Abnormal Anther Development Leads to Lower Spikelet Fertility in Rice (Oryza Sativa L.) Under High Temperature During the Panicle Initiation Stage

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Abstract

**Background:** Decreased spikelet fertility is often responsible for reduction in grain yield in rice (*Oryza sativa* L.). In this study, two varieties with different levels of heat tolerance, Liangyoupeijiu (LYPJ, heat susceptible) and Shanyou63 (SY63, heat tolerant) were subjected to two temperature treatments for 28 days during the panicle initiation stage in temperature/relative humidity-controlled greenhouses: high temperature (HT; 37/27°C; day/night) and control temperature (CK; 31/27°C; day/night) to investigate changes in anther development under HT during panicle initiation and their relationship with spikelet fertility.

**Results:** HT significantly decreased the grain yield of LYPJ by decreasing the number of spikelets per panicle and seed setting percentage. In addition, HT produced minor adverse effects in SY63. The decreased spikelet fertility was primarily attributed to decreased pollen viability and anther dehiscence, as well as poor pollen shedding of the anthers of LYPJ under HT. HT resulted in abnormal anther development (fewer vacuolated microspores, un-degraded tapetum, unevenly distributed Ubisch bodies) and malformation of pollen (obscure outline of the pollen exine with a collapsed bacula, disordered tectum, and no nexine of the pollen walls, uneven sporopollenin deposition on the surface of pollen grains) in LYPJ, which may have lowered pollen viability. Additionally, HT produced a compact knitted anther cuticle structure of the epidermis, an un-degraded septum, a thickened anther wall, unevenly distributed Ubisch bodies, and inhibition of the confluent locule, and these malformed structures may be partially responsible for the decreased anther dehiscence rate and reduced pollen shedding of the anthers in LYPJ. In contrast, the anther wall and pollen development of SY63 were not substantially changed under HT.

**Conclusions:** Our results suggest that disturbed anther walls and pollen development are responsible for the reduced spikelet fertility and grain yield of the tested heat susceptible variety, and noninvasive anthers and pollen formation in response to HT were associated with improved heat tolerance.

**Background**

Plant responses to temperature stress are receiving increased attention due to growing awareness about global warming. Several studies have revealed that high temperature (HT) stress at different periods of reproductive growth has detrimental effects on rice yield, mainly via decreasing pollen viability, spikelet fertility and the grain filling percentage [1–4]. Importantly, almost all spikelets were found to be sterile when high temperature stress occurred at the early microspore stage after meiosis [5]. With temperature increases induced by global warming in the future, it is expected that developing panicles will experience HT stress more frequently. Therefore, it is important that measures are taken to study the mechanism of HT injury on rice panicle development and implement reasonable procedures to alleviate high temperature injury during panicle initiation in the future.
Abnormal panicle development is one of the main factors limiting grain yield formation under HT stress. At panicle initiation, HT resulted in attenuated differentiation of secondary branches and an increased number of degraded branches, and fewer spikelets per panicle, as well as reduced grain length, grain width, grain area, and grain weight [3]. Additionally, decreased pollen viability was found to be one of the main factors responsible for spikelet sterility under HT during panicle initiation [3]. Reductions in pollen viability are tightly associated with abnormal anther development [6–9]. Reduced spikelet fertility under HT at the flowering stage was attributed to abnormalities in anther dehiscence [10], pollen shedding and pollen germination [4, 11]. Pollen production, the amount of pollens on the stigma, in vivo pollen germination on the stigma, and hidden stigmas were positively associated with spikelet fertility under HT at the flowering stage [1, 2, 12–14]. These previous reports show that the normal development of reproductive organs is a necessary perquisite for spikelet fertility and grain yield formation under heat stress.

Anther development plays an important role in pollen viability and spikelet fertility; however, anther development is frequently affected by HT stress [2, 15, 16]. Anther development can be divided into 14 stages according to the cellular features of anthers [17], and the meiosis stage and microspore development stage were found to be sensitive to HT stress in rice [5, 6, 18]. Several studies have reported that high temperature resulted in disordered pollen development in wheat, sorghum, tomato and maize [7, 19–21], which led to ovoid pollens with dehydrated exine [20], shrunken and collapsed pollen grains with rough exine walls, and collapsed germinal apertures [21]. Deng et al. [6] found that tetrad cells failed to separate under HT during the meiosis stage, while meiocytes adhered to each other. However, the process of pollen development and its relationship with spikelet fertility and yield in rice exposed to HT stress during panicle initiation are not well understood.

The anther wall consists of four layers: epidermis, endothecium, middle layer, and tapetum. At the tetrads stage (stage 8 of anther development), the middle layer disappears and the tapetum is degraded. At the vacuolated microspore stage (stage 10 of anther development), the tapetum thins and microspores become vacuolated and adhere tightly to the tapetum. At the pollen maturity stage (stage 13 of anther development), only the epidermis and endothecium remain, pollen grain filling is completed, and the stomium is split for dehiscence [8]. HT stress often disturbs anther development [6, 8, 22, 23]. Sato et al. [22] found that HT disrupted the development of the endothecium, epidermis, and stomium at stage 13 in Lycopersicon esculentum Mill. Zhang et al. [23] observed that cells of the epidermis were arranged loosely in mature rice anthers exposed to high temperature at the heading stage.

