The role of Oryza sativa L. ‘Milyang 44’ husks on the resistance to two rice stink bugs

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To elucidate the resistance mechanisms of the rice (Oryza sativa L.) cultivar ‘Milyang 44’ against rice stink bugs, we compared the number of stylet sheaths, husk perforations, and feeding marks on the surface of the grains caused by Leptocorisa chinensis and Cletus punctiger on Milyang 44 and the control cultivar, i.e., ‘Aichinokaori SBL’. We also examined the cross-sectional structure of the rice husks. We found that the number of stylet sheaths per panicle was higher in Milyang 44 than in Aichinokaori SBL for both rice stink bug species, except in one test involving C. punctiger. However, Milyang 44 had significantly less damage per number of stylet sheaths than Aichinokaori SBL, resulting in a lower percentage rates of pecky rice grains in Milyang 44. Interestingly, there was no difference in the percentage rates of pecky rice between the two cultivars after removing one third of the husks. Histological analysis showed that the sclerenchymatous cell wall containing lignin of husk was thicker in Milyang 44 than in Aichinokaori SBL, suggesting that the husk of Milyang 44 plays an important role in its resistance to these two rice stink bug species.

Key Words: rice stink bugs, host plant resistance, Milyang 44, stylet sheaths, husk, Leptocorisa chinensis, Cletus punctiger.
mechanisms of Milyang 44 against rice stink bugs may be related to both the maturation of the spikelet and the preferences of rice stink bugs. However, the details of these mechanisms remain unclear.

Therefore, in this study, we observed the presence of stylet sheaths and perforations on the rice husks and feeding marks on the grains caused by *L. chinensis* and *C. punctiger* on Milyang 44 and a control cultivar to elucidate the mechanisms of damage suppression. We also compared the histological structures of the rice husks between the two cultivars using phloroglucinol/HCl staining to detect the distribution of lignin. Finally, we compared the percentage rate of pecky rice using panicles with pruned spikelets to determine whether the rice husks play an important role in protecting the plant from rice stink bug damage.

### Materials and Methods

#### Feeding treatment to test cultivars using rice stink bugs and their degree of damage

The test cultivar Milyang 44 and the control cultivar Aichinokaori SBL were damaged by exposing them to rice stink bugs, following the individual feeding treatment of Sugiura *et al.* (2017). *C. punctiger* is known to have no specific sucking sites on the spikelets (Kawamura 1993, Takeuchi *et al.* 2004), and *L. chinensis* is known to suck specifically on the hooking part of the grain (Furuie and Kiyota 1993, Kawamura 1993, Takeuchi *et al.* 2004). Two rice stink bug species were captured in the field at the Aichi Agricultural Research Center (Nagakute, Japan) and used for the individual feeding treatment in every year of examination. The rice cultivars were planted in pots (1/10,000 a) and the following fertilizers were applied: 8.0 g N m$^{-2}$, 4.3 g P$_2$O$_5$ m$^{-2}$, and 3.3 g K$_2$O m$^{-2}$. The pots were then set outdoors. The panicles were processed in the feeding treatment at 20 DAH because Nakamura *et al.* (2017) previously revealed that Milyang 44 exhibited clear resistance at 20 DAH. Two panicles were chosen from three test pots, each per cultivar, and all other panicles were removed at 20 DAH. Male adults of any age of each rice stink bug species were then released severally into an organdy mesh bag (50 cm wide by 60 cm long) that had been placed over the panicles and upper leaves of the test plants. The feeding treatment was conducted for 6 d in 2013 using five adult rice stink bugs and for 7 d in 2015 using four adult rice stink bugs. After the feeding treatment, the test plants were sprayed with dinotefuran, and the adult rice stink bugs were removed from the plants.

The panicles were harvested at maturity and dried, following which the number of pecky rice grains was visually counted and the percentage rates of pecky rice grains was calculated. In addition, the mean total number of spikelets and total number of pecky rice on the two whole panicles on each plant were calculated. Data for the percentage rate of pecky rice grains were arcsine-transformed and analyzed using a *t*-test in Microsoft Excel 2012 Statistical Analysis (Social Survey Research Information Co., Ltd., Tokyo, Japan) with a significance level of *P* < 0.05.

#### Investigation of physical damage to the husks and grains

To investigate whether rice stink bugs attacked each grain, all of the spikelets (2,433) from one of the two panicles from each set that was used in the individual feeding treatment were stained with 1% methylene blue (Takeuchi *et al.* 2004) and observed under a binocular dissection microscope to detect any stylet sheaths from the rice stink bugs on the surface of each spikelet. The husks were then separated from the grains to observe for perforations on the husks and feeding marks on the grain surfaces. Following Takeuchi *et al.* (2004), a feeding mark was defined as a hole that was formed in the middle of a pecky mark on the grain by the rice stink bug stylet. These traces of damage were used to estimate the feeding patterns of each species of rice stink bug. The percentage rate of successful sucking was then calculated as the number of feeding marks from each stylet sheath.

