, T_{max} and T_{min} of 2019 were also initially high and became low later. Notably, the highest T_{max} (38–39°C) was found in 2016. Although T_{mean} was generally higher in 2018 than 2017, T_{max} was lower and T_{min} was higher in 2018 than 2017 because the 5 days HT treatment was conducted in a glasshouse with air-conditioner in case of 2018. T_{max} was temporarily decreased at High2 and High4 in 2017.

Chalky ratio of wild type cultivars including HTS and HTR was very low over an entire grain filling period, suggesting that 5 days HT treatment in wild type was inefficient for the evaluation of temperature response (Figure 3(a)). The cultivar Hatuboshi is well known as HTS (Wakamatsu et al., 2007) but did not work as a check variety in our experiments, probably because our single column (main stem) cultivation method in pots might enable rice panicles to get enough photosynthate under high illuminance as documented by Kobata et al. (2004) and hinder possible genotypical differences in source abilities under HT. Meanwhile, chalky ratio of the mutant was largely

varied from zero to above 0.5 from one year to another year (Figure 3(b)). In 2016 the treatment was preliminary conducted at the early grain filling stage and the chalky ratio was increased from 0.3 to above 0.5 along with the developmental stage. In 2017, the chalky ratio was gradually increased and reached the peak (0.45) at 17 DAF and then steeply decreased thereafter except temporary decrease at High2 and High4 regimes. Meanwhile, the chalky ratio was much low over the grain filling period in 2018. In 2019, chalky ratio was initially increased but thereafter variably decreased.

Most critical meteorological factor and developmental stage for chalkiness

The comparison between Figures 2 and 3 suggested T_{max} is more causative to chalkiness than T_{mean} and T_{min} in the mutant. Therefore, all the chalky ratio data of 5 days HT treatment from 2016 to 2019 were combined and compared with T_{mean} , T_{max} , and T_{min} (Figure 4). The results demonstrated that chalky ratio was positively and

