Cleistogamy Decreases the Effect of High Temperature Stress at Flowering in Rice

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Abstract: Rice sterility due to a high temperature at flowering is a serious agricultural problem that has been associated with global warming. The flowering stage in rice plants is most vulnerable to high temperature stress. Closed flowering rice plants may better withstand high temperature stress. The aim of this study was to determine the role of cleistogamy (closed flowering) in avoiding high temperature-induced sterility. Cleistogamy was induced by moderate heat treatment at 30°C during the panicle development stage. Both cleistogamous and chasmogamous (ordinary open flowering) rice plants, which possess the same genetic background, were subjected to 38°C or 36°C for 4 h just before flowering, and the percentage of fertility, number of pollen grains on a stigma, number of germinated pollen grains on a stigma, and temperatures inside and outside of the closed spikelets were examined. The cleistogamous rice plants showed a higher fertility percentage and a larger number of germinated pollen grains on a stigma than the chasmogamous rice plants. The temperature inside the closed flowering spikelets was 1.8°C lower than that outside the spikelets. The cleistogamous rice plants thus showed avoidance to high temperature stress at 38°C at flowering. On the basis of these results, we concluded that cleistogamy was advantageous to rice pollination and fertilization at high temperatures because of glume cooling.

Key words: Chasmogamous rice plants, Cleistogamous rice plants, Closed flowering rice, Fertility percentage, Germinated pollen grains, High temperature, spw1-cls.

Global warming is affecting rice production in Japan. A high temperature during ripening deteriorates rice quality by causing cracked grains and immature grains with a white portion and deep ditch (Morita, 2008). Concurrently, high temperature-induced spikelet sterility has also become a concern in southern Japan (Hasegawa et al., 2008).

Sterility due to physiological damage in reproductive organs significantly decreases rice yield. High temperature stress causes sterility at flowering than at booting. Several studies conducted using indica rice varieties (Satake and Yoshida, 1978) have revealed that: (1) the flowering stage of plants is most vulnerable to high temperatures; (2) high temperature stress tolerance is rapidly acquired, generally within an hour after flower opening; and (3) reduction in the number of pollinated and germinated pollen grains on stigma results in sterility. In japonica rice varieties, pollination and germination of pollen grains on the stigma are also important restricting factors of fertility at high temperatures (Matsui et al., 2001a). Pollen grain swelling has been shown to be the driving force for the opening of anthers through septum rupture, whereas a high temperature inhibits anther dehiscence (Matsui et al., 2000). Anther structure in high temperature-tolerant rice has been associated with well-developed cavities and thick locule walls (Matsui et al., 2001b). Early anther dehiscence at the beginning of flower opening and early morning flowering before the rise in air temperature to avoid high temperature stress have been suggested as suitable characteristics of high temperature-tolerant rice varieties (Nishiyama and Blanco, 1981; Ishimaru et al., 2010). These studies showed that a high temperature affects fertility at flowering, and that failure in anther dehiscence, pollination, and germination of pollen grains causes high temperature damage.

Closed flowering, also known as cleistogamy, is defined as a method of self-fertilization without flower opening. A
cleistogamous rice mutant, spw1-cls, which follows a nearly perfect closed flowering style under warm conditions during reproductive growth stage and a high fertility rate without failing other agronomic traits has been previously reported (Yoshida et al., 2007; Ohmori et al., 2012). spw1-cls has received notable attention as the rice with gene containment because in these days, genetically modified (GM) crops have been cultivated around the world and the possibilities of outcrossing these with non-GM plants have raised public concern (Dale et al., 2002). In spw1-cls, lodicules, which are necessary for pushing out the lemma to cause flower opening, are morphologically mutated (Hoshikawa, 1989; Yoshida et al., 2007). Characteristically, spw1-cls shows varied flowering styles depending on the growth temperature during panicle development. Constant warm temperature during the day drives the mutant into cleistogamy, whereas cool temperatures would induce it into chasmogamy (Ohmori et al., 2009). The temperature sensitivity of spw1-cls provides a unique experimental system in which both open- and closed-type flowers with the same genetic background can be generated. It is possible to evaluate the effects of cleistogamy on biotic/abiotic stresses using this system.