To demonstrate the unique feeding patterns of each species, the stylet sheaths from *L. chinensis* on the palea of the hooking part were photographed using a UMO6 digital microscope (MicroLinks Technology Corporation, Kaohsiung, Taiwan) and the perforations made by *C. punctiger* in the middle area of the spikelets were observed using a TM3030 scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan). Cross sections were also taken from the middle of the spikelets using a blade and prepared to examine the hooking part, and the husks without their grains were sliced into sections to observe the perforations. Both types of sections were fixed onto the microscope stage using carbon tape and inserted into the sample chamber without any pretreatment, such as gold coating, and the reflected electron images were obtained under vacuum conditions using 15 kV of accelerating voltage. The total number of spikelets, number of stylet sheaths, number of feeding marks, stylet sheaths per spikelet, and percentage rate of successful sucking (number of feeding marks per number of stylet sheaths x 100) from one of the panicles from each plant were averaged. Data for the percentage rate of successful sucking were then arcsine-transformed and analyzed using a *t*-test with a significance level of *P* < 0.05.

#### Observation of the cross-sectional structure of the rice husks

Milyang 44 and the control cultivar Aichinokaori SBL were grown in the same way as for the individual feeding treatment in 2013. Three panicles selected from each of three plants were harvested at 10 and 20 DAH. Next, spikelets on the uppermost three branches of these three panicles were sampled and dried to obtain decomposed husks. These husk segments were fixed in FAA (formalin:acetic acid:70% ethanol; 1:1:18) and then rinsed twice using 0.1 M phosphate buffer (pH 7.0) for 30 min each. They
were then dehydrated in a series of graded acetone, embedded in a water-soluble methacrylate resin (Technovit® 7100; Kulzer Co., Ltd.), and polymerized at 45°C. The middle area of six randomly selected spikelets was then cut into 6-μm-thick sections. The lignin in these sections was detected using phloroglucinol/HCl staining (Jensen 1962). A 1% (w/v) staining solution was prepared by adding phloroglucinol in 75% ethanol, and the sections were soaked in this solution for 1 h and then dipped in 6 M HCl for 10 min prior to observation. It was observed that the lignified tissues were stained red when visualized under white light. The sections from each spikelet were photographed and the thickness of each red-stained sclerenchymatous cell wall of five successive cells, as indicated by arrowheads in Fig. 4, was measured using the ImageJ program (ImageJ, Version 1.52k). Data for the thickness of the sclerenchymatous cell wall in the palea of the hooking part at 20 DAH were analyzed using a t-test with a significance level of \( P < 0.01 \), and those of the lemma at 10 and 20 DAH were analyzed using an analysis of variance (ANOVA) with a significance level of \( P < 0.01 \) or a Tukey’s HSD test with a significance level of \( P < 0.05 \).

**Degree of damage caused by rice stink bugs using panicles with pruned spikelets**

Milyang 44 and the control cultivar Aichinokaori SBL were grown in pots (1/10,000 a), using the same protocols that were used for the individual feeding treatment in 2013. At 1–3 DAH, one-third part of the tips were pruned in the two panicles per plant from those spikelets that had flowered, using three pots per test. The panicles with pruned spikelets were then covered with paper bags (13 cm wide by 35 cm long) and were allowed to ripen in a glass chamber. At 20 DAH, the paper bags and unpruned spikelets were removed from the test plants, and feeding treatments with *L. chinensis* and *C. punctiger* were conducted using the group feeding treatment method (Sugiura et al. 2017).

After five days of insect feeding within the mesh cages that contained each rice stink bug species, the plants were sprayed with dinotefuran and placed inside another glass chamber to ripen. The mature panicles were then harvested and threshed, and the spikelets were collected.

The spikelets were stained with 1% methylene blue (Takeuchi et al. 2004), following which they were separated into the husks and grains, and the grain surface was examined for feeding marks under a binocular dissection microscope. The pecky rice grains were then categorized according to their area with feeding marks on the grain surface as follows: naked grain (area not covered by husk following pruning of the spikelets), covered by husk (area covered with the remainder of the husk following pruning of the spikelets), both, or indeterminable (unclear whether naked grain or covered by husk). The mean total number of spikelets, number of pecky rice grains, and percentage rate of pecky rice grains per panicle on each plant were calculated. Data for the percentage rate of pecky rice grains were arcsine-transformed and analyzed using a t-test with a significance level of \( P < 0.05 \).