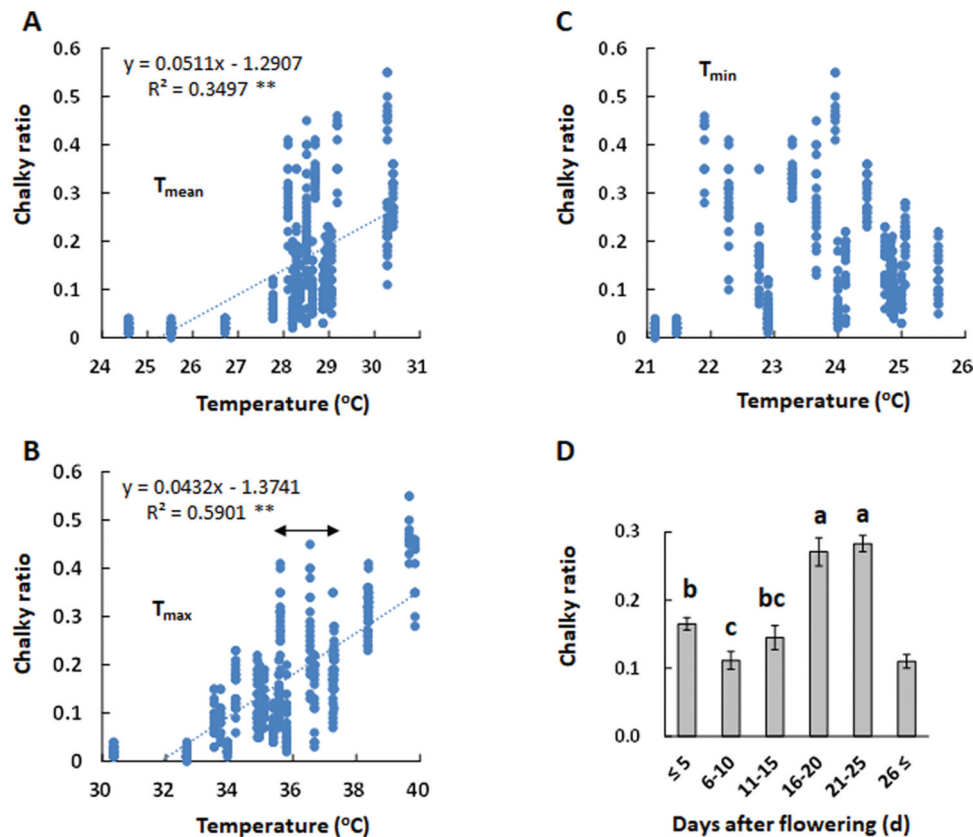


Figure 4. Correlation between chalky ratio and air temperatures and chalky ratio comparison between different grain filling periods. Chalky ratio was plotted against averaged values of (a) daily mean temperature (T_{mean}), (b) daily maximum temperature (T_{max}), and (c) daily minimum temperature (T_{min}) within 5 days HT treatments. All data in four consecutive years from 2016 to 2019 were combined ($n = 394$). ** indicates significance at $p < 0.01$. Horizontal arrow in the panel (b) indicates the range of 35.5–37.5°C of T_{max} . (d) Chalky ratio within the range of 35.5–37.5°C of T_{max} ($n = 139$), in which a large variation in chalky ratio was found, was compared between six different grain filling periods (mean $\pm$ SE). Multiple comparisons were conducted using the Kruskal Wallis test with Steel-Dwass ($p < 0.01$). Different letters indicate significant differences between the grain filling periods. Note that the sixth period (26 $\leq$ days after flowering) was excluded for statistical analysis due to small number of data. Number of data for ≤ 5 , 6–10, 11–15, 16–20, 21–25, 26 $\leq$ days after flowering is 48, 21, 28, 20, 20, and 2, respectively.

significantly correlated with T_{max} ($r^2 = 0.59$) and with T_{mean} ($r^2 = 0.35$) while no correlation was found with T_{min} (Figure 4). T_{mean} greater than 28°C increased the chalky ratio with a large variation. T_{max} was largely correlated with chalky ratio except for the range of 35.5–37.5°C, in which a large variation in chalky ratio was found from 0.02 to 0.45. Thus, we sorted data within the range of T_{max} from 35.5°C to 37.5°C into each grain filling stage with 5 days intervals. The chalky ratio was significantly higher during 5 days from 16 to 20 DAF and 21 to 25 DAF than the other periods (Figure 4(d)), suggesting that the late grain filling period around 20 DAF, that is timing changing from a storage product accumulation stage to a maturation stage (Wu et al., 2016), is the most sensitive to HT within T_{max} of 35.5–37.5°C. It should be noted that the mean flowering date of all caryopses in a panicle is several days behind the first flowering date in the panicle. In the present study of 5 days HT treatments, chalkiness was

evaluated at a panicle basis (a mean value of all grains in a panicle), while the developmental stage (DAF) of a panicle was counted from the first flowering date. Thus, a single caryopsis may be most susceptible to HT at several days earlier than 20 DAF.

Impacts of long-term heat stress on chalkiness

To confirm the significance of T_{max} , we analyzed the chalky ratio under 30 days of continuous temperature treatments in 2017 and 2018. The chalkiness of HT-treated mutant grown outside in 2017 (T_{max} 35.9°C and T_{mean} 28.1°C; H treatment, Figure 5(b)) was significantly higher than that of the wild types. At phytotron in 2017 (T_{max} 28.2°C and T_{mean} 23.5°C; L treatment, Figure 5(a)), the chalky ratio of the mutant was significantly higher than the wild type but the value was much lower than that of H treatment in 2017. Furthermore, chalky ratio of the mutant in phytotron

