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Pollen germination and *in vivo* fertilization in response to high-temperature during flowering in hybrid and inbred rice

Wanju Shi[1,2] | **Xiang Li**[3] | **Ralf C. Schmidt**[4] | **Paul C. Struik**[2] | **Xinyou Yin**[2] | **S.V. Krishna Jagadish**[1,5]

[1] International Rice Research Institute, DAPO Box 7777, Metro Manila, Philippines

[2] Centre for Crop Systems Analysis, Wageningen University & Research, PO Box 430, 6700 AK Wageningen, The Netherlands

[3] State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, Guangzhou 510642, China

[4] Bayer CropScience NV Innovation Center—Research, Technologiepark 38, 9052 Zwijnaarde, Ghent, Belgium

[5] Department of Agronomy, Kansas State University, 3706 Throckmorton Ctr., Manhattan, KS 66506, USA

Correspondence
S.V. Krishna Jagadish, Department of Agronomy, Kansas State University, 2004 Throckmorton Plant Sciences Center, 1712 Claflin Road, Manhattan, KS 66506-5501, USA.
Email: kjagadish@ksu.edu

Xinyou Yin, Centre for Crop Systems Analysis, Wageningen University & Research, PO Box 430, 6700 AK Wageningen, The Netherlands.
Email: xinyou.yin@wur.nl

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Abstract

High-temperature during flowering in rice causes spikelet sterility and is a major threat to rice productivity in tropical and subtropical regions, where hybrid rice development is increasingly contributing to sustain food security. However, the sensitivity of hybrids to increasing temperature and physiological responses in terms of dynamic fertilization processes is unknown. To address these questions, several promising hybrids and inbreds were exposed to control temperature and high day-time temperature (HDT) in Experiment 1, and hybrids having contrasting heat tolerance were selected for Experiment 2 for further physiological investigation under HDT and high-night-time-temperature treatments. The day-time temperature played a dominant role in determining spikelet fertility compared with the night-time temperature. HDT significantly induced spikelet sterility in tested hybrids, and hybrids had higher heat susceptibility than the high-yielding inbred varieties. Poor pollen germination was strongly associated with sterility under high-temperature. Our novel observations capturing the series of dynamic fertilization processes demonstrated that pollen tubes not reaching the viable embryo sac was the major cause for spikelet sterility under heat exposure. Our findings highlight the urgent need to improve heat tolerance in hybrids and incorporating early-morning flowering as a promising trait for mitigating HDT stress impact at flowering.

KEYWORDS

fertilization, flowering, high day-time temperature, high night-time temperature, *in vivo* pollen germination, rice

1 | INTRODUCTION

Hybrid rice plays a pivotal role in sustaining food security due to its high productivity under favourable conditions, as demonstrated consistently throughout China (Cheng et al., 2007). Outside of China, hybrid rice has increased steadily, up to 6.36 million hectares in 2014, mostly planted in tropical and subtropical rice-growing countries, such as the Philippines, India, Bangladesh, and Indonesia (Xie & Peng, 2016). However, the planting area of hybrid rice in these countries is limited and often fluctuates because of unfavourable weather conditions (Xie & Peng, 2016). It has been projected that global temperature will continue to increase steadily during the 21st century, accompanied by more frequent and more intense heat episodes and warmer nights (Intergovernmental Panel on Climate Change [IPCC], 2013). Although typical heat episodes occur in short durations, when they coincide with critical flowering stages, they can pose a serious threat to spikelet fertility and therefore induce yield loss (Jagadish, Craufurd, & Wheeler, 2007). To date, heat-induced spikelet sterility during flowering has been documented in rice fields from different rice-growing regions, for example, China (Tian et al., 2010), Japan (Hasegawa et al., 2011), and Laos and Southern India (Ishimaru et al., 2016).

Developing heat-tolerant varieties is a sustainable strategy to cope with the challenges arising from increasing temperature (Challinor et al., 2014). Given the unpredictable occurrence of high-temperature stress in the tropics and subtropics, hybrid rice should essentially have both high-yielding potential and heat stress tolerance to sustain higher yields in these areas. To that end, identifying genetic

variation and understanding physiological mechanisms underlying the variation are essential to support breeding for heat tolerance. A series of phenotyping studies have identified a wide genetic variation in rice in their response to high-temperature at flowering among indica and/or japonica ecotypes (Jagadish, Craufurd, & Wheeler, 2008; Matsui, Omasa, & Horie, 2001; Shi, Ishimaru, Gannaban, Oane, & Jagadish, 2015). Also, some studies have assessed the performance of hybrid rice under high-temperature conditions in China, showing sensitivity of hybrid rice to increasing day-time temperature during flowering (Fu et al., 2015; Hu, Zhang, Zhu, Lin, & Xiang, 2012). Our previous study has confirmed the serious vulnerability of tropical and subtropical hybrid rice to high night-time temperature (HNT) during reproductive growth (Shi et al., 2016) and postflowering phase (Shi et al., 2017). However, there is little information regarding the tolerance of tropical and subtropical hybrid rice to high day-time temperature (HDT). In addition, it is not clear whether there is any difference in, and interaction between, the effects of HDT and HNT on spikelet fertility during flowering.

Flowering includes pollination, pollen germination, and fertilization. Any stress taking place during this stage is likely to cause sterility and ultimately induce yield loss. The flowering stage is considered to be the most sensitive stage to temperature fluctuations (Jagadish et al., 2007; Matsui et al., 2001; Satake & Yoshida, 1978). During high-temperature exposure, anther dehiscence of rice is inhibited from decreased ability of pollen grains to swell, resulting in a lower number of pollen grains adhering to the stigma (Jagadish et al., 2010; Matsui, Omasa, & Horie, 2000; Prasad, Boote, Allen, Sheehy, & Thomas, 2006). Immediately after landing on the stigma, pollen grains start to germinate followed by pollen tube growth inside the pistil to reach the female gametophyte inside the ovule. Even when a sufficient number of pollen grains are shed on the stigma, pollen germination can sometimes be poor, and pollen tube growth could be impeded under heat stress (Satake & Yoshida, 1978; Tang et al., 2008). In rice, all processes including flower opening, anther dehiscence, pollination, pollen germination, and pollen tubes reaching the ovary usually take about 45 min (Jagadish et al., 2010). Subsequent fertilization typically occurs within 1.5 to 4.0 hr after flower opening (Cho, 1956). Considering the normal flowering pattern in rice (between 10:00 and 12:00 hr; Sheehy, Elmido, Centeno, & Pablico, 2005), there is a significant chance of a double-fertilization process after pollen tubes penetrate the embryo sac to coincide with hotter temperatures around noon. However, all previous studies on spikelet sterility caused by HDT during flowering have mainly concentrated on pollination and pollen germination, whereas its impact on fertilization remains unknown. With recent success, breeders are working towards introducing the *early-morning flowering* trait in rice plants that allows spikelets to flower during early hours of the day when the temperature is cooler (Ishimaru et al., 2010). The introduction of the early-morning flowering trait can potentially minimize heat stress damage on pollen viability (Satake & Yoshida, 1978), but the fertilization process after the completion of pollination and pollen germination will still be vulnerable to high-temperature during late morning and early noon.

The objective of this study is to investigate the high-temperature effects on the fertilization process in rice in the context of evaluating the early-morning flowering strategy to improve tolerance to high-temperature stress. Two experiments were conducted. In Experiment 1, we evaluated some promising tropical and subtropical hybrids to assess their difference in heat tolerance to HDT compared with some popular high-yielding inbreds. Pollen germination was also tested in Experiment 1 to explore its contribution to sterility under HDT stress exposure. On the basis of the results of Experiment 1, contrasting hybrids were selected to be further examined in Experiment 2 on impact of independent HNT, HDT, and their combination (HNDT) on spikelet fertility. More importantly, by using an advanced experimental set-up for *in vivo* imaging of double fertilization, we had a clear visual observation on the dynamic fertilization process inside the intact ovule, thereby specifically filling knowledge gaps in identifying the effect of high-temperature on the *in vivo* fertilization process.