Normal and timely degeneration of the tapetum is essential for microspore development, pollen adhesion and germination on the stigma, and pollen viability [5, 8, 9, 24]. The reported effects of HT on the initiation of tapetum degeneration have not been consistent in previous studies. Suzuki et al. [9] observed advanced tapetum degeneration and pollen sterility in snap bean plants exposed to HT conditions; however, HT delayed tapetum degeneration in cotton [25] and rice [6], resulting in low pollen viability. In addition, Endo et al. [5] observed that 3 days of HT treatment during meiosis did not affect the tapetum degradation process in rice, but pollen viability was decreased by this treatment. These data show the
The inconsistency of reported tapetum degeneration responses to HT conditions and demonstrate that the effect of HT on tapetum degeneration requires further study.

Anther dehiscence is essential for pollen spreading and pollination, which are tightly associated with spikelet fertility [10, 11]. Several studies revealed that the anther dehiscence percentage in rice decreased under HT during flowering [10, 11]. There are several cell layers of anther wall between the locule and the lacuna (the space between the septum adhering to the anther wall and the stomium). Matsui et al. [26] observed thickened cell layers and delayed locule opening in response to HT, leading to decreased spikelet fertility. However, Bagha [27] found that the cell layers were not affected by HT, and failure in lysis of the septum cell wall prevented anther locules from opening during panicle initiation in rice. Therefore, probing the relationship between anther dehiscence and anatomic structures is an important step toward a deeper understanding of the effects of heat stress on spikelet fertility.

In a previous study, we found that rice cultivar Liangyoupeijiu (LYPJ) is susceptible to HT during panicle initiation, whereas Shanyou63 (SY63) is more tolerant to HT [3]. However, the relationship among anther development, morphological features and spikelet fertility under HT are not yet clear. Therefore, the objective of this study was to illustrate changes in anther development under HT during panicle initiation and their relationship with spikelet fertility.

**Results**

**Effects of growth temperature on rice yield formation**

HT treatment significantly decreased the grain yield, seed setting percentage, and spikelets per panicle of LYPJ plants, but it had no significant effects on the grain yield or yield components of SY63 plants (Table 1). The grain yield of LYPJ plants was reduced by 84% under HT treatment compared with that measured under the CK treatment, and this change was accompanied by reductions in spikelets per panicle (35%), seed setting percentage (69%), and 1000-grain weight (17%). The change in the grain yield of SY63 plants (18%) was smaller than that of LYPJ plants due to small reductions in 1000-grain weight (7% in SY63) and grain setting percentage (20% in SY63). HT had no significant effects on the number of panicles per plant or biomass of either cultivar (Table 1).
Table 1
Effects of high temperature treatment on yield and yield components in LYPJ and SY63

| Cultivars | Treatment | Yield  | Panicles | Spikelets | Seed setting percentage | 1000-grain weight | Biomass  |
|-----------|-----------|--------|----------|-----------|-------------------------|-------------------|----------|
|           |           | g plant\(^{-1}\) | No. plant\(^{-1}\) | No. panicle\(^{-1}\) | % | g | g plant\(^{-1}\) |
| LYPJ      | CK        | 20.7 ± 1.4 \(^a\) | 11.3 ± 0.3 \(^a\) | 153.2 ± 5.2 \(^a\) | 53.3 ± 0.8 \(^b\) | 22.3 ± 0.6 \(^b\) | 87.0 ± 1.3 \(^a\) |
|           | HT        | 3.3 ± 0.8 \(^b\) | 11.0 ± 0.0 \(^a\) | 100.2 ± 6.5 \(^c\) | 16.4 ± 4.5 \(^c\) | 18.5 ± 0.1 \(^c\) | 81.4 ± 0.7 \(^ab\) |
| SY63      | CK        | 25.5 ± 0.8 \(^a\) | 11.3 ± 0.3 \(^a\) | 115.3 ± 5.7 \(^bc\) | 81.1 ± 1.0 \(^a\) | 24.2 ± 0.5 \(^a\) | 75.3 ± 3.4 \(^bc\) |
|           | HT        | 21.0 ± 1.4 \(^a\) | 11.0 ± 0.6 \(^a\) | 130.9 ± 2.2 \(^ab\) | 65.1 ± 4.7 \(^ab\) | 22.5 ± 0.5 \(^ab\) | 69.7 ± 3.2 \(^c\) |

CK control temperature; HT high temperature. Data are the average of three replicates ± standard error of the mean (n = 3). The different superscript lower-case letters indicate significance at \(P<0.05\) across the different treatments and varieties.

Effects of HT on fertility and anther dehiscence

More inviable pollen grains (colored in green, Fig. 2C) and anthers with many pollen grains (colored in yellow, Fig. 2G) were observed in LYPJ plants under HT treatment compared with those observed under the CK treatment (Fig. 2A and E); however, no obvious difference in the numbers of green pollens or yellow anthers were found between SY63 plants exposed to the CK and HT treatments (Fig. 2B, D, F and H). Accordingly, HT treatment markedly decreased pollen viability by 46% (Fig. 2I) and spikelet fertility by 69% (Fig. 2J) in LYPJ plants, but no significant reduction was observed in SY63 plants exposed to the CK and HT treatments. The anther dehiscence rate was decreased by 5% in LYPJ plants under HT treatment in comparison with those exposed to the CK treatment, and the pollen shedding percentage of the anthers was decreased by 11% (Fig. 2K and L), while SY63 plants exposed to HT treatment did not show significant differences in either trait. Additionally, the absolute values of the pollen viability, spikelet fertility, anther dehiscence rate, and pollen shedding percentage of the anthers of SY63 plants were 42%, 50%, 7% and 27% higher, respectively, than those of LYPJ plants under HT treatment (Fig. 2I, J, K and L).