**Results**

**Degree of damage caused by individual feeding treatment**

The percentage rates of pecky rice grains caused by *L. chinensis* and *C. punctiger* attacks were significantly lower for Milyang 44 than for Aichinokaori SBL in both 2013 and 2015 (Table 1). The 2-year average number of pecky rice grains per day caused by each individual of *L. chinensis* was 1.4 for Milyang 44 and 2.6 for Aichinokaori SBL, while that caused by *C. punctiger* was 1.1 for Milyang 44 and 2.5 for Aichinokaori SBL.

**Physical damage to the husks and grains**

The stylet sheaths of *L. chinensis* were clustered within the hooking part in both Milyang 44 and Aichinokaori SBL.

**Table 1.** The number and percentage of pecky rice after individual feeding treatment for two rice stink bugs, *Leptocorisa chinensis* and *Cletus punctiger*

| Insect species | Year | Cultivar      | Total number of spikelets* | Number of pecky rice* | Ratio (%)b | Percentage rate of pecky rice (%) | Ratio (%)b | Number of pecky rice per bug per dayc |
|----------------|------|---------------|---------------------------|-----------------------|------------|----------------------------------|------------|--------------------------------------|
| *L. chinensis* | 2013 | Milyang 44    | 201±27                    | 34±3                  | (42)       | 17**                             | (33)       | 1.1                                  |
|                |      | Aichinokaori SBL | 155±23                    | 80±12                 |            | 52                               |            | 2.7                                  |
|                | 2015 | Milyang 44    | 215±15                    | 44±5                  | (63)       | 20*                              | (65)       | 1.6                                  |
|                |      | Aichinokaori SBL | 223±30                    | 70±5                  |            | 32                               |            | 2.5                                  |
| *C. punctiger* | 2013 | Milyang 44    | 190±33                    | 30±7                  | (41)       | 16**                             | (39)       | 1.0                                  |
|                |      | Aichinokaori SBL | 182±22                    | 74±10                 |            | 41                               |            | 2.5                                  |
|                | 2015 | Milyang 44    | 183±24                    | 32±4                  | (48)       | 18*                              | (55)       | 1.2                                  |
|                |      | Aichinokaori SBL | 212±64                    | 68±24                 |            | 32                               |            | 2.4                                  |

*a* The number represents the means ± standard deviation of two total panicles on each plant after individual feeding treatment.

*b* Percentage rate compared with Aichinokaori SBL.

*c* Number of pecky rice/6 d/5 insects (2013) & number of pecky rice/7 d/4 insects (2015).

* and ** indicate significant difference by t-test with 5% and 1% confidence levels, respectively. Data were arcsine-transformed before the analyses.
While those of *C. punctiger* were distributed throughout the entire spikelet area in both cultivars. Observation of the stylet sheaths, perforations on the husks, and feeding marks on the grains under a binocular dissection microscope revealed that *L. chinensis* tried to insert its stylet into the gap in the hooking part (**Fig. 3A–3C**) between the palea and lemma 100% in Milyang 44 and 97% in Aichinokaori SBL, with the palea (outside the hooking part) and lemma being perforated in the remaining 3% of cases (**Fig. 2**). Inside the hooking part (**Fig. 3A–3C, 3F; 3F is the horizontal section of the hooking part (the area is similar to the square shown in 3C)), the stylet sheaths were found lying along (**Fig. 3D**) or penetrating the palea. By contrast, *C. punctiger* perforated the palea (outside the hooking part) and lemma of all spikelets in both rice cultivars (**Fig. 2**). Moreover, all of the perforations were found under the stylet sheaths in the cleavage between the lines of the tubercles in both cultivars (n = 65 for Milyang 44 n = 104 for Aichinokaori SBL following the individual feeding treatment in 2015) (**Fig. 3E; enlarged view of the palea outside the hooking part (similar to the square shown in Fig. 3A)**).

The number of stylet sheaths per panicle was higher in Milyang 44 than in Aichinokaori SBL for both rice stink bug species, with the exception of *C. punctiger* in 2015 (**Table 2**). However, the percentage rate of successful sucking in Milyang 44 was only 23%–68% of that in Aichinokaori SBL for both rice stink bug species, which was a significant difference (**Table 2**).