2 | MATERIALS AND METHODS

2.1 | Materials and crop husbandry

In Experiment 1, promising tropical and subtropical hybrids from a private company (H1–H3) and the International Rice Research Institute (IRRI) (Mestizo 1 [H4], Mestizo 3 [H5], Mestizo 21 [H6], and Mestizo 31 [H7]), high-yielding inbreds (PSBRc4, NSIC Rc222, and HHZ12-DT10-Sal1-DT1 [HHZ12]), and the best heat-tolerant check N22, an *aus* variety from India and a popular variety IR64 as susceptible check, were chosen to determine their responses to HDT. Seeds of all entries were first exposed to 50 °C in the oven for 3 days to break their dormancy and then incubated at about 30 °C room temperature for 2 days. After that, the germinated seeds were sown in seeding trays on October 17, 2013, followed by transplanting one 14-day-old seedling into each plastic pot (23 cm in diameter and 25 cm in depth) filled with 6-kg clay loam soil on October 31, 2013. As basal fertilizer, 2.0 g of ammonium sulfate ($(NH_4)_2SO_4$), 1.0 g of single superphosphate, and 1.0 g of potassium chloride (KCl) were applied in each pot, and an additional 2.0 g of $(NH_4)_2SO_4$ was used for topdressing at 25 days after transplanting. Plants were grown in the greenhouse with natural environmental conditions (temperature, photoperiod, relative humidity, and radiation) at IRRI, Los Baños (14°11′N, 121°15′E, 21 m above sea level), Philippines, before transferring them to walk-in growth chambers for imposing various temperature treatments described below. MINCERs (Micrometeorological Instrument for Near Canopy Environment of Rice, developed by the National Institute of Agrobiological Sciences, Japan; Yoshimoto et al., 2012) were placed in the greenhouse to record the actual temperature and relative humidity at the plant level at 15-min intervals. The recorded actual temperature, relative humidity, and vapour pressure deficit (VPD) in the greenhouse during the period of experiments are shown in Supporting Information Table S1. The VPD was calculated by using the equation presented in the website (http://cronklab.wikidot.com/calculation-of-vapour-pressure-deficit). Photoperiod was about 12 hr during the greenhouse phase of the experiment. Both pests and diseases were effectively controlled.

In Experiment 2, hybrids with contrasting responses to high-temperature impact on spikelet fertility as observed in Experiment 1 (H2,

H5, and H6) and heat-tolerant IR64 near-isogenic line (HT NIL) introgressed with a Chromosome 4 fragment from N22 (Ye et al., 2012) together with the parents (N22 and IR64) were selected. Seeds were sown on August 7, 2014, and seedlings were transplanted 2 weeks later. Crop husbandry was the same as in Experiment 1.

2.2 | Temperature treatments

When the main tillers of the plants showed the first signs of flowering (external appearance of anthers), 18 pots per genotype and per treatment were randomly moved into walk-in growth chamber (3.3-m length × 3.2-m width × 2.7-m height; 10.56-m^2 ground area in each chamber) facility at IRRI to impose temperature treatments (Experiment 1). Plants were exposed to control temperature at 30 °C and HDT at 38 °C for 6 hr·day^{-1} (08:00 to 14:00 hr), lasting for six consecutive flowering days. The transition from night to day temperatures was for 3 hr (from 05:00 to 08:00 hr), and that from day to night temperatures was from 14:00 to 18:00 hr, after which the chamber temperature was set to 23 °C as night temperature till 06:00 hr, the next day. The 6-hr day-temperature treatment was applied with an aim to cover the major flowering period within a given flowering day and to make sure that >90% spikelets that flowered on that day were exposed to high-temperature (Jagadish et al., 2007). In the controlled-environment walk-in chambers, six independent units of 1-kW high-intensity discharge lamps were fixed in each chamber to provide ≥650 μmol·m^{-2}·s^{-1} of photosynthetic photon flux density at the crop canopy for 11 hr of photoperiod and 215 μmol·m^{-2}·s^{-1} for 1 hr during the day–night changeover period, resulting in 12 hr of photoperiod. The relative humidity was controlled at 70%. MINCERs were placed in the middle of the chamber at plant canopy (about 130 to 150 cm above the floor and adjusted slightly between genotypes depending on their canopy height) to record the actual temperature and relative humidity at 15-min intervals. Actual temperature, relative humidity, and VPD are shown in Supporting Information Table S1. After 6 days of high-temperature stress, the majority of spikelets from the targeted main-tiller panicles completed flowering under stress exposure, and the remaining unflowered spikelets were marked and excluded from the determination of spikelet fertility. All plants were then transferred back to the greenhouse until grain maturity.

In Experiment 2, 18 plants per genotype whose main tillers started to flower were randomly moved into four independent walk-in growth chambers for six consecutive days of exposure to temperature treatments. Plants were exposed to four temperature treatments: control (day/night, 31/23 °C), HDT (38/23 °C), HNT (31/30 °C), and HNDT (38/30 °C). Both day-time and night-time temperatures were maintained for 11 hr from 06:00 to 17:00 hr and from 18:00 to 05:00 hr, respectively, with a 1-hr transition period between day and night. The longer duration of stress exposure on each day in Experiment 2 was to ensure all the key flowering and fertilization processes during day-time were stressed, allowing us to observe the entire time course of the fertilization process under a similar level of heat stress. Because flowering in rice extends until close to noon, with a known 4-hr time frame needed for postflowering fertilization events to be completed (Cho, 1956), the treatment structure followed in Experiment 2 is essential to dissect the impact on the entire fertilization process, minimizing bias or stress escape. The set-up for the other environmental factors within the walk-in growth chambers, including photosynthetic photon flux density, photoperiod, and relative humidity, were the same as in Experiment 1. After the treatment, the plants were moved back to the greenhouse until they reached grain maturity.

In both Experiments 1 and 2, three units of 0.2-mm-diamater copper constantan thermocouples (polytetrafluoroethylene twin twisted pair thermocouple, RS Components Corporation, Northamptonshire, UK) were inserted into the lemma and palea of three independent spikelets per genotype to measure spikelet tissue temperature in each chamber maintained at different temperature treatments. The measurements were taken from spikelets selected randomly on primary tillers (not the targeted main-tiller spikelets), a day after flowering. Each thermocouple was inserted into new spikelet during morning hours, before temperature treatment was initiated. Spikelet tissue temperatures were monitored every 5 s, and means over 5 min were recorded by a data logger (CR 1000 data logger, Campbell Scientific Inc., Logan, UT).

2.3 | Observation of spikelet fertility

At the maturity stage, spikelet fertility from 12 plants (pots) per genotype and per treatment was estimated from the targeted main-tiller panicles in Experiments 1 and 2. Individual spikelet was pressed by thumb and forefinger to determine whether it was fertilized or not. Spikelets with enlarged ovule (Shi et al., 2015), partially filled spikelets (spikelets with incomplete grain filling), and fully filled spikelets were grouped into fertilized spikelets. Thus, spikelet fertility was calculated as the ratio of fertilized spikelets to total number of spikelets from a panicle. The heat stress index (%) for spikelet fertility was calculated following Tao et al. (2008).

2.4 | Observation of *in vivo* pollen germination

On the first day of temperature exposure at the flowering stage, spikelets that just began to flower (opening of lemma and palea) after transferring into the chambers were carefully marked, and more than 20 spikelets from six plants (pots) per genotype and per treatment were randomly sampled into FAA (50% absolute ethanol, 5% acetic acid, 27% formaldehyde, and 18% sterilized water) fixative following the protocol by Rang, Jagadish, Zhou, Craufurd, and Heuer (2011), at about 1 hr after their flowering from each genotype and each temperature treatment in Experiment 1. The spikelets were vacuumed for 1 hr followed by washing with 50% ethanol and deionized water. Thereafter, the sampled spikelets were carefully dissected using a stereomicroscope (Olympus SZX7, Olympus Corp, Japan). Isolated stigmas were cleared in 8 N NaOH for 3–5 hr at room temperature and subsequently stained with 2% aniline blue dissolved in 0.1 M K_2HPO_4 for 5–10 min. The total number of pollen grains and the number of germinated pollen grains on the stigma were recorded to determine the percentage of pollen germination.