Here, we report the effects of 4-h treatments at either 38°C or 36°C for 4 h (1000 – 1400). Materials and Methods

1. Plant materials and high temperature treatment conditions

Fig. 1 shows a schematic diagram of the high temperature treatment conditions applied to spw1-cls plants. The rice plants were grown at 26°C day (0600 – 1800)/20°C night (1800 – 0600) under a natural light chamber until the panicle initiation stage (panicle length: 1 mm), then in the artificially lit chamber at 26°C light (12 h: 0600 – 1800, 400 μmol/s·m²)/20°C dark (12 h: 1800 – 0600). To induce cleistogamy (closed flower), the plants were grown from panicle initiation to heading at 30°C for an additional 4 h around noon (1000 – 1400) for 4 weeks. The chasmogamous rice plants (opened flower; regular flower) were continuously grown at 26°C during the day (0600 – 1800)/20°C night (1800 – 0600) in a natural light chamber until heading. Two days after heading, both types of rice plants were treated at either 38°C or 36°C for 4 h (1000 – 1400).

A rice mutant, spw1-cls, was generated from Taichung 65 by treatment with a chemical mutagen (MNU: N-methyl-N-nitrosourea; Yoshida et al., 2007). Twenty mutant seeds of the F6 generation were sown circularly in a 1/5,000–a pot. Four pots were used for each 38°C or 36°C high-temperature treatment of cleistogamous or chasmogamous spw1-cls plants. The specified spikelets, namely, 3rd, 4th, and 5th spikelets from the top spikelet at the primary
branches on the upper three branches and the top spikelet on the secondary branches on the same upper primary three branches were used because they grew together and hardly resulted in degeneration (Ito, 1980). Since most of the specified spikelets started flowering at around 1130 two days after heading, high-temperature treatments were initiated at 1000.

2. Sampling of spikelets, anthers, and stigmas

Over 200 spikelets were used for fertility analysis; from 15 – 25 spikelets that flowered during treatments, the stigmas were collected at noon during treatments and stained with acetocarmine dye solution, and the number of pollen grains and germinated pollen grains on the stigmas were counted under a microscope (Satake and Koike, 1983). In cleistogamous rice plants, we collected the stigmas from spikelets in which the anthers reached the top of the palea and lemma during high-temperature treatment; the anther filaments were checked before they elongated at the beginning of the treatment.

The fertility percentage of the chasmogamous spw1-cls was determined for flowered and unflowered spikelets during the 38°C treatment by marking on the surface of the spikelets using felt-tip pens at the end of 38°C treatment. A part of the spikelets of chasmogamous spw1-cls flowered during high-temperature treatments in the same way as an ordinary rice plants. About one fourth of the specified spikelets of chasmogamous spw1-cls flowered during the 38°C treatment, and most of the spikelets flowered after the end of 38°C treatment. We also examined the fertility percentages at 36°C. Spikelet fertility was also determined at harvest.

3. Measurements of the number of pollen grains in the anthers and on the stigma

Only the empty pollen grains in which engorged starch granules were not observed on a stigma were defined as germinated pollen grains. The number of pollen grains in an anther was counted as follows. The anthers were picked up at 1000, the beginning of high temperature treatment, from the spikelets, two days after heading. At 1000, the anthers with pollen grains were nearly fully engorged before anther dehiscence (Koike and Satake, 1987). An anther was dipped in a droplet of 50% (w/w) glycerol and iodide-potassium iodide (I2-KI) solution, the anther walls were broken using insect pins, and all pollen grains were spread out on a microscope slide glass along the narrow path marked with a manicure pen, and then counted.

4. Measurements of the temperature inside and outside the spikelets

During the 38°C or 36°C treatments, air temperatures outside and inside of spikelets were measured using thermocouple devices (Chino; φ: 0.3-mm sheathed thermocouple NCF600, the thermo sensor was at the top portion of the device) as shown in Fig. 6. A thermocouple device was stacked through from a small hole at the base part of a lemma where anthers situated. Temperatures inside and outside of 10 spikelets were measured at 1100. In the case of exterior measurements, a thermocouple device was placed under a shaded aluminum foil sheet to avoid heating by direct light. In Fig. 6, the average temperatures and standard deviations (SD) were calculated from the results of the 3-min measurements conducted at 3-s intervals.