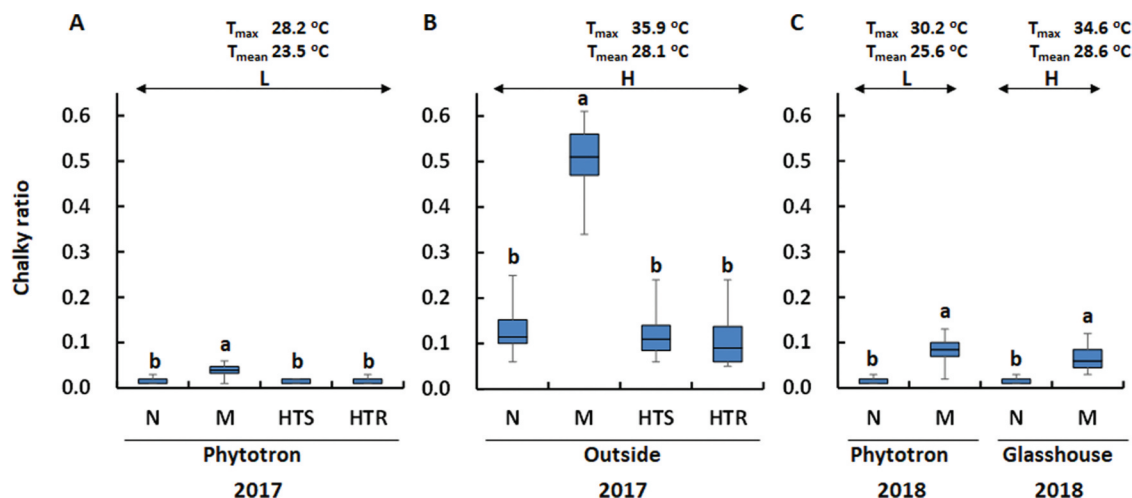


Figure 5. Chalky ratio of 30 days temperature treatments. Chalky ratio was compared between wild type (N, Nipponbare), the *fla11-2* mutant (M), and HT-susceptible (HTS, Hatsuboshi) and – resistant (HTR, Aichi 123) cultivars grown under different environments. (a) Phytotron in 2017; averaged temperatures of daily maximum (T_{max}) and daily mean temperature (T_{mean}) during 30 days of continuous temperature treatments in a grain filling was 28.2°C and 23.5°C, respectively. (b) Outside in 2017; T_{max} , 35.9°C; T_{mean} , 28.1°C. (c) Phytotron in 2018 (left); T_{max} , 30.2°C; T_{mean} , 25.6°C; Glasshouse in 2018 (right); T_{max} , 34.6°C; T_{mean} , 28.6°C. L and H indicate continuous low- and high-temperature treatments. Multiple comparisons were conducted using the Kruskal Wallis test with Scheffe ($p < 0.01$) (panels A and C) and were controlled for using the Bonferroni correction ($p < 0.01$) (panel B). Different letters indicate significant differences. The ends of the box are the upper and lower quartiles, the horizontal line inside the box is the median, and the whiskers outside the box extend to the highest and lowest observations.

(L treatment) and glasshouse (H treatment) in 2018 was significantly larger than that of the wild type (Figure 5(c)) but the value was again much lower than that of H treatment in 2017. It was pointed that the T_{mean} of field condition in 2017 (28.1°C) and glasshouse in 2018 (28.6°C) (H treatments) was almost similar to each other but T_{max} was 1.3°C higher in the former than the latter. This difference in T_{max} might be the most critical for chalkiness under 30 days of continuous temperature treatment. Meanwhile, the diurnal temperature change was different between the field (large sinusoidal change) and glasshouse (narrow fluctuating change) conditions, exemplified in Figure 6. In this example of a typical sunny summer day, T_{mean} was similar to each other (28.7°C vs. 28.9°C) but T_{max} and T_{min} were higher and lower in the field than the glasshouse, respectively (Figure 6(a)). Thus, the number of days with T_{max} above 34°C was much less in the glasshouse than the field from July to October (Figure 6(b)). Furthermore, integrated impact of HT, for example, calculated as cumulated excess temperature above 30°C (Ext_{30}) was much different between the glasshouse and the field conditions (Figure 6(a)) and daily mean Ext_{30} was varied even at the same T_{max} in the field especially at high T_{max} (Figure 6(b)). Note that the determination coefficient of regression of chalky ratio to daily mean Ext_{30} ($R^2 = 0.57$, data not shown) was not higher than that to T_{max} ($R^2 = 0.59$, Figure 4(b)) in the 4-year 5 days HT treatments. However, further precise analyses on T_{max} , integrated impact of HT as well as other additional