2.5 | Whole-mount observation of *in vivo* fertilization

In Experiment 2, individual flowering spikelets were marked at the initiation of flowering on the first day of temperature treatments by using the acrylic paint tagging technique (Jagadish et al., 2008) for control and HDT treatments. As day-time temperature had a predominant effect on spikelet fertility (see Section 3) presumably because pollination and the subsequent fertilization processes mainly occurred during the day-time, we conducted the observations on the effects of only HDT on fertilization. About 30 to 50 spikelets from six plants (pots) per genotype and per treatment were systematically collected at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 8.0, 12.0, and 24.0 hr, after flowering was initiated. The spikelet samples were collected into FAA fixative and vacuumed for 1 hr followed by washing with 70% ethanol and stored in 70% ethanol at 4 °C until microscopic observation.

The embryo sac of rice is enclosed within the nucellus, integument, and ovary wall, thus posing a technical challenge for its observation using conventional paraffin sectioning (Zeng, Hu, Lu, Li, & Liu, 2007). To facilitate observation of cells and nuclei in different stages of their development within the embryo sac without continuous sections of the sample and to obtain a clear visual image, a simple and effective eosin B staining procedure for embryo sac scanning using a laser scanning confocal microscope (Zeng et al., 2007), designated as WE-CLSM (whole-mount eosin B staining confocal laser scanning microscopy), was applied in our study. In detail, the ovaries from the spikelets were carefully dissected in 70% ethanol under a stereomicroscope (Olympus SZX7, Olympus Corp, Japan). Then, they were sequentially rehydrated in 50%, 30%, and 10% ethanol and distilled water for 20 min. To facilitate dyeing of the samples, the ovaries were pretreated in 2% aluminium potassium sulfate for 20 min. They were then stained with 10 mg/L of eosin B solution (dissolved in 4% sucrose) for 10 to 12 hr at room temperature. After completion of all the above steps, the ovaries underwent thorough dehydration. Specifically, after being treated with 2% aluminium potassium sulfate for 20 min to remove partial dye from the ovary walls, the ovaries were rinsed two to three times with distilled water. Then the samples were dehydrated with a series of ethanol solutions (10%, 30%, 50%, 70%, and 90%) for 20 min individually and then with 100% ethanol for 20 min for two to three times. Finally, the dehydrated ovaries were carefully transferred into a mixture of absolute ethanol and methyl salicylate (1:1) for 1–2 hr, and then they were kept in pure methyl salicylate for at least 1 hr before microscopic observation.

The stained ovaries were carefully placed on a glass concavity slide and mounted with pure methyl salicylate before being covered with a coverslip. Then samples were scanned by a Leica SPE laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany) at excitation wavelength of 543 nm, and emitted light was detected between 550 and 630 nm. The images of embryo sac were recorded, and the abnormality of their structure and fertilization was determined.

2.6 | Statistical analysis

A two-way analysis of variance was used to assess the effects of genotypes, temperature treatment, and their interaction on spikelet fertility and percentage of pollen germination. Spikelet temperature was analysed by a one-way analysis of variance. Together with correlation and regression analyses, the above analyses were performed using Genstat (Version 16, Rothamsted Experimental Station, Harpenden, UK).

3 | RESULTS

3.1 | Spikelet tissue temperature

In Experiment 1, the range of spikelet tissue temperatures across the tested genotypes under control conditions and heat stress was between 31.0 and 33.3 °C, and between 36.0 and 38.5 °C, respectively (Table 1). The spikelet tissue temperatures under the control condition were slightly higher than the target air temperature, whereas they were close to the target air temperature under the HDT condition. In contrast, spikelet tissue temperatures from Experiment 2 were similar to the target air temperature across all temperature treatments in day, night, and combined stress treatments (Table 2). Overall, spikelet tissue temperature differed significantly among genotypes (Tables 1 and 2). As measured spikelet tissue temperature did not differ much from air temperature, we used air temperature in all our analysis, unless specified otherwise.

3.2 | Spikelet fertility

In Experiment 1, plants were exposed to two temperature treatments, that is, control (30 °C) and HDT (38 °C) for 6 hr·day^{-1} for 6 days at flowering. Significant genotype, treatment, and interaction between genotype and treatment effects ($P < .001$) were recorded for spikelet fertility, on the targeted main-tiller panicles. Specifically, under the

TABLE 1 Spikelet tissue temperature of checks (N22 and IR64), selected common high-yielding inbreds (NSIC Rc222, HHZ12, and PSBRc4), and seven rice hybrids (H1 to H7), which were exposed to control (30 °C) and higher day-time temperature (HDT, 38 °C) from 08:00 to 14:00 hr for 6 hr at the flowering stage for six consecutive days

Genotype	Control (*M* ± *SD*)	HDT (*M* ± *SD*)
N22	33.3 ± 0.8	38.4 ± 0.3
IR64	32.5 ± 0.7	37.7 ± 0.3
NSIC Rc222	31.2 ± 0.1	37.1 ± 1.4
HHZ12	32.1 ± 0.2	38.0 ± 0.6
PSBRc4	33.0 ± 1.0	36.0 ± 0.9
H1	32.2 ± 0.4	38.0 ± 0.3
H2	31.6 ± 0.4	38.5 ± 0.7
H3	32.9 ± 1.3	37.6 ± 0.6
H4	31.0 ± 0.4	36.1 ± 0.7
H5	32.8 ± 1.0	37.0 ± 0.7
H6	33.0 ± 1.4	38.0 ± 0.3
H7	32.0 ± 0.6	36.2 ± 0.9
Significance	*	***

*Significance at 5%.

***Significance at 0.1%.

TABLE 2 Spikelet tissue temperature of checks (N22 and IR64), heat-tolerant IR64 near-isogenic line (HT NIL), and three hybrids (H2, H5, and H6) exposed to control (31/23 °C [day/night]), higher night-time temperature (HNT, 31/30 °C), higher day-time temperature (HDT, 38/23 °C), combined higher day-time and night-time temperatures (HNDT, 38/30 °C) at the flowering stage for six consecutive flowering days

	Day-time (*M* ± *SD*)				Night-time (*M* ± *SD*)			
Genotype	Control	HNT	HDT	HNDT	Control	HNT	HDT	HNDT
N22	31.2 ± 0.1	29.6 ± 1.2	38.1 ± 0.1	37.6 ± 0.3	22.5 ± 0.1	28.2 ± 0.4	23.1 ± 0.1	28.3 ± 0.2
IR64	29.9 ± 0.0	29.4 ± 0.3	37.7 ± 1.0	38.4 ± 0.4	22.4 ± 0.1	29.4 ± 0.2	22.4 ± 0.4	29.4 ± 0.4
HT NIL	30.2 ± 0.3	30.0 ± 0.7	38.0 ± 0.4	38.0 ± 0.4	22.6 ± 0.3	29.6 ± 0.6	22.1 ± 0.0	28.9 ± 0.2
H2	30.7 ± 0.3	31.6 ± 0.3	39.3 ± 0.5	38.1 ± 0.4	22.7 ± 0.2	29.5 ± 0.1	22.2 ± 0.1	30.0 ± 0.2
H5	31.0 ± 0.3	30.6 ± 0.4	37.2 ± 0.3	38.1 ± 0.3	22.9 ± 0.1	29.6 ± 0.2	23.0 ± 0.1	30.4 ± 0.4
H6	30.0 ± 0.3	29.4 ± 0.3	38.6 ± 1.1	38.6 ± 0.2	22.9 ± 0.1	29.4 ± 0.2	22.5 ± 0.3	29.1 ± 0.4
Significance	***	***	**	NS	**	***	***	***

Note. Both day and night temperatures were maintained for 11 hr from 06:00–17:00 and 18:00–05:00 hr, respectively. NS = nonsignificant.