5. Statistical analysis

A statistical analysis was performed with Student’s t-test using paired samples as means for all measurements.

Results

1. Fertility of spw1-cls without high temperature treatments

In the control conditions (without high temperature treatment after heading) with and without additional 30°C 4-h treatments from panicle initiation to heading, the fertility percentages were 96.3% (n = 8 panicles; SE = 1.8) and 95.3% (n = 10 panicles; SE = 2.1), respectively. The number of pollen grains in the anthers of spw1-cls was 1,744 (n = 20 spikelets; SE = 37) and 1,733 (n = 20 spikelets; SE = 28), respectively.

2. The fertility after high-temperature treatment

Fig. 2 shows the fertility percentages of chasmogamous
The fertility percentage of chasmogamous panicles was 58.1% (\(n = 22\) panicles; SE = 5.9) and that of the cleistogamous was 77.9% (\(n = 28\) panicles; SE = 4.5). A significant difference was observed at the 5% level using the \(t\)-test.

Of the 22 chasmogamous panicles, 11 panicles had only unflowered spikelets, while the remaining 11 panicles had both flowered and unflowered spikelets after the 38ºC treatment. Fig. 3 shows the fertility percentages of the flowered and unflowered spikelets of chasmogamous spw1-cls after a 38ºC treatment. The fertility percentages of the flowered and unflowered spikelets were 42.6% (\(n = 11\) panicles; SE = 11.3) and 71.1% (\(n = 11\) panicles, SE = 5.7), respectively, and a significant difference was seen at the 5% level using the \(t\)-test. No cleistogamous spw1-cls spikelets flowered during and after the 38ºC treatment.

As shown in Fig. 4, after the 36ºC treatment for 4 h, the fertility percentages of chasmogamous and cleistogamous spw1-cls were 86.2% (\(n = 26\) panicles; SE = 2.7) and 92.5% (\(n = 19\) panicles; SE = 2.3), respectively. Thus, the 36ºC treatment for 4 h resulted in comparable fertility percentages in both chasmogamous and cleistogamous spw1-cls, with small differences in fertility percentages between them. During the 36ºC treatment, out of the 276 spikelets in 26 panicles, only 17 spikelets in 3 panicles flowered in chasmogamous spw1-cls, and a significant difference was not observed in the fertility percentages between flowered and unflowered spikelets (data not shown).

3. Number of pollen grains

To examine the causal reason behind the reduction in floret fertility, we counted the number of the pollen grains on the stigmas. For chasmogamous spw1-cls, we counted the numbers of pollen grains in the spikelets that flowered after the 38ºC treatment. For the cleistogamous spw1-cls, we examined the spikelets in which the filaments reached the top of the lemma, which represented the most dehisced anthers. Fig. 5(left) shows the number of pollen grains on the stigmas after the 38ºC treatment. It was 182 (\(n = 23\) spikelets; SE = 10.2) and 162 (\(n = 14\) spikelets; SE = 14.9), in the chasmogamous and cleistogamous spw1-cls spikelets respectively. The number of pollen grains in the chasmogamous spikelets was slightly larger than that in the cleistogamous ones. These data demonstrate that the anthers dehisced without difficulty after the 38ºC treatment in both chasmogamous and cleistogamous spw1-cls.

As shown in Fig. 5(right), the number of germinated pollen grains on the stigmas of chasmogamous spw1-cls spikelets was 7 (\(n = 23\); SE = 1.2) and that of cleistogamous ones was 19 (\(n = 14\); SE = 1.1). A three-fold higher number of pollen grains germinated on the stigmas of cleistogamous spw1-cls than on those of chasmogamous ones, and a significant difference was seen at the 5% level using the \(t\)-test.
data showed that the difference in temperature regimes at panicle initiation to heading to induce the opening and closing flowering phenotype did not result in any differences in the fertility percentages and the number of pollen grains in the anthers. Cleistogamous *spw1-cls* showed a 20% higher percentage of fertility after the 38ºC treatment for 4 h relative to that observed in the chasmogamous ones. This suggests that under high temperature condition, open flowering worked against the fertility of spikelets. The lower fertility of chasmogamous *spw1-cls* was mainly caused by the lower fertility of spikelets that flowered during treatment. In addition, the heat-treated pollen grains might have lost their ability to germinate.