Scanning electron microscopic and light microscopic observations of anther characteristics

Anther and microspore characteristics were analyzed by scanning electron microscopy. As shown in Fig. 3, almost all pollens were round-shaped under the CK treatment (Fig. 3A and C), and the pollen surface was smooth (Fig. 3E and G); however, HT treatment resulted in shriveled and collapsed pollen grains with a hollow germinal aperture (Fig. 3B and F), especially in LYPJ plants. Additionally, typically
granular sporopollenins on the pollen surface were observed under the CK treatment (Fig. 3I and K); however, sporopollenin deposition was abnormal under HT treatment, especially in LYPJ plants (Fig. 3J). The Ubisch bodies were evenly distributed and had typically sharp protrusions under the CK treatment (Fig. 3M and O); however, HT resulted in unevenly distributed Ubisch bodies with blunt protrusions in LYPJ plants (Fig. 3N) and had no effect on SY63 plants (Fig. 3P). The surface of the epidermis showed genetic variation between LYPJ (Fig. 3Q) and SY63 plants (Fig. 3S). HT treatment resulted in a compact knitted anther cuticle structure on the epidermis of LYPJ plants (Fig. 3R, V) in comparison with CK plants (Fig. 3Q and U). Generally, HT treatment had no substantial effect on epidermis characteristics (Fig. 3T and X) in comparison with those of plants exposed to the CK treatment (Fig. 3S and W).

Observations were carried out at 4 stages of anther development via light microscopy. At stage 8b, there were no obvious abnormal changes between the plants exposed to the CK and HT treatments for both varieties (Fig. 4A, B, C and D). At stage 9, when microspores are normally released from the tetrad, the tapetum of LYPJ and SY63 plants closely adhered to the endothecium under CK conditions (Fig. 4E and F), whereas the tapetum was separated from the endothecium in LYPJ plants, but not in SY63 plants, under HT treatment (Fig. 4G). At stage 11, condensed and degenerated tapetal cells and vacuolated microspores were observed in LYPJ and SY63 plants under CK conditions (Fig. 4I and J). In LYPJ plants under HT treatment, the anther wall layers were disordered and broken, microspores were degraded and appeared irregularly shaped, the tapetum became less condensed, and the lacuna between the septum and stomium became smaller (Fig. 4K), but these structural changes were not observed in SY63 plants under HT treatment (Fig. 4L). At stage 13 in LYPJ plants under CK conditions, most of the middle layer and endothecium of the anther wall were degraded, and deeply stained pollen grains and confluent locules were observed (Fig. 4M). However, in LYPJ plants grown under HT treatment, the anthers were smaller, the tapetal cells were visible, the anther wall was shrunken and irregularly shaped, and there were fewer fertile pollens per anther locule (Fig. 4O and M). However, no significant differences were observed in SY63 plants under HT treatment in comparison with CK plants (Fig. 4N and P).

**Transmission electron microscopic observations at stage 10**

Anther ultra-structures in stage 10 were subjected to further observation and quantification via transmission electron microscopy (Fig. 5). Spherically vacuolated microspores were closely adhered to the tapetum under the CK conditions for both cultivars (Fig. 5A, B, E, F, I and J). However, HT treatment led to the collapse of microspores in LYPJ plants, as well as degradation of the cytoplasm and organelles (Fig. 5G). In addition, the tapetum in LYPJ plants was vacuolated and hypertrophic under HT treatment (Fig. 5C, K and S) in comparison with that observed under the CK treatment (Fig. 5A, I and Q). In comparison with CK plants (Fig. 5M and Q), LYPJ plants grown under HT treatment showed unevenly distributed Ubisch bodies (Fig. 5O and S) and an obscure outline of the pollen exine with three malformed layers (collapsed bacula, disordered tectum, and no nexine) (Fig. 5O). Generally, the characteristics of the microspores and anther walls mentioned above were not significantly changed in SY63 plants under HT treatment (Fig. 5D, H, L, P and T).
Quantitatively, HT treatment significantly increased the average area of tapetum cells by 136% in LYPJ plants under HT treatment in comparison with the CK treatment (Table 2, Fig. 5Q and S), and it had no effect on the tapetum cells of SY63 plants (Table 2, Fig. 5R and T). The tapetum cell area of LYPJ plants was twice as that of SY63 plants under HT treatment, but the tapetum cell areas of LYPJ and SY63 plants were similar under the CK conditions (Table 2, Fig. 5Q and S). In LYPJ plants, the number of vacuolated microspores per locule was significantly decreased from 7.2 under the CK treatment to 1.5 under HT treatment (Table 2, Fig. 5A and C), but this number was reduced by only one in SY63 plants (Table 2, Fig. 5B and D).

Table 2
The effects of high temperature treatment on anther characteristics at stage 10 in LYPJ and SY63

| Cultivars | Treatment | Average area of tapetum cell | Number of vacuolated microspores | Number of stomium cells | Number of septum cells between bundle sheath and stomium and sub-stomial lacuna |
|-----------|-----------|-----------------------------|---------------------------------|-------------------------|---------------------------------------------------------------------------------|
| LYPJ      | CK        | 40.5 ± 1.7 b                | 7.2 ± 0.3 b                      | 5.5 ± 0.3 a             | 2.8 ± 0.2 a 0.9 ± 0.1 ab                                                      |
|           | HT        | 95.7 ± 4.1 a                | 1.5 ± 0.0 c                      | 4.8 ± 0.2 a             | 3.0 ± 0.0 a 1.1 ± 0.1 a                                                       |
| SY63      | CK        | 44.4 ± 5.3 b                | 8.2 ± 0.2 a                      | 5.5 ± 0.3 a             | 2.3 ± 0.2 a 0.5 ± 0.1 c                                                       |
|           | HT        | 48.5 ± 1.9 b                | 7.2 ± 0.3 b                      | 4.3 ± 0.3 a             | 2.5 ± 0.3 a 0.8 ± 0.0 bc                                                      |

CK control temperature; HT high temperature; Data are the average over three replicates ± standard error of the mean (n = 3). The different superscript lower-case letters indicate significance at $P<0.05$ across the different treatments and varieties.