**Significance at 1%.

***Significance at 0.1%.

control condition, spikelet fertility was more than 80% in checks and high-yielding inbred varieties, but slightly lower in all hybrids except for H1 (Figure 1a). Spikelet fertility across the tested genotypes decreased under HDT stress, and there was significant genotypic variation in response to HDT ($P < .001$). Under HDT exposure, tolerant check N22 had 12.4% reduction in spikelet fertility compared to 64.9% in susceptible check IR64. Across the three inbreds, an average decline of 15.0% in fertility was recorded with exposure to HDT stress. The largest decrease of spikelet fertility was documented across all hybrids except for H2, with an average of 48.2%.

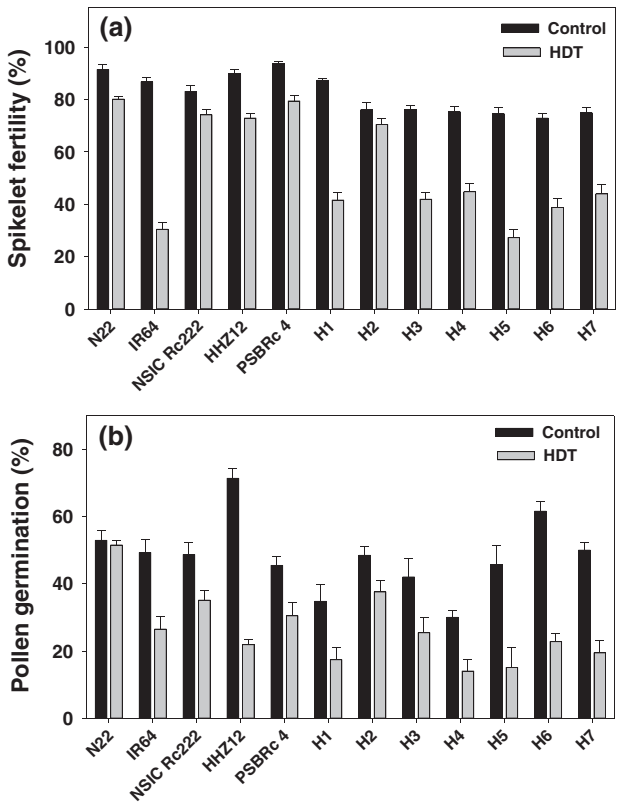

FIGURE 1 Spikelet fertility (a) and percentage of pollen germination on the stigma (b) of checks (N22 and IR64), selected common high-yielding inbreds (NSIC Rc222, HHZ12, and PSBRc4), and seven rice hybrids (H1 to H7), which were exposed to control (30 °C) and high day-time temperature (HDT, 38 °C) at the flowering stage for six consecutive flowering days in Experiment 1. Bars indicate standard errors of the means

In Experiment 2, the plants were exposed to independent and combined HNT and HDT, along with control temperature. There were significant effects of genotype, temperature treatments, and interaction between genotype and temperature ($P < .001$). Spikelet fertility was not significantly affected when exposed to HNT in checks and HT NIL, whereas there was a moderately significant decline in all tested hybrids (Table 3). In contrast, there was a highly significant decrease in spikelet fertility when plants were exposed to HDT and HNDT across all genotypes, with an average fertility of only 14.7% and 15.4% under HDT and HNDT exposure, respectively. Interestingly, there was a significant increase in spikelet fertility of H6 at HNDT over HDT. Regression analysis was undertaken to test the relative importance of day-time temperature (Tday) and night-time temperature (Tnight), as well as their interaction (Tday * Tnight) on spikelet fertility. Using air temperature or spikelet tissue temperature essentially gave similar results (Table 4). Overall, Tday was more damaging than Tnight as absolute values of the negative coefficients of Tday were generally higher than those of Tnight (Table 4). Moreover, the effect of Tday on spikelet fertility was significant for all genotypes, whereas that of Tnight was significant only in some genotypes, indicating genotypic variation in responses to Tday and Tnight. Spikelet fertility of hybrids in particular was, to some extent, further affected by Tnight. Moreover, of the hybrids, H5 and H6 showed a significant Tday * Tnight interaction, suggesting that although the effect of Tday was dominant, Tnight notably interacted with Tday in determining spikelet fertility in these two genotypes.

A strong positive correlation was observed for the heat stress index between two independent sets of plants exposed to HDT in Experiments 1 and 2, indicating genotypic consistency in tolerance/susceptibility to heat ($P < .05$; $n = 5$; Supporting Information Figure S1) in two independent experiments. However, the heat stress index was higher in Experiment 2 than in Experiment 1, presumably due the different durations of high-temperature (11 hr in Experiment 2 vs. 6 hr in Experiment 1).

TABLE 3 Spikelet fertility of checks (N22 and IR64), heat-tolerant IR64 near-isogenic line (HT NIL), and three hybrids (H2, H5, and H6) exposed to control (31/23 °C [day/night]), higher night-time temperature (HNT, 31/30 °C), higher day-time temperature (HDT, 38/23 °C), combined higher day-time and night-time temperatures (HNDT, 38/30 °C) at the flowering stage for six consecutive flowering days (Experiment 2)

Genotype	Control (*M* ± *SE*)	HNT (*M* ± *SE*)	HDT (*M* ± *SE*)	HNDT (*M* ± *SE*)
N22	93.0 ± 0.7	91.8 ± 0.7	24.5 ± 5.0	29.0 ± 1.8
IR64	88.2 ± 0.8	86.5 ± 0.8	12.1 ± 1.5	9.7 ± 1.7
HT NIL	83.4 ± 1.7	80.7 ± 1.7	19.1 ± 2.6	20.2 ± 2.0
H2	69.5 ± 2.1	52.5 ± 2.5	15.4 ± 3.9	9.3 ± 2.9
H5	70.3 ± 2.1	40.6 ± 6.6	4.6 ± 1.5	3.8 ± 0.8
H6	73.4 ± 2.0	66.4 ± 2.1	12.7 ± 1.7	20.9 ± 1.4
Genotype (G)		***		
Treatment (T)		***		
G*T		***		

***Significance at 0.1%.

TABLE 4 Regression analysis (Y = a + b1 * Tday + b2 * Tnight + b12 * Tday * Tnight) for the spikelet fertility for six rice genotypes grown under control (31/23 °C [day/night]), higher night-time temperature (HNT, 31/30 °C), higher day-time temperature (HDT, 38/23 °C), or combined higher night-time and day-time temperature (HNDT, 38/30 °C) for six consecutive flowering days (based on data of Experiment 2)

				Coefficients				*P* value			
Factor	Genotype	No. of observations	R^2	Intercept	Tday	Tnight	Tday * Tnight	Intercept	Tday	Tnight	Tday * Tnight
Air temperature	N22	48	.93	482.9 ± 102.7	−12.5 ± 3.0	−3.8 ± 3.8	0.1 ± 0.1	.000	.000	.333	.301
	IR64	48	.99	420.9 ± 49.0	−10.5 ± 1.4	0.2 ± 1.8	0.0 ± 0.1	.000	.000	.917	.787
	HT NIL	48	.96	433.5 ± 76.7	−11.0 ± 2.2	−2.8 ± 2.9	0.1 ± 0.1	.000	.000	.329	.345
	H2	48	.87	523.4 ± 109.8	−12.8 ± 3.2	−9.3 ± 4.1	0.2 ± 0.1	.000	.000	.028	.068
	H5	48	.85	879.5 ± 134.7	−23.0 ± 3.9	−22.5 ± 5.0	0.6 ± 0.1	.000	.000	.000	.000
	H6	48	.95	585.8 ± 68.6	−15.8 ± 2.0	−10.6 ± 2.6	0.3 ± 0.1	.000	.000	.000	.000
Spikelet temperature	N22	48	.93	716.4 ± 121.6	−18.2 ± 3.6	−13.9 ± 4.7	0.4 ± 0.1	.000	.000	.005	.010
	IR64	48	.99	516.3 ± 40.1	−13.7 ± 1.2	−6.2 ± 1.5	0.2 ± 0.0	.000	.000	.000	.000
	HT NIL	48	.96	425.5 ± 66.6	−10.8 ± 1.9	−4.1 ± 2.5	0.1 ± 0.1	.000	.000	.114	.135
	H2	48	.87	284.8 ± 99.0	−5.8 ± 2.8	−0.9 ± 3.9	0.0 ± 0.1	.006	.043	.820	.826
	H5	48	.85	1,101.3 ± 142.7	−29.7 ± 4.1	−30.6 ± 5.2	0.8 ± 0.2	.000	.000	.000	.000
	H6	48	.95	533.1 ± 56.7	−14.2 ± 1.6	−11.0 ± 2.1	0.3 ± 0.1	.000	.000	.000	.000

Note. Estimates ± standard error of the estimates.