meteorological factors such as solar radiation would be necessary.

Critical developmental stages for chalkiness

Nagato and Ebata (1965) demonstrated that the most critical developmental stages for MW and WB were 0–10 DAF and that for BW was 0–20 DAF. By contrast, Tashiro and Wardlaw (1991) found that the most sensitive stage to HT (36°C/31°C) was 12–20 DAF for MW and 16–24 DAF for WB. Recently, Ishimaru et al. (2019) have carried out 5 days heat stress (33°C/27°C) from 5 to 20 DAF and showed that 5–10 DAF was the most critical for combined chalk (MW+WB) and MW, while WB, BW, white belly occurred mostly at 15–20 DAF. One possible reason for such discrepancy with the present study might be the difference in sampling and scoring methods. The above-mentioned three studies seemed to analyze only superior caryopses at specific position within a panicle and counted chalky grain number regardless of chalky degree differences after categorizing chalky types (qualitative traits) by visual inspection, which requires training and experience for accurate and coherent assessment (Yoshioka et al., 2007). Meanwhile, the present study used imaging analysis to quantify the chalky area using all grains in a panicle, which is high-throughput and thoroughly quantitative. As seen in Figure 1, the chalky ratio varies widely but linearly in each panicle,

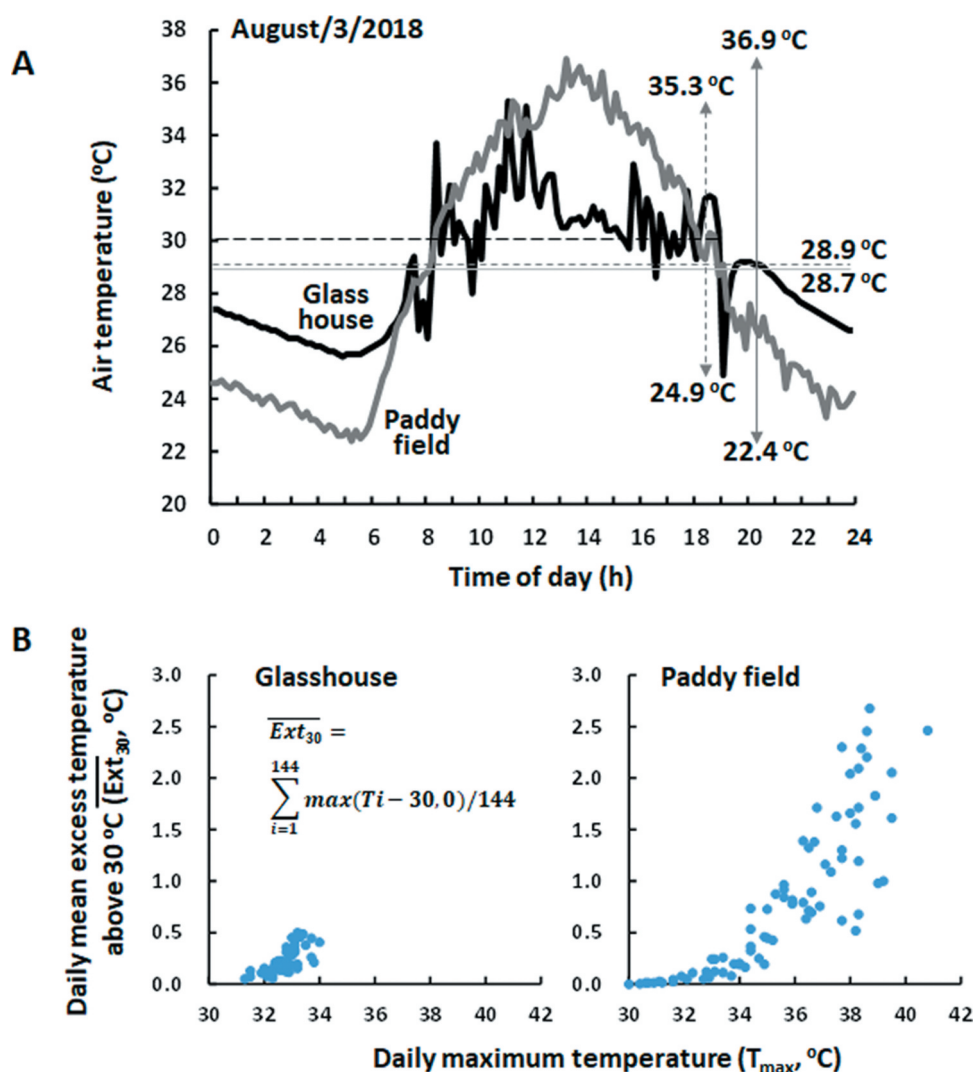


Figure 6. Examples of diurnal change in air temperature in a paddy field and an air-conditioned glasshouse and comparison between daily maximum temperature and daily mean excess temperature above 30°C. (a) Temperatures of 3 August 2018 were exemplified for comparison between a paddy field (gray line) and an air-conditioned glasshouse (black line). Solid and dotted horizontal lines (daily mean temperature) and vertical arrows (range between daily maximum and minimum temperatures) are shown for the paddy field and the glass house, respectively. 