HT had no effect on the number of stomium cells for either variety (Table 2 and Fig. 6), but HT reduced the size of the sub-stomial lacunas between septum cells and stomium cells in LYPJ plants. In addition, septum cells were not degraded under HT treatment in LYPJ plants (Fig. 6A and C), but SY63 plants had larger sub-stomial lacunas and degraded septum cells (Fig. 6B and D).
HT did not significantly affect the number of septum cells between the bundle sheath and sub-stomial lacuna or the number of septum cells between the stomium and locule (Table 2). SY63 plants had fewer septum cells between the bundle sheath and sub-stomial lacuna, as well as between the stomium and locule, in comparison with LYPJ plants (Table 2 and Fig. 6).

Discussion

The effects of HT during panicle initiation on yield formation

Most previous studies investigated damage induced by HT during the flowering stage [2, 13, 14, 28–30], and there have been few investigations of the effects of HT during panicle initiation on rice yield formation [2, 3]. Therefore, in this study, we assessed the detrimental effects of HT during panicle initiation in rice.

We found that HT treatment during panicle initiation seriously decreased yield (84%) and yield components, with the exception of the number of panicles per plant, in LYPJ plants; however, HT had a smaller effect on yield formation in SY63 plants in comparison with that observed in LYPJ plants (Table 1). In the HT-susceptible genotype LYPJ, the seed setting percentage showed the largest decline among yield components under HT treatment, indicating that the seed setting percentage was more vulnerable to HT in comparison with the other components, which was in agreement with previous reports [3, 31]. In the present study, the heat-tolerant variety SY63 had less yield damage in response to HT, which may be attributed to the high, stable seed setting percentage of this genotype under HT treatment (reduced by 20% in SY63 and 69% in LYPJ). It is important to note that the LYPJ plants had a relatively low seed setting percentage under the CK conditions in this study (Table 1), which may have been due to the natural high temperature (maximum temperature of more than 35°C for five consecutive days) during early flowering (data not shown). It has been reported previously that the LYPJ genotype is susceptible to heat during flowering [13].

In the present study, LYPJ had very low spikelet fertility under HT (Fig. 2J), and we also found very few half-grains with filling initiation (0.5% in LYPJ and 1.4% in SY63). These findings showed that it was spikelet fertility, rather than the grain filling process, that was responsible for the reductions in seed setting percentage under HT during panicle initiation. These results are similar to those reported in a recent study by Cheabu et al. [32], in which spikelet fertility was the major restriction for observed reductions of grain yield under HT from booting to maturity.

The intrinsic factors responsible for low spikelet fertility under HT include low pollen productivity, low pollen viability, poor anther dehiscence and pollen reception, and poor pollen germination [1, 2, 26]. In this study, HT during panicle initiation significantly decreased the anther dehiscence rate (Fig. 2K). This finding is in accordance with reports by Jagadish et al. [29] and Kobayashi et al. [10], in which rice plants were exposed to HT during the flowering stage. However, Endo et al. [5] and Wu et al. [3] reported that the
anther dehiscence rate was not substantially influenced by heat stress during panicle initiation. Moreover, HT treatment was performed for 15 days with a mean daytime maximum temperature of 36.1°C by Wu et al. [3], whereas HT treatment was performed for 3 days with a mean daytime maximum temperature of 39°C by Endo et al. [5]. Therefore, these data suggest that the response of the anther dehiscence rate to HT depends on the developmental stage during which plants are exposed to HT and the intensity of the HT treatment. Significant decreases in pollen viability (reduced by 46%), the pollen shedding percentage of the anthers (reduced by 11%) and the anther dehiscence rate (reduced by 5%) were observed in LYPJ plants under HT treatment (Fig. 2C, G, I, K and L), indicating that pollen viability was more vulnerable to HT in comparison with the other components (Fig. 2I, K and L). Therefore, we considered that it was altered pollen viability, rather than changes in pollen shedding or anther dehiscence, that was mainly responsible for the lower spikelet fertility of HT-susceptible cultivar LYPJ under HT treatment during panicle initiation (Fig. 2I, J and K). However, subjecting SY63 plants to HT treatment had no significant effect on any of these traits (Fig. 2). In comparison with LYPJ, the process from anther dehiscence to complete dispersal of pollen grains from the anthers was relatively rapid in SY63 under both tested temperatures (9.0 min under CK and 16.2 min under HT for LYPJ, 2.2 min and 2.5 min for SY63, data not shown). Recently, Wu et al. [13] found that enclosed stigmas of SY63 plants contributed to high spikelet fertility under HT treatment. Therefore, higher spikelet fertility (67%) of HT-tolerant SY63 plants under HT treatment during panicle initiation may be attributed to higher pollen viability (85%), a higher anther dehiscence rate (98%), a shorter period of time required for complete dispersal of pollen grains from anthers, better pollen shedding from anthers (94%), and enclosed stigmas (Fig. 7).