3.3 | Pollen germination and its relationship with spikelet fertility

The percentage of pollen germination was significantly affected by genotypes, temperature treatment, and their interaction ($P < .001$) in Experiment 1. Under control conditions, the pollen germination ranged from 30.0% to 71.4% across all genotypes (Figure 1b). There was a significant decline in percentage of pollen germination with HDT in all genotypes except for the tolerant check N22, with an average of 46.8% of reduction across cultivars. The percentage of pollen germination was significantly correlated with spikelet fertility with the high-temperature condition ($P < .01$; $n = 12$), whereas it was not strongly associated with spikelet fertility at the control condition (Figure 2).

3.4 | Fertilization observation

By applying the WE-CLSM, the *in vivo* imaging of the double-fertilization process was successfully observed for rice exposed to both control and HDT. About 0.5 to 1 hr after flowering across all genotypes (Supporting Information Tables S2), the tip of pollen tubes passed through the micropylar pole (Figure 3a), discharging its content through interaction with one of the synergids, which looked like a horn (Figure 3b). The horn-like structure was seen extending and getting closer to the two polar nuclei and egg cell, allowing the release of two sperm cells to migrate towards the female gametes (one of the released sperm cell moved towards the egg cell and the other one

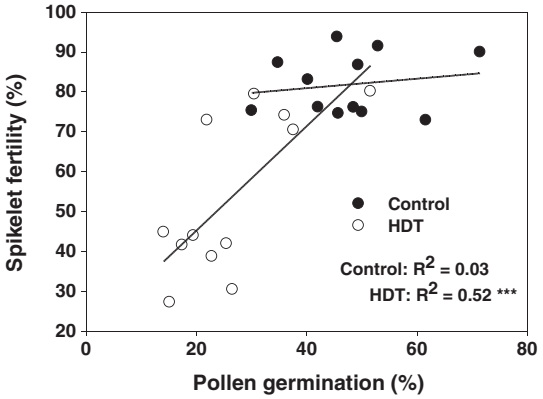

FIGURE 2 The relationship between spikelet fertility and pollen germination of all tested genotypes that were exposed to control (30 °C) and high day-time temperature (HDT, 38 °C) at the flowering stage for six consecutive flowering days (Experiment 1). The significance of the correlation is represented as ***$P < .001$

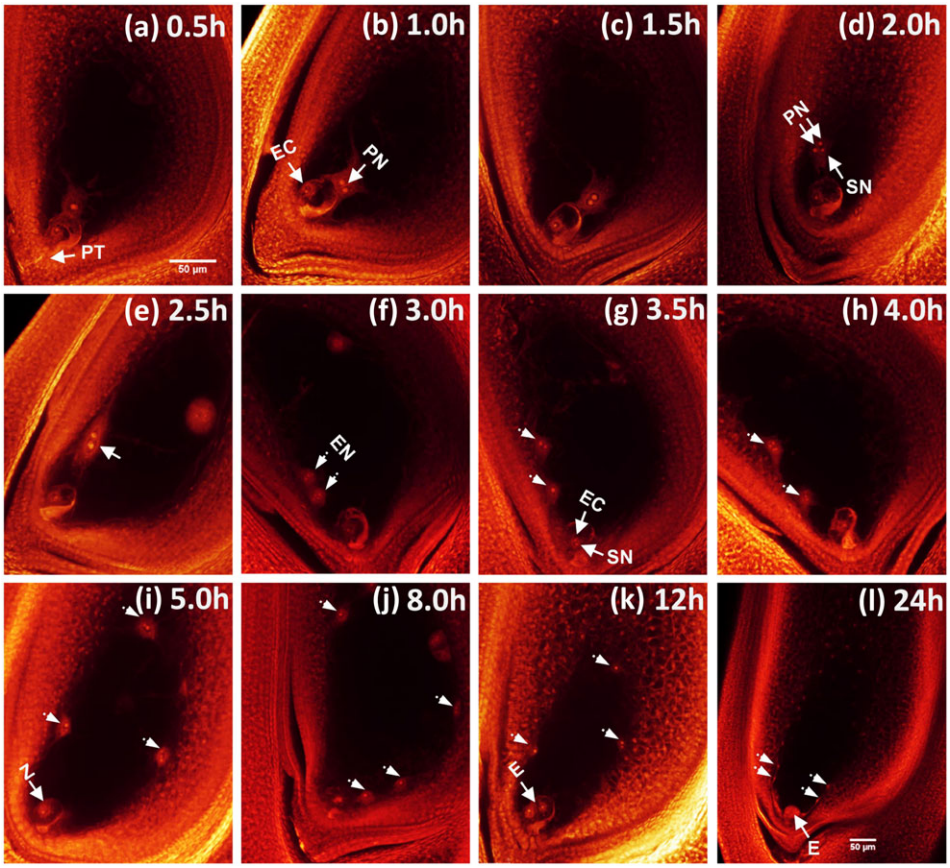

FIGURE 3 Processes leading to fertilization and zygote formation in IR64 plants exposed to control temperature (30 °C) coinciding with flowering (Experiment 2). (a) At 0.5 hr after flowering (h), PT was passing through the micropyle. (b) At 1 hr, PT penetrated into the one of the synergids and a horn-like structure gets closer to two PN and EC. (c, d) From 1.5 to 2.0 hr, two PN together with one SN (shown in 2.0 hr) started migration. (e) At 2.5 hr, the fusion of PN and SN occurred (indicated by an arrow). (f–h) From 3.0 to 4 hr, free EN is shown from the first division of the primary EN. (i) At 5 hr, Z shown with large nucleolus was seen from the fusion of EC and SN. (k) At 12.0 hr, pre-embryo with two cells. (l) At 24 hr, E together with plenty of free EN. Bars = 50 µm. The bars for pictures from a to k are the same. E, embryo; EC, egg cell; EN, endosperm nucleus; PT, pollen tube; PN, polar nuclei; SN, sperm cell nuclei; Z, zygote

TABLE 5 Percentage of spikelets categorized into various classes during fertilization in checks (N22 and IR64), heat-tolerant IR64 near-isogenic line (HT NIL), and three hybrids (H2, H5, and H6) exposed to control (31/23 °C [day/night]) and high day-time temperature (HDT, 38/23 °C) during flowering (Experiment 2)

Genotype	Treatment	Total number of spikelets used for observation	Spikelets with normal fertilization (%)	Spikelets without pollen tubes reached its embryo sac (%)	Spikelets with arrested fertilization (%)	Unclear samples and abnormal embryo sac (%)
N22	Control	446	94.8	2.5	0.9	1.8
	HDT	306	19.0	77.1	2.3	1.6
IR64	Control	319	91.2	2.5	0.3	6.0
	HDT	341	2.3	73.3	16.7	7.6
HT NIL	Control	533	89.1	8.6	0.2	2.1
	HDT	384	16.4	78.4	4.2	1.0
H2	Control	356	81.2	14.6	2.2	2.0
	HDT	306	9.8	81.4	4.6	4.2
H5	Control	412	73.3	23.8	0.5	2.4
	HDT	307	5.9	83.4	3.3	7.5
H6	Control	459	88.7	6.3	0.4	4.6
	HDT	530	9.8	87.4	1.3	1.5