30°C of the air temperature was shown with a broken horizontal line. (b) Daily mean excess temperature above 30°C (calculated as equations in the left panel with 10 min intervals data) was plotted against daily maximum temperature during 3 July 2019 and 5 October 2019 for the glasshouse and paddy field.

probably well representing the actual chalky degree variation within a panicle and being caused in part by the variation in flowering dates and superior/inferior caryopsis differences. Nonetheless, chalky type categorization is crucial because white core type (formed at early grain filling) and WB/BW types (formed at late grain filling) differently affect milling quality and market value (Lyman et al., 2013) and because assimilate transport routes (through dorsal side) and starch accumulating site within endosperm tissue change during ripening stages (Hoshikawa, 1993), providing information on when and how the rice plant is exposed to heat stress. Consequently, chalky type-specific and caryopsis

position (within a panicle)-specific image analysis should be required as future works.

Preharvest sprouting of the *flo11-2* mutant

Usually the wild type 'Nipponbare' and the *flo11-2* mutant reach panicle emergence (heading) at around 10 August under a natural field condition in Kyoto, Japan. After this date, the air temperature gradually decreases day by day. When a short daylength treatment was applied during mid-June, the heading of both the wild type and the mutant was accelerated to mid-July. The T_{mean} averaged over 30 DAH was