The relationship of pollen viability with anther characteristics

Pollen sterility caused by heat stress has been associated with abnormal anther development in sorghum [19], wheat [20], tomato [22], cotton [25], dwarf bean [9], and rice [2, 33]. In our study, HT treatment disrupted the morphologic structures of the anther wall and spherical microspores in LYPJ (Figs. 3, 4 and 5). Specifically, HT treatment resulted in malformation of the pollen structure (obscure outline of the pollen exine, collapsed bacula, disordered tectum, and no nexine) in LYPJ (Fig. 5G and O) at stage 10. Additionally, we observed aborted pollens at stage 13 in LYPJ under HT treatment (Fig. 4O), manifesting as a shriveled and collapsed pollen surface with a hollow germinal aperture and uneven sporopollenin deposition. Previous studies found that the pollen surface severely shriveled under HT treatment in sorghum and maize, and this change was accompanied by poor pollen viability [19, 21]. In the present study, heat stress did not obviously alter anther development or anther structure in the heat-tolerant variety SY63 (Figs. 3, 4 and 5). These data indicate that abnormal pollen formation was responsible for low pollen viability under HT treatment during panicle initiation, and the heat tolerance of SY63 may be attributed to normal anther development (Fig. 7).

Additionally, we found that Ubisch bodies had blunt protrusions and an uneven distribution on the inner surface of the anther wall in LYPJ plants under HT treatment (Figs. 3N and 5O). HT also resulted in tight wrinkles of the knitted anther cuticle on the epidermis of LYPJ plants (Fig. 3V). Similarly, Uzair et al. [34]
found marginal differences in the patterning of nano-ridges on the outer surface, as well as in the
distribution of Ubisch bodies, on the inner surface of anthers in rice *ptc2* mutants, which resulted in
decreased pollen viability in comparison with that of wild-type plants. However, in SY63 plants, HT had no
substantial effect on Ubisch bodies or anther cuticles (Fig. 3). Ubisch bodies carry a sporophytically
produced structural protein that is essential for pollen development [35]. The cuticle on the outer surface
of the anther serves as a barrier and protects the microspore/pollen grain from various environmental
stresses [36]. These data suggest that well-developed Ubisch bodies and cuticles contribute to the heat
tolerance of SY63 under HT during panicle initiation, whereas alterations in Ubisch bodies and cuticle
formation may result in pollen sterility in LYPJ (Fig. 7).

In this study, the single-cell tapetum area of LYPJ was larger at stage 10 under HT treatment in
comparison with that observed under CK conditions. In LYPJ plants exposed to HT treatment, the tapetum
was still observed at stage 13 due to slow degradation, but the tapetum had completely disappeared at
this stage in LYPJ plants under CK conditions and in SY63 plants under HT and CK conditions (Fig. 4).
These results show that HT disrupted tapetum degradation in LYPJ. Similarly, halted and incomplete
tapetum degradation was reported in rice *ptc2* mutants [34] and rice plants under chilling [37], and as well
in tomato and cotton plants under heat stress [25, 38]. Regarding causes for abnormal tapetum
degradation, Mamun et al. [37] revealed that vacuolation and hypertrophy of the tapetum under chilling
was caused by osmotic imbalance, which was triggered by the reabsorption of callose breakdown
products in the absence of OsMST8 activity. Min et al. [25] found that delayed programmed cell death of
the tapetum was mainly due to inactivation of starch synthase in cotton under HT treatment. These
findings suggest that different regulatory mechanisms govern tapetum degradation in different
organisms; however, the mechanism underlying tapetum degeneration retardation in rice under HT is not
yet clear.

The tapetum, the innermost layer in the anther wall, serves as an active nutrient source for neighboring
microspores [34, 39], and abnormal tapetum degeneration results in pollen sterility in photoperiod and
thermosensitive genic male-sterile rice [40, 41] and rice mutants [34]. These previous reports indicate that
termination of secretory-type tapetum development and disruption of tapetal functions is partly
responsible for pollen viability. In the present study, tapetum degradation in LYPJ was inhibited by HT
treatment, whereas SY63 showed nearly tapetum degradation under the same conditions (Fig. 5R and T).
Additionally, we observed several differences in the characteristics of the anther walls of LYPJ and SY63
plants following HT treatment. For example, the tapetum did not adhere to the endodermis (Fig. 4G) at
stage 9 in LYPJ, at which point tapetum degradation was initiated. In addition, LYPJ plants had a tightly
knitted anther cuticle on the epidermis (Fig. 3R and V). These heat-induced changes in anther
development and tapetum degradation may partly explain the high pollen sterility rate of LYPJ plants. In
contrast, the well-developed anthers of SY63 plants enhanced their heat tolerance in terms of pollen
viability (Fig. 7).

The relationship among anther structures, anther
dehiscence and pollen shedding
It has been reported that pollen reception (pollen numbers on stigma) influences spikelet fertility under HT treatment [1, 13, 29]. Wu et al. [4] also found that HT treatment at the heading stage led to poor pollen shedding in heat-susceptible cultivars. In this study, we observed that HT treatment during panicle initiation had a negative effect on anther dehiscence and the pollen shedding percentage of the anthers in LYPJ plants; however, SY63 plants showed stable anther dehiscence and pollen shedding under HT and CK conditions (Fig. 2K and L). It has been reported that poor pollen shedding may be a disadvantage for successful reproduction under HT treatment [12].