Note. The total number of observed spikelets is the sum of collected spikelets starting from 0.5 to 24 hr after flowering. Spikelets with normal fertilization indicate spikelets having similar processes as in the control condition at a particular timepoint. Spikelets with arrested fertilization indicate that the fertilization process was stopped and was not the same as in control conditions.

towards polar nuclei—not visible in our pictures). After that, cytoplasm content around polar nuclei became denser (Figure 3c). From then until 2 or 3 hr after flowering in different genotypes, polar nuclei together with sperm nucleus moved closer to the wall of the embryo sac (Figure 3d,e), and the primary endosperm nucleus was formed from the fertilization of polar nuclei and sperm nucleus. Then the primary

endosperm nucleus started its first free-nuclear division (Figure 3f), and there was genotypic variation in the timing when division was started (Supporting Information Table S2). From then on, the primary endosperm nucleus continued its second or third division (Figure 3g–j). In 5 hr after flowering, the sperm nucleus fused with the egg nucleus, forming a larger nucleolus providing initial signals of zygote formation (Figure 3i). Twelve hours after flowering, the zygote was seen to undergo its first division, and a two-celled embryo was formed, while the primary endosperm nucleus had completed three or more rounds of divisions (Figure 3k). At 24 hr after flowering, the size of the embryo sac was enlarged with plenty of free nuclei distributed around the wall of the embryo sac, and simultaneously the zygote continued its division (Figure 3l).

On the basis of the *in vivo* fertilization observations, spikelets were classified into four categories to distinguish the differences between control and HDT exposures (Table 5). Under the control condition, more than 73.3% of the spikelets had normal fertilization, and the next large group was the spikelets without pollen tubes reaching the embryo sac, accounting for 2.5% to 23.8% of spikelets across all genotypes (Table 5). There were only 0.2% to 2.2% of samples in which pollen tubes reached the embryo sac, but then were arrested with no further progress. After exposure to HDT stress, the spikelets with normal fertilization largely decreased and ranged between 2.3% and 19.0% (Table 5). There was an average of 80.2% samples without pollen tubes reaching to the micropylar pole or without bright horn-like structure, indicating lack of penetration of pollen tube into the synergid in all genotypes (Figure 4—showing the process at 0.5 and 1.0 hr after flowering). In contrast, the spikelets with arrested fertilization increased under the HDT condition compared to that in the control condition even though it was less frequent among the various fertilization classifications.

4 | DISCUSSION

In view of constraints affecting hybrid rice development under current and projected increase in frequency of heat episodes in tropical and subtropical rice-growing countries, we studied experimentally the impact of high-temperature stress on hybrids in comparison with inbred rice genotypes. When designing experiments involving temperature effects, transpiration cooling could be a confounding factor due to different VPDs, which is determined by interaction between day temperature and relative humidity (Julia & Dingkuhn, 2013; Yan et al., 2010). Considering tissue temperature in plants has been highlighted (Sheehy, Mitchell, Beerling, Tsukaguchi, & Woodward, 1998; Yoshimoto et al., 2011), to account for this interaction. There are indications of genotypic difference in panicle temperature under highly variable environments such as extreme heat and low relative humidity (Julia & Dingkuhn, 2013). However, in our study, spikelet tissue temperature measured across all genotypes and both experiments was close to air temperature, by following an established experimental set-up (i.e., HDT and moderately high relative humidity) wherein VPD is maintained at low levels (Jagadish et al., 2010; Shi et al., 2015). Additionally, it has been reported that the tolerant genotype with

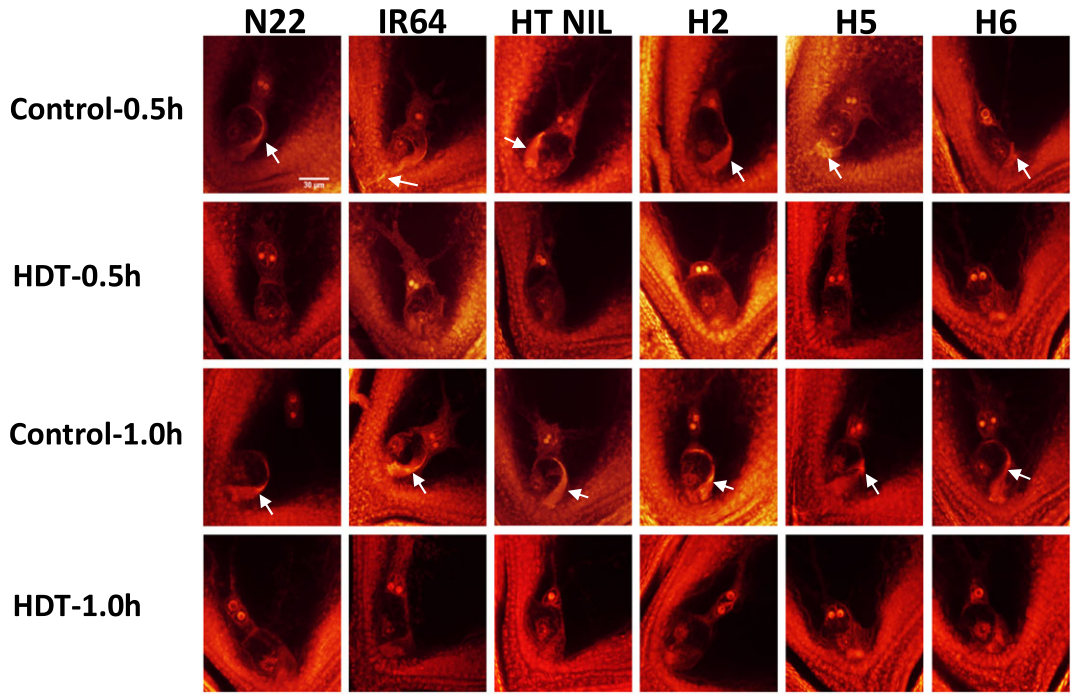

FIGURE 4 Processes happening at 0.5 and 1.0 hr after flowering in two rice genotypes (checks N22 and IR64), heat-tolerant IR64 near-isogenic line (HT NIL), and three hybrids (H2, H5, and H6) after exposure to control temperature (30 °C) and high day-time temperature (HDT, 38 °C) at the flowering stage (Experiment 2). Control-0.5h: Highlighted pollen tubes were passing through the micropylar (in IR64 and H5) or soon after it penetrates into the synergid and a bright horn was formed (indicated by arrows). Control-1.0h: Horn-like structure extended into the middle of two polar nuclei and egg cell, allowing the release of two sperm cell nuclei. HDT-0.5h and HDT-1.0h: The bright pollen tubes or horn-like structure was not observed in the embryo sac, indicating the pollen tubes did not reach the embryo sac. Bar = 30 μm

higher spikelet fertility had higher absolute spikelet tissue temperature compared to susceptible genotype (Coast, Ellis, Murdoch, Quiñones, & Jagadish, 2015; Shi et al., 2015). Our study also showed that the spikelet fertility of tolerant check N22 was much higher than that of other genotypes under high-temperature, with no obvious difference in spikelet tissue temperature between N22 and other genotypes (Tables 1 and 2). These data indicate that genotypic resilience is not merely associated with avoiding the hot microclimate, but is also mostly due to its reproductive physiology (such as number of pollen grains and pollen germination on the stigma).