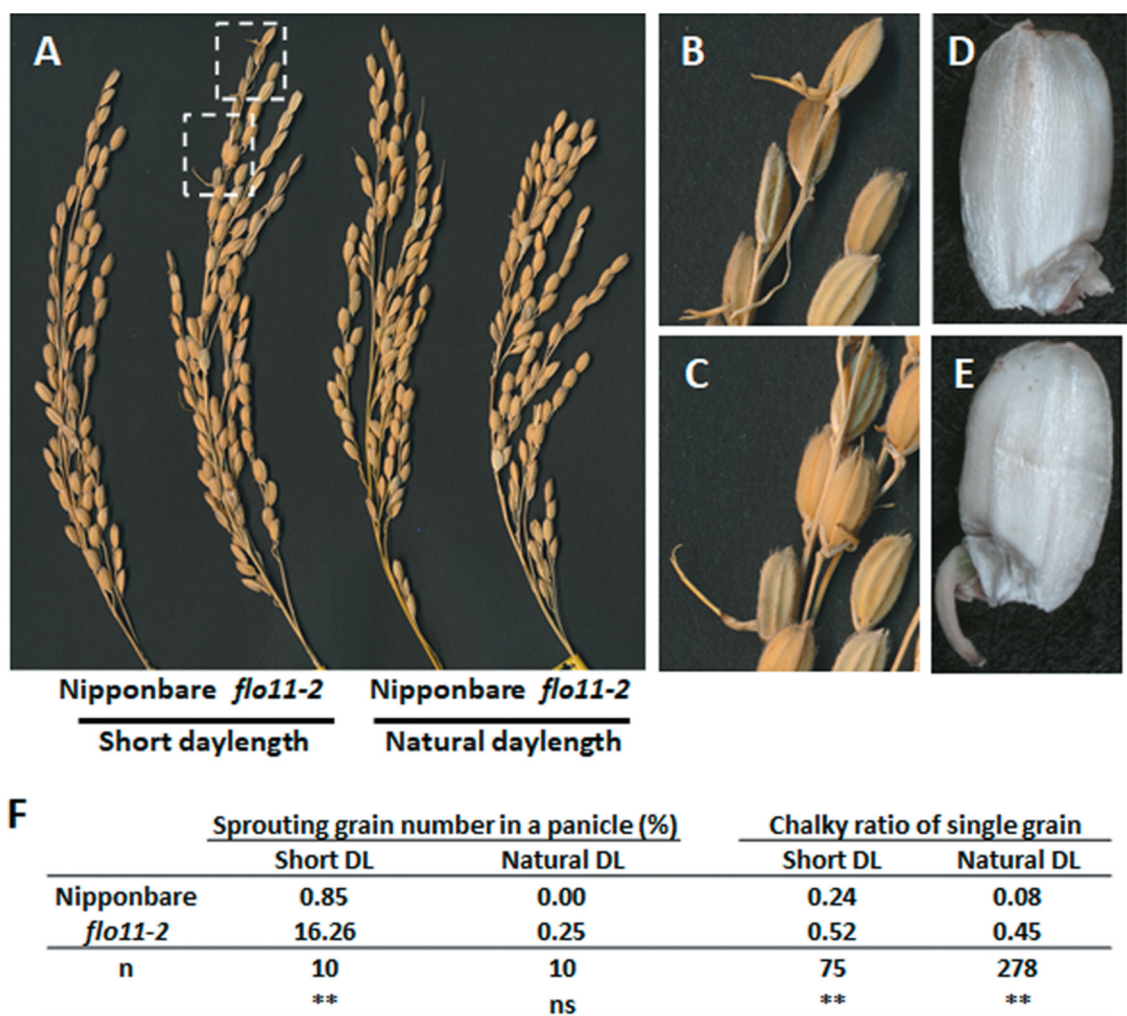


Figure 7. Preharvest sprouting of the *flo11-2* mutant panicle matured at HT in 2018. (a) Representative panicles of the wild type (Nipponbare) and the *flo11-2* mutant matured at HT exposed by heading acceleration of short-daylength treatment or at cool temperature of natural environment in a paddy field of Kyoto, 2018. (b,c) Magnified images of the *flo11-2* mutant panicles surrounded with dashed rectangulars in panel (a). (d, e) Representative brown rice of the sprouting *flo11-2* mutant seeds. (f) Sprouting grain number in a panicle and chalky ratio of single grains of the wild type (Nipponbare) and the *flo11-2* mutant were compared. Short DL (short daylength), Natural DL (natural daylength). Short daylength treatment was applied during 14 to 20 June by covering the plant canopy with a black sheet from 5:00pm to 7:00am. In 2018, full heading date, average of daily mean and daily maximum temperatures during 30 days after full heading were 17 July, 28.5°C, 35.4°C, respectively for the short daylength treatment and 11 August, 26.4°C, 33.5°C, respectively for the natural daylength treatment. The full heading date was same between the wild type and the *flo11-2*. t-test based on Welch. **: $P < 0.01$; ns: not significant. Short DL: Short daylength; Natural DL: Natural daylength. Chalky ratio of Natural DL was from Table 3.