The existence of the tapetum at flowering may halt anther dehiscence [26]. Our microscopic observations demonstrated that the tapetum did not degenerate until stage 13 (anther maturity stage) in LYPJ plants under HT treatment (Table 2, Figs. 4O, 5C, K and 6C), and the anther wall (i.e., more remaining cell layers) remained between the locule and the lacuna. Similarly, Matsui et al. [26] reported that locules were kept closed by parenchyma and endothecium cells at anthesis due to the remaining anther wall cell layers in rice, which subsequently led to poor pollen shedding under HT treatment. However, Bagha [27] reported that the cell layers of the anther wall in rice were not affected by HT treatment during panicle initiation, while failure in lysis of the septum cell wall inhibited anther locule opening. In our study, lysis failure of the septum cell wall was also observed in LYPJ plants under HT treatment at anthesis (Fig. 4O), and the septum cell wall degraded in LYPJ plants under CK conditions (Fig. 4M), as well as in heat-tolerant SY63 plants under both CK and HT conditions (Fig. 4N and P). Therefore, our study suggests that both anther wall degradation and septum cell wall lysis together regulate anther dehiscence under HT conditions during panicle initiation. HT treatment during panicle initiation inhibited anther wall degradation and septum cell wall lysis in heat-susceptible LYPJ plants and subsequently resulted in low anther dehiscence in comparison with that of heat-tolerant genotype SY63 (Fig. 7).

Anther locules are opened via the stomium splitting. In our study, HT treatment did not affect the number of stomium cells in LYPJ or SY63 plants, and the two varieties had similar numbers of stomium cells under CK and HT (Table 2 and Fig. 6). Similarly, Bagha [27] did not find differences in stomium cell abundance between various genotypes grown under CK and HT conditions. Therefore, these results suggest that inhibition of anther dehiscence and pollen shedding at stage 13 under HT treatment may be attributed to septum cell lysis rather than stomium splitting.

In this study, we also found that the number of septum cells in SY63 plants was lower than that of LYPJ plants under both CK and HT conditions, and HT treatment resulted in a slight increase in the abundance of septum cells in both varieties (Table 2). This result was consistent with the results of Bagha [27], who reported that the number of septum cells was not affected by HT in heat-susceptible or heat-tolerant varieties; moreover, the heat-tolerant variety had fewer septum cells. Therefore, a lower number of septum cells may be a favorable characteristic for anther dehiscence in heat tolerant varieties, and this factor may have contributed to the high heat tolerance of SY63 (Fig. 7). Our study did not investigate the cause for the cessation of septum splitting in LYPJ plants under HT treatment. Inhibition of septum cell wall lysis may be attributed to low cell wall invertase activity caused by HT treatment [27]. The underlying physiological mechanism for regulation of septum splitting under HT merits further investigation.
Our study found that HT inhibited pollen shedding of the anthers (Fig. 2G and L). There are two likely reasons for our observation of inhibited shedding under HT conditions. First, Ubisch bodies on the inner surface of the anther wall show non-wettability due to the distribution of hydrophobic substances, and a continuous hydrophobic layer appears on the inner surface due to locule shrinkage after dehiscence [42]. Therefore, the occurrence of Ubisch bodies decreased the sticking properties of the pollen to the locule wall [43], and decreased sticking favors pollen dispersal [42]. HT induced uneven distribution of Ubisch bodies in heat-susceptible LYPJ plants (Fig. 3N), which may be one of the reasons for the reduction in pollen shedding of the anthers (Figs. 2L and 7). Second, Pacini et al. [44] indicated that anther stomata may play a crucial role in anther dehydration in rice, which may favor anther dehiscence and pollen shedding. Recently, in ICE1 (ice1-2) Arabidopsis mutants, Wei et al. [45] found that decreased stomata density and abnormal stomata development were not advantageous for anther dehydration and anther water movement, so the epidermis did not shrink to dehisce, regardless of the existence of stomium cells in the anther; these changes resulted in low pollen dehiscence, low pollen viability and a low germination percentage. Therefore, to elucidate the mechanism underlying poor pollen shedding, more investigations, such as studies of anther stomata development and the physiological changes associated with anther dehiscence in dehisced anthers, should be performed under HT conditions.

Conclusions

HT treatment during panicle initiation significantly decreased the grain yield of heat sensitive variety LYPJ by decreasing both the number of spikelets per panicle and the seed setting percentage. The reduction in the seed setting percentage of LYPJ was mainly attributed to lower spikelet fertility, which was caused by decreased pollen viability, reduced anther dehiscence and poor pollen shedding of the anthers. HT treatment had only minor adverse effects on the grain yield of heat-tolerant variety SY63, which was attributed to stable pollen viability, anther dehiscence, and pollen shedding of the anthers. Abnormal anther development and malformation of pollen together resulted in shrunken pollen grains in LYPJ plants under HT treatment, which may account for the observation of poor pollen viability. Abnormal anther epidermis, thickened anther walls, un-degraded septum, inhibition of confluent locule formation and unevenly distributed Ubisch bodies at pollen maturity were partially responsible for the decreased anther dehiscence rate and reduced anther pollen shedding of LYPJ plants under HT treatment. HT treatment did not substantially change the development of the anther wall and microspores at the late stage of pollen formation in SY63 plants.