Thus, it is necessary to investigate how spikelet fertility is associated with reproductive physiology under stress. To date, our study is the first to evaluate this association in the context of the performance of tropical and subtropical hybrids in response to HDT. We first observed the vulnerability of these hybrids to HDT during the flowering stage, which is in agreement with the previous studies working on evaluating the heat tolerance of hybrid rice grown in China (Fu et al., 2015; Hu et al., 2012; Tong, Li, Duan, Deng, & Tian, 2008; Zhang et al., 2014). It is worth noting that hybrids having heat stress tolerance to HDT in the above studies and even our study account for only a small portion of all tested hybrids. Moreover, substantial differences in the sensitivity of spikelet fertility to HDT were identified within investigated hybrid rice and selected best performing modern inbred indica varieties and a heat-tolerant check. Hybrids showed greater decreases in spikelet fertility over the inbreds exposed to HDT at the flowering stage. Madan et al. (2012) showed that the large yield advantage of one hybrid over an inbred cultivar (IR64) at 29 and 35 °C disappears at 38 °C as sterility significantly increased. In line with our results, a study that compared one inbred japonica variety with two hybrids showed higher heat susceptibility in hybrids at the heading stage (Zhang et al., 2014). From the above studies and our own evaluation, it can be concluded that high-temperature is a major factor in regulating the stability of hybrid rice production, with hybrid rice being more sensitive to increasing temperature than indica and japonica inbreds. These findings, therefore, highlight the urgent need to address the damage caused by HDT on hybrids and develop heat-tolerant hybrids by utilizing the genetic advances made using inbreds and landraces (Ye et al., 2015). In Experiment 2, HDT and HNDT significantly decreased spikelet fertility in all tested genotypes, whereas HNT moderately decreased spikelet fertility in only three hybrids (Table 3). Regression analysis also demonstrated that day temperature was dominant in deciding spikelet fertility of rice rather than night temperature (Table 4). This is in agreement with the results of Yin, Kropff, and Goudriaan (1996) on phenological development to flowering in response to day and night temperatures and with Ishimaru et al. (2016) on the spikelet sterility in the fields of heat-vulnerable regions in Laos and Southern India.

High-temperature during flowering has been identified to affect anther dehiscence, pollination, and pollen germination, causing spikelet sterility (Jagadish et al., 2010; Matsui et al., 2000; Prasad et al., 2006). Our result (Figure 2) was in line with previous reports that spikelet sterility under HDT exposure was strongly associated with lower numbers of pollen grains germinating on the stigma. Only one pollen tube can succeed in penetrating the embryo sac from the micropyle because the others that arrive later cannot enter the micropyle as the signals to guide the pollen tubes are stopped. However, the elongation of the pollen tubes is more favourable when several pollen tubes are in close proximity to each other as compared with isolated ones (Hoshikawa, 1989). Thus, a certain number of germinated pollen grains are required for maintaining the spikelet fertility under the HDT condition, and Yoshida, Satake, and Mackill (1981) identified this minimum number of germinated pollen grains to be 10. After pollen grains are germinated on the stigma and pollen tubes penetrate the embryo sac, the double-fertilization process is immediately initiated. However, this *in vivo* fertilization process has never been clearly described in previous research to pinpoint relative changes under both control and high-temperature conditions. Our results clearly demonstrated that increased temperature during flowering caused spikelet sterility due to disturbance on the prefertilization process, as spikelets without pollen tube reaching the embryo sac accounted for the largest proportion among all observations (Table 5, Figure 4, and Supporting Information Figure S2). Our findings add a more detailed mechanistic explanation to previous observations that high-temperature affects pollen viability and germination on the stigmatic surface and along its journey to the ovary. In contrast, the fertilization process in both control and HDT conditions was less affected by temperature stress as spikelet with arrested fertilization accounted for a small proportion compared with spikelets without pollen tubes reaching the ovary. Our study is the first to prove the hypothesized statement that temperatures ≥38 °C occurring 1 hr after flowering had minimal impact on fertility (Jagadish et al., 2007; Satake & Yoshida, 1978; Yoshida et al., 1981). Furthermore, our results imply that shifting the flower opening to early-morning cooler conditions (Bheemanahalli et al., 2017; Ishimaru et al., 2010) is an effective strategy and should be considered as a potential trait to improve heat stress resilience in hybrids.

In summary, our study supports previous findings and indicates higher degree of heat susceptibility in tropical and subtropical hybrids compared to high-yielding inbred varieties. We identified a novel mechanism of high-temperature impact during flowering; that is, the fertilization process was minimally affected by HDT, with physiological disturbances in the prefertilization phase identified to be the primary cause for heat-induced spikelet sterility. Thus, introducing the early-morning flowering trait will be an effective heat stress escape strategy as the most vulnerable flowering would occur under cooler morning hours and the later fertilization process, though taking place at high-temperature around noon, would be less affected, as demonstrated from our findings. Although this mechanism may explain our result in H6 that HNT might alleviate the negative effect of the following-day HDT, it does not explain a direct negative effect of HNT seen with three hybrids. The latter effect merits further detailed physiological and mechanistic investigations.

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ORCID

Xinyou Yin http://orcid.org/0000-0001-8273-8022

S.V. Krishna Jagadish http://orcid.org/0000-0002-1501-0960

REFERENCES

Bheemanahalli, R., Sathishraj, R., Manoharan, M., Sumanth, H. N., Muthurajan, R., Ishimaru, T., & Jagadish, S. V. K. (2017). Is early morning flowering an effective trait to minimize heat stress damage during flowering in rice. *Field Crops Research*, *203*, 238–242.

Challinor, A. J., Watson, J., Lobell, D. B., Howden, S. M., Smith, D. R., & Chhetri, N. (2014). A meta-analysis of crop yield under climate change and adaptation. *Nature Climate Change*, *4*, 287–291.

Cheng, S., Cao, L., Zhuang, J., Chen, S., Zhan, X., Fan, Y., … Min, S. (2007). Super hybrid rice breeding in China: Achievements and prospects. *Journal of Integrative Plant Biology*, *49*(6), 805–810.

Cho, J. (1956). Double fertilization in *Oryza sativa* L. and development of the endosperm with special reference to the aleurone layer. *Bulletin of the National Institute of Agricultural Sciences* (Japan), *6*, 61–101.

Coast, O., Ellis, R. H., Murdoch, A. J., Quiñones, C., & Jagadish, S. V. K. (2015). High night temperature induces contrasting responses for spikelet fertility, spikelet tissue temperature, flowering characteristics and grain quality in rice. *Functional Plant Biology*, *42*(2), 149–161.

Fu, G., Zhang, C., Yang, Y., Xiong, J., Yang, X., Zhang, X., … Tao, L. (2015). Male parent plays a more important role in heat tolerance in three-line hybrid rice. *Rice Science*, *22*(3), 116–122.

Hasegawa, T., Ishimaru, T., Kondo, M., Kuwagata, T., Yoshimoto, M., & Fukuoka, M. (2011). Spikelet sterility of rice observed in the record hot summer of 2007 and the factors associated with its variation. *Journal of Agricultural Meteorology*, *67*(4), 225–232.

Hoshikawa K. (1989) Growing rice plant. In: An anatomical monograph, Nosan Gyoson Bunka Kyokai (Nobunkyo), Tokyo, Japan.

Hu, S., Zhang, Y., Zhu, D., Lin, X., & Xiang, J. (2012). Evaluation of heat resistance in hybrid rice. *Chinese Journal of Rice Science*, *26*, 751–756 (in Chinese with English abstract).

IPCC. (2013). Working Group I contribution to the IPCC fifth assessment report on climate change 2013: The physical science basis, summary for policymakers. Retrieved from www.climatechange2013.org/images/report/WG1AR5%20SPM%20FINAL.pdf.

Ishimaru, T., Hirabayashi, H., Ida, M., Takai, T., San-Oh, Y. A., Yoshinaga, S., … Kondo, M. (2010). A genetic resource for early-morning flowering trait of wild rice *Oryza officinalis* to mitigate high-temperature-induced spikelet sterility at anthesis. *Annals of Botany*, *106*(3), 515–520.