26.1°C and 28.5°C for the natural and the short daylength treated conditions, respectively, in 2018. The chalky ratio was increased to 0.24 from 0.08 in the wild type and to 0.52 from 0.45 in the *flo11-2* mutant by HT conditions (short daylength treatment) (Figure 7), suggesting that starch granule formation and accumulation in the *flo11-2* mutant were significantly changed by HT conditions. In 2018, it was an extremely hot summer followed by a rainy autumn. Monthly average of $T_{\max}$ in July, August, and September were 35.0°C, 35.0°C, and 27.4°C, respectively, in 2018, compared to 31.5°C, 33.3°C, and

28.8°C, respectively, for the previous 30 years average, according to the Kyoto local meteorological observatory (<https://www.jma-net.go.jp/kyoto/>). This weather condition may have accelerated seed maturation but delayed harvest in 2018. Because of the slow desiccation of grains especially in the short daylength treated plants, the duration from heading to harvest was 10 days longer than the typical duration, i.e. 45 days at 22°C of T_{mean} . When harvested, the panicle of the *flo11-2* mutant from the short daylength treatment in 2018 showed a significantly high degree of preharvest sprouting, which damaged grain

quality (Figure 7). In 2019, preharvest sprouting was not observed (data not shown). Preharvest sprouting is inhibited by dormancy, which is arisen from both embryonic and maternal tissues, and is dependent on environmental and genetic factors (Gubler et al., 2005). Major genes associated with seed dormancy are known to be related to the biosynthesis, catabolism, perception, and signal transduction of abscisic acid (ABA) (G. A. Lee et al., 2017). The *flo11-2* mutant has three homozygous mutations which cause non-synonymous substitutions or frameshift in the coding sequence, including Bet v I allergen family protein, hypothetical protein, and cpHSP70-2 (Tabassum et al., 2020). Because the three proteins are seemingly not related with ABA metabolism and signaling, the high preharvest sprouting coupled with high chalky ratio could be not the direct effect of the mutations but the indirect effect through the poor starch accumulation. Note that the seeds of rice variety Ketaktara (Kataktara) ripened at 30°C showed lower dormancy than that ripened at 25°C (Hayashi & Hidaka, 1979). These results suggest that rice grains with a high chalky ratio have a risk of lower yields and quality under unusual but possible future climatic conditions.

Conclusion

In the present study, we isolated a unique mutant in which the grain appearance, as well as the growth, development, and yield were similar to that in the wild type under cool temperature conditions, but more chalky grains appeared than that of the wild type under HT conditions. This highly temperature-dependent chalkiness has not been found before. Using this sensitivity, the importance of $T_{\max}$ rather than T_{mean} and $T_{\min}$ and the developmental stage around 20 DAF on chalkiness was demonstrated. In addition, it was found that the *flo11-2* mutant with a high chalky ratio was vulnerable to preharvest sprouting, which has never been reported before for chalky grains, but could cause significant yield loss after extremely hot and dry summers followed by rainy autumns. The *flo11-2* mutant is, therefore, a useful model for studies into the effects of HT conditions on rice grain yield and quality.

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Author contributions

TKT and TA conceived and designed the experiments. RT, TD, HI, RM, YD, TA, and TKT performed the experiments. HI, RM, TA, and TKT analyzed the data. RT and TKT wrote the paper. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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