Methods

Plant materials and growth conditions

A pot experiment was performed during the rice growth season from May to October 2016 at the experimental station of Huazhong Agricultural University, Wuhan, China (30°29' N, 114°22' E). Two rice genotypes, LYPJ and SY63 were used in this study. Seeds of LYPJ were purchased from Mingtian Seed Co., Ltd., Nanjing city in Jiangsu province, China; Seeds of SY63 were purchased from Chichengsannong
Seed Co., Ltd., Suining city in Sichuan province, China. Seeds of LYPJ were sown in plastic seeding trays with loam soil on 8 May after breaking dormancy at 50 °C for 48 h. Staggered nursery sowing at a 7-day interval was performed for SY63 to synchronize the panicle initiation time of LYPJ and SY63. Four-leaf seedlings were transplanted into a 12 L plastic pot (25.5 cm height × 24.4 cm top diameter) on 5 June, which contained a mixture of 10 kg clay soil with the following properties: pH 6.6, 10.5 g organic matter kg⁻¹, 1 mg total N kg⁻¹, 8.1 mg Olsen P kg⁻¹, and 113.8 mg exchangeable K kg⁻¹. Phosphate fertilizer (1.5 g P pot⁻¹) in the form of calcium superphosphate and potassium fertilizer (1.5 g K pot⁻¹) in the form of potassium chloride were applied as basal fertilizer. A total of 1.80 g N pot⁻¹ was applied as urea with three splits: 0.72 g N pot⁻¹ as basal fertilizer, 0.36 g N pot⁻¹ top-dressed 12 days after transplanting, and 0.72 g N pot⁻¹ applied as panicle fertilizer. The potted rice plants were randomly arranged in three replicates under natural ambient conditions, and all pots were maintained in approximately 2 cm of water from sowing to maturity. Pots in the greenhouse were manually moved every 7 days to avoid positional effects. Pests, diseases, birds, and weeds were intensively controlled.

**High temperature treatment**

The facility for the temperature treatments contained two individual greenhouses (4 m length × 4 m width × 4.5 m height). As described by Wu et al. [3], each greenhouse was equipped with an air conditioner, a wetting machine, two ventilators, and four sensors for monitoring temperature and relative humidity. All greenhouses and equipment were connected to a central auto-controlled system (Auto-Greenhouse Monitoring and Data Management System, Version 3.00, Auto, China).

The temperature treatments were performed during panicle initiation. For the high temperature (HT) treatment, the daytime temperatures were set at 37°C from 07.30–19.30 h, whereas the daytime temperature was 31°C for the control temperature (CK) treatment. The nighttime temperature for both temperature treatments was set at 27°C from 19.30–07.30 h. The daytime relative humidity was set at 80% for both treatments, and the nighttime relative humidity was set at 90%. Air temperature and relative humidity were recorded 5 cm above the canopy in the greenhouse using a stand-alone sensor (HOBO, H08-003-02, Onset Computer Corporation, Bourne, MA, USA). The rice plants were moved to the controlled facility at the onset of panicle initiation according to apical dissection and leaf age. Plants were subjected to HT treatment throughout the whole panicle initiation period. The actual daytime average maximum temperature under HT treatment was 39.1 °C, which was 8.0 °C higher than that of the CK treatment. The actual mean daytime temperature during the whole HT duration was 36.9 °C, which was 6.4 °C higher than that of the CK treatment (Fig. 1A). The mean daytime relative humidity of the CK treatment was 73.0% during the entire treatment. The mean daytime relative humidity of the HT treatment was 80.6% during the entire treatment (Fig. 1B). The mean nighttime temperature was 27.4 °C under the CK treatment and 28.0 °C under the HT treatment during the whole HT duration. The mean nighttime relative humidity was 95.6% under the CK treatment and 93.7% under the HT treatment during the whole HT duration. When the young panicle of the main tiller emerged from the flag leaf sheath, the HT treatment was terminated and all plants were moved to natural ambient conditions and grown to maturity.
Yield and yield components, spikelet fertility

At maturity, three plants from three pots were harvested for measurement of yield traits. Plants were separated into leaf blades, stems (sheath and culms) and panicles. After recording panicle number, all spikelets were hand-threshed and separated into filled and unfilled grains (including half-filled and empty grains) by submerging them in tap water. The unfilled grains floating at the surface of the water were separated into partially filled and empty grains by pressing each of them between the thumb and the forefinger and checking them by opening the lemma and palea [1]. Empty grains without an embryo were considered to be sterile. All leaves, stems, panicle rachis, filled grains, half-filled grains and empty grains were separately collected and oven-dried at 80°C to a constant weight. The total biomass (g plant\(^{-1}\)) was the total dry weight of the aboveground parts, and all filled grains were weighed to determine grain yield (g plant\(^{-1}\)). Panicles per plant, spikelets per panicle, seed setting percentage (%), 1000-grain weight (g), and spikelet fertility (%) were calculated.

Pollen viability, anther dehiscence rate, pollen shedding percentage of anthers

Several spikelets were collected from three panicles one day prior to anthesis, immediately frozen in liquid nitrogen and stored at -80 °C. All indehiscent anthers were excised from 20 non-opening spikelets. Pollen grains were scattered on a slide with 60 µL Alexander solution (Bioshap, China) by manual squeezing using tweezers. Ten minutes later, the Alexander-stained pollen grains were examined at 10 × magnification using an inverted fluorescence microscope (Nikon Ti-SR, Japan). Pollen stained reddish purple was considered fertile, whereas pollen stained green was considered sterile [46]. The percentage of pollen viability was calculated as the percentage of reddish-purple pollen grains to all pollen grains (%).