Ishimaru, T., Xaiyalath, S., Nallathambi, J., Sathishraj, R., Yoshimoto, M., Phoudalay, L., & Samson, B. (2016). Quantifying rice spikelet sterility in potential heat-vulnerable regions: Field surveys in Laos and Southern India. *Field Crops Research*, *190*, 3–9.

Jagadish, S. V. K., Craufurd, P. Q., & Wheeler, T. R. (2007). High-temperature stress and spikelet fertility in rice (*Oryza sativa* L.). *Journal of Experimental Botany*, *58*, 1627–1635.

Jagadish, S. V. K., Craufurd, P. Q., & Wheeler, T. R. (2008). Phenotyping parents of mapping populations of rice for heat tolerance during anthesis. *Crop Science*, *48*, 1140–1146.

Jagadish, S. V. K., Muthurajan, R., Oane, R., Wheeler, T. R., Heuer, S., Bennett, J., & Craufurd, P. Q. (2010). Physiological and proteomic approaches to address heat tolerance during anthesis in rice (*Oryza sativa* L.). *Journal of Experimental Botany*, *61*(1), 143–156.

Julia, C., & Dingkuhn, M. (2013). Predicting temperature induced sterility of rice spikelets requires simulation of crop-generated microclimate. *European Journal of Agronomy*, *49*, 50–60.

Madan, P., Jagadish, S. V. K., Craufurd, P. Q., Fitzgerald, M., Lafarge, T., & Wheeler, T. R. (2012). Effect of elevated CO_2 and high-temperature on seed-set and grain quality of rice. *Journal of Experimental Botany*, *63*, 3843–3852.

Matsui, T., Omasa, K., & Horie, T. (2000). High-temperature at flowering inhibits swelling of pollen grains, a driving force for thecae dehiscence in rice (*Oryza sativa* L.). *Plant Production Science*, *3*(4), 430–434.

Matsui, T., Omasa, K., & Horie, T. (2001). The difference in sterility due to high-temperatures during the flowering period among japonica-rice varieties. *Plant Production Science*, *4*(2), 90–93.

Prasad, P. V. V., Boote, K. J., Allen, L. H., Sheehy, J. E., & Thomas, J. M. G. (2006). Species, ecotype and cultivar differences in spikelet fertility and harvest index of rice in response to high-temperature stress. *Field Crops Research*, *95*(2), 398–411.

Rang, Z., Jagadish, S. V. K., Zhou, Q. M., Craufurd, P. Q., & Heuer, S. (2011). Effect of high-temperature were recorded for spikelet fertility,Regression analysis fertility in rice. *Environmental and Experimental Botany*, *70*(1), 58–65.

Satake, T., & Yoshida, S. (1978). High-temperature-induced sterility in indica rice at flowering. *Japanese Journal of Crop Science*, *47*(1), 6–17.

Sheehy, J., Elmido, A., Centeno, G., & Pablico, P. (2005). Searching for new plants for climate change. *Journal of Agricultural Meteorology*, *60*, 463–468.

Sheehy, J. E., Mitchell, P. L., Beerling, D. J., Tsukaguchi, T., & Woodward, I. F. (1998). Temperature of rice spikelets: Thermal damage and the concept of a thermal burden. *Agronomie*, *18*, 449–460.

Shi, W., Ishimaru, T., Gannaban, R. B., Oane, W., & Jagadish, S. V. K. (2015). Popular rice (*Oryza sativa* L.) cultivars show contrasting responses to heat stress at gametogenesis and anthesis. *Crop Science*, *55*(2), 589–596.

Shi, W., Yin, X., Struik, P. C., Solis, C., Xie, F., Schmidt, R. C., … Jagadish, S. V. K. (2017). High day-time and night-time temperature affect grain growth dynamics in contrasting rice genotypes. *Journal of Experimental Botany*, *58*, 5233–5245.

Shi, W., Yin, X., Struik, P. C., Xie, F., Schmidt, R. C., & Jagadish, S. V. K. (2016). Grain yield and quality responses of tropical hybrid rice to high night-time temperature. *Field Crops Research*, *190*, 18–25.

Tang, R., Zheng, J., Jin, Z., Zhang, D., Huang, Y., & Chen, L. (2008). Possible correlation between high-temperature-induced floret sterility and endogenous levels of IAA, GAs and ABA in rice (*Oryza sativa* L.). *Plant Growth Regulation*, *54*(1), 37–43.

Tao, L., Tan, H., Wang, X., Cao, L., Song, J., & Cheng, S. (2008). Effects of high-temperature stress on flowering and grain-setting characteristics of Guodao 6. *Acta Agronomica Sinica*, *34*(4), 609–674.

Tian, X., Matsui, T., Li, S., Yoshimoto, M., Kobayasi, K., & Hasegawa, T. (2010). Heat-induced floret sterility of hybrid rice (*Oryza sativa* L.) cultivars under humid and low wind conditions in the field of Jianghan Basin, China. *Plant Production Science*, *13*, 243–251.

Tong, Z., Li, S., Duan, W., Deng, Y., & Tian, X. (2008). Temperature-driven climatic factors and their impact on the fertility of hybrid rice at anthesis. *Chinese Journal of Eco-Agriculture*, *16*(5), 1163–1166 (In Chinese with English abstract).

Xie, F., & Peng, S. (2016). History and prospects of hybrid rice development outside of China. *Science Bulletin*, (35), 3858–3868.

Yan, C., Ding, Y., Wang, Q., Liu, Z., Li, G., Muhammad, I., & Wang, S. (2010). The impact of relative humidity, genotypes and fertilizer application rates on panicle, leaf temperature, fertility and seed setting of rice. *The Journal of Agricultural Science*, *148*(03), 329–339.

Ye, C., Argayoso, M. A., Redoña, E. D., Sierra, S. N., Laza, M. A., Dilla, C. J., … Hernandez, J. E. (2012). Mapping QTL for heat tolerance at flowering stage in rice using SNP markers. *Plant Breeding*, *131*(1), 33–41.

Ye, C., Tenorio, F. A., Redoña, E. D., Morales-Cortezano, P. S., Cabrega, G. A., Jagadish, S. V. K., & Gregorio, G. B. (2015). Fine-mapping and validating qHTSF4. 1 to increase spikelet fertility under heat stress at flowering in rice. *Theoretical and Applied Genetics*, *128*(8), 1507–1517.

Yin, X., Kropff, M. J., & Goudriaan, J. (1996). Differential effects of day and night temperature on development to flowering in rice. *Annals of Botany*, *77*, 203–213.

Yoshida S., Satake T., & Mackill D. J. (1981). High-temperature stress in rice. IRRI Research Papers series 67. International Rice Research Institute, Manila, Philippines.

Yoshimoto, M., Fukuoka, M., Hasegawa, T., Matsui, T., Tian, X., Vijayalakshmi, C., & Tarpley, L. (2012). MINCERnet: A global research alliance to support the fight against heat stress in rice. *Journal of Agricultural Meteorology*, *68*, 149–157.

Yoshimoto, M., Fukuoka, M., Hasegawa, T., Utsumi, M., Ishigooka, Y., & Kuwagata, T. (2011). Integrated micrometeorology model for panicle and canopy temperature (IM2PACT) for rice heat stress studies under climate change. *Journal of Agricultural Meteorology*, *67*, 233–247.

Zeng, Y., Hu, C., Lu, Y., Li, J., & Liu, X. (2007). Diversity of abnormal embryo sacs in indica/japonica hybrids in rice demonstrated by confocal microscopy of ovaries. *Plant Breeding*, *126*(6), 574–580.

Zhang, Z., Wang, Q., Lang, Y., Wang, C., Zhu, Q., & Yang, J. (2014). Effects of high-temperature stress at heading stage on pollination and fertilization of different types of rice variety. *Acta Agronomica Sinica*, *40*(2), 273–282.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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