As shown in Fig.5, the number of germinated pollen grains on a stigma of chasmogamous and cleistogamous *spw1-cls* subjected at 38ºC for 4 h.

|                       | Chasmogamous *spw1-cls* (n = 23 spikelets) | Cleistogamous *spw1-cls* (n = 14 spikelets) |
|-----------------------|-------------------------------------------|--------------------------------------------|
| The number of pollen grains on a stigma | 15                                      | 10                                        |
| The number of germinated pollen grains on a stigma | 7                                       | 15                                        |

SE: Standard Error, 'ns': not significant, *: 5% significant by t-test.

4. **Temperature inside and outside the spikelets**

We measured the temperature inside and outside the spikelets using a thermocouple device. Fig. 6 shows that during the 38ºC treatment, the detected air temperature outside and inside the cleistogamous spikelets was 38.3ºC (± 0.11, SD) and 36.5ºC (± 0.13, SD), respectively (n = 10 replications). The temperature difference between the outside and inside was 1.8ºC, with the interior temperature lower than that of the exterior. Meanwhile, in the 36ºC treatment, the air temperature outside and inside the spikelet was 35.8ºC (± 0.19, SD) and 35.5ºC (± 0.42, SD), respectively (n = 10 replications), with a difference of 0.3ºC.

**Discussion**

In the control conditions with and without additional 30ºC 4-h treatments from panicle initiation to heading, no changes in the fertility percentages and the number of pollen grains in an anther were observed. The 36ºC treatment could also be regarded as a control condition because of the observed normal fertility percentages. The data showed that the difference in temperature regimes at panicle initiation to heading to induce the opening and closing flowering phenotype did not result in any differences in the fertility percentages and the number of pollen grains in the anthers.

Cleistogamous *spw1-cls* showed a 20% higher percentage of fertility after the 38ºC treatment for 4 h relative to that observed in the chasmogamous ones. This suggests that under high temperature condition, open flowering worked against the fertility of spikelets. The lower fertility of chasmogamous *spw1-cls* was mainly caused by the lower fertility of spikelets that flowered during treatment. In addition, the heat-treated pollen grains might have lost their ability to germinate.

As shown in Fig.5, the number of germinated pollen grains on a stigma of chasmogamous *spw1-cls* was 7, about one third of that of the cleistogamous plants. A sufficient number of pollen grains on a stigma were observed in both flowering types, based on a previous report that at least 100 pollen grains were required for pollination.
In *indica* rice varieties, less than 9 germinated pollen grains on a stigma resulted in sterile spikelets (Satake and Yoshida, 1978). In *japonica* rice varieties, more than 5 to 10 germinated pollen grains on a stigma are necessary for normal fertility (Satake and Koike, 1983). No significant differences in the number of pollen grains on a stigma and effective pollination between the cleistogamous and the chasmogamous rice plants during high temperature treatments were observed in this study. These findings suggest that anther dehiscence was apparently normal, which were contrary to the previously reported changes in flowering caused by high temperature (Satake and Yoshida, 1978; Matsui et al., 2000). The reason behind the small difference in air temperature between outside and inside at 36°C compared to that at 38°C is uncertain. However, in bright and slight windy paddy fields, the air temperature inside the rice spikelet gradually decreased compared to the outside when the air temperature exceeded 30°C, and the interior of the spikelet was approximately 2°C lower than the external environment in the open-air growth cabinet at a 40°C temperature (Nishiyama, 1981). Latent heat by transpiration from the stomata of the glume at 38°C seems to be a plausible reason for the lower sterility in cleistogamous rice than in chasmogamous plants, and thus cleistogamous rice plants seem to have an advantage at flowering under high temperature stress as compared with chasmogamous plants.

Field trials in heat-vulnerable regions are further required to verify the practical effectiveness of cleistogamy against high temperature-induced sterility.

This is the first report that shows that cleistogamy is beneficial during flowering under high temperature conditions. High temperature stress avoidance is currently an important agricultural trait to cope with global warming.

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