The opening spikelets of the main panicles were collected from 10.30–11.30 h. The collection was repeated three times for each replicate. Six anthers from one spikelet were collected for stereo microscopical observation (Olympus, SZX2-ILLT, Japan). Anthers with opened apical and/or basal pores were recorded as dehisced. The anther dehiscence rate (%) was calculated as the ratio of dehisced anthers to total anthers.

At the flowering stage, anthers that had completely shed their pollen were white, and anthers on which many pollen grains remained were yellow. The pollen shedding percentage of the anthers (%) was expressed as the percentage of white anthers among all anthers collected. Dehiscent and indehiscent anthers were assessed.

Observation of anther structures via light and scanning electron microscopy

Young panicles were selected during the booting stage based on the distance between the collar of the 2nd leaf and the flag leaf collar [47]. According to the divisions of the anther development period [17], the top three spikelets of the panicle were collected at stage 8b (the distance between the collars of the 2nd leaf and the flag leaf was – 2 cm, indicating that the flag leaf collar inside the sheath of the 2nd leaf was
2 cm below the 2nd leaf collar), stage 9 (the distance was 0 cm), stage 11 (+ 7 cm), and stage 13 (+ 13 cm), respectively. The collected spikelets were placed in fixation solution (50% ethanol, 5% glacial acetic acid, and 3.7% formaldehyde) for 24 h at room temperature and rinsed with 70% ethanol twice after removing the fixation solution. Fixed anthers were embedded in paraffin after dehydration in an ethanol concentration series (50%, 75%, 4 h in each; 85%, 90%, 2 h in each; 95%, 1 h; 100%, 30 min), clearing via xylene and paraffin infiltration. The 3-µm thin sections were prepared with a microtome (RM2016, Leica, Germany) and stained with 0.5% toluidine blue solution. Images were captured with an inverted fluorescence microscope (Nikon Ti-SR, Japan).

Samples for scanning electron microscopy observation were prepared according to the methods described by Min et al. [25] with minor modification. Mature anthers from opening spikelets were sampled and pre-fixed in fixation tubes containing 2 mL of 2.5% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) overnight at 4°C. Next, the anthers were dehydrated in a graded series of ethanol solutions (30%, 50%, 70%, 10 min in each; 80%, 100%, 8 min in each) and immersed three times in isoamyl acetate (10 min in each). The fixed samples were processed via critical point drying using liquid CO₂ and gold coating, followed by observation using a scanning electron microscope (JSM-6390/LV, JEOL, Japan).

Observation of the ultrastructure of anthers using transmission electron microscopy

Samples were prepared for transmission electron microscopy observation according to methods described by Cao et al. [48]. At the 10th anther development stage (the collar of the flag leaf was 5 cm above the collar of the 2nd leaf), 30 spikelets attached to the top five primary branches were sampled from three panicles, after which approximately 100 anthers were fixed in a fixation tube containing 2 mL of 2.5% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) and vacuumed for 15 min. The tubes containing the anthers were stored at 4 °C overnight. Subsequently, the anthers were washed three times with 0.1 M phosphate buffer (pH 7.2) and post-fixed with 1% (w/v) osmium tetroxide (SPI, SPI Chem, West Chester, PA, United States) for 2 h at room temperature. The samples were washed three times with 0.1 M phosphate buffer at room temperature (30 min each time) and dehydrated via a graded acetone series (30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 20 min each). The dehydrated samples were infiltrated with gradient acetone-Spurr resin solution (3:1 v/v, 1:1 v/v, 1:3 v/v, Spurr resin, 6 h each), after which each sample was placed into Spurr resin solution, which was polymerized with Spurr resin at 60 °C for 48 h. Ultra-thin sections (90–110 nm) were cut from the polymerized block with a microtome (UC6, Leica, USA) and mounted onto copper grids. The sections were stained with 2% (w/v) uranyl acetate solution (SPI, SPI Chem, West Chester, PA, United States) for 30 min at room temperature, following by observation using transmission electron microscopy (TEM) (H-7650, Hitachi, Tokyo, Japan) and a CCD camera (Model 832 ORIUS, Gatan, America).

The TEM images were used to quantify the average tapetum area per cell, number of vacuolated microspores per locule, stomium cells per cross-section, septum cells between the bundle sheath and substomial lacuna, and septum cells between the stomium and locule via image analysis software (National
Stomium cells were distinguished according to whether their cell walls had smooth cuticles; non-stomium epidermal cells had crenulate cuticles [27]. In addition, vacuolated microspores, pollen exine, the distribution of Ubisch bodies, and the morphologic structure of the anther walls were also observed and characterized using the TEM images. Two anthers for each replicate were used for TEM observations, and two slices of each anther were used to characterize anatomical structures.

**Statistics analysis**

The value for a given trait was expressed as the mean of three replicates with standard error (SE) using the SigmaPlot 10.0 software package (SPSS Inc., Chicago, IL, USA). Differences between different treatments were evaluated using the least significant difference (LSD) test at a 5% probability level using Statistix 9 software package (Analytical software, Tallahassee, FL, USA).

**Abbreviations**

CK
Control temperature; HT:High temperature

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All the data on the present study has been included in the tables and/or figures form in this manuscript; and the datasets used and/or analyzed in this study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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Authors’ contributions

QH and KC conceived the research, designed the experiments, analyzed the data, and wrote the manuscript. QH carried out pot experiments. WW and QL assisted in both sampling and physiological determinations in the laboratory. SP and JH gave valuable suggestions during the whole pot experiments. All authors read and approved the final manuscript.

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