Histological analysis of the husks

In the sucking sites of *L. chinensis* at 20 DAH, each sclerenchymatous cell wall that stained red (i.e., the parts in which lignin was distributed) was significantly thicker in Milyang 44 than in Aichinokaori SBL (**Figs. 4A, 4B, 5A, t = 8.93, df = 10**). On the other hand, the interactions between days after heading and cultivar (DAH × C) were significant for the thickness of sclerenchymatous cell wall (**Fig. 5B**) in the sucking sites of *C. punctiger*. The thickness of each sclerenchymatous cell wall in those sucking sites was also significantly thicker in Milyang 44 than in Aichinokaori SBL at both 10 DAH and 20 DAH (**Figs. 4C–4F, 5B**). In addition, compared with 10 DAH, sclerenchymatous cell wall in both varieties was significantly thicker at 20 DAH (**Figs. 4C–4F, 5B**). However, there was no significant difference between Milyang 44 at 10 DAH and Aichinokaori SBL at 20 DAH (**Fig. 5B**).

Degree of damage caused by rice stink bugs using panicles with pruned spikelets

Nearly all of the grains of both cultivars were damaged by both rice stink bug species, with no significant differences (**Table 3**). The area where the pecky rice grains were
most frequently (66% or more) categorized was naked grain. Less than 6% of pecky rice grains were categorized as covered by husk (Table 3).

Discussion

Feeding patterns and successful sucking rates of the two rice stink bug species

Milyang 44 was confirmed to be resistant to both C. punctiger and L. chinensis resulting from individual feeding treatment. The stylet sheaths of L. chinensis occurred mainly on the palea of the hooking part, whereas those of C. punctiger occurred across the lemma and palea (Fig. 1); this finding is in accordance with previous studies (Furuie and Kiyota 1993, Kawamura 1993, Takeuchi et al. 2004). On the other hand, the number of stylet sheaths per panicle was higher in Milyang 44 than in Aichinokaori SBL for both rice stink bug species, with the exception of C. punctiger in 2015 (Table 2), indicating that they made more sucking attempts on Milyang 44 than on Aichinokaori SBL. However, the sucking success rates of both species were significantly lower on Milyang 44 than on Aichinokaori SBL, resulting in a lower percentage of pecky rice grains in Milyang 44 (Table 2).

It has previously been shown that resistant rice cultivars against brown planthopper (Nilaparvata lugens) receive more probing punctures and feeding marks than susceptible varieties (Reddy and Kalode 1985, Sogawa and Pathak 1970). Sogawa and Pathak (1970) found that although the resistant cultivar ‘Mudgo’ did not have any obvious mechanical barrier to feeding by the brown planthopper and provided the insects with better access to feeding sites than the susceptible varieties ‘IR8’ and ‘Taichung 1’, brown planthoppers did not prefer and excreted very little honey-dew on Mudgo, probably because of a lack of feeding stimuli or the presence of one or more taste repellents for brown planthoppers. However, in the case of Milyang 44, there were few feeding marks on the grain, indicating that the stylet did not reach the feeding site. Therefore, we consider

Table 2. The number of stylet sheaths and feeding marks and percentage rate of successful sucking by each rice stink bugs in individual experiment

| Insect species | Year | Cultivar       | Total number of spikelets | Number of stylet sheaths | Stylet sheaths/spikelet | Number of feeding marks | Percentage rate of successful sucking (%) | Ratio (%) |
|----------------|------|---------------|---------------------------|--------------------------|-------------------------|-------------------------|-------------------------------------------|-----------|
| L. chinensis   | 2013 | Milyang 44    | 114 ± 15                 | 176 ± 22                 | 1.58 ± 0.39             | 22 ± 12                 | 12**                                      | (26)      |
|                |      | Aichinokaori SBL | 84 ± 19                  | 92 ± 9                   | 1.12 ± 0.26             | 45 ± 7                  | 49                                        |           |
|                | 2015 | Milyang 44    | 110 ± 24                 | 101 ± 48                 | 0.89 ± 0.35             | 24 ± 12                 | 24**                                      | (46)      |
|                |      | Aichinokaori SBL | 118 ± 31                 | 63 ± 19                  | 0.56 ± 0.23             | 32 ± 5                  | 51                                        |           |
| C. punctiger   | 2013 | Milyang 44    | 95 ± 31                  | 87 ± 53                  | 0.98 ± 0.56             | 12 ± 4                  | 15**                                      | (23)      |
|                |      | Aichinokaori SBL | 79 ± 15                  | 40 ± 5                   | 0.51 ± 0.08             | 27 ± 10                 | 66                                        |           |
|                | 2015 | Milyang 44    | 93 ± 15                  | 44 ± 18                  | 0.49 ± 0.25             | 22 ± 9                  | 50*                                       | (68)      |
|                |      | Aichinokaori SBL | 118 ± 62                 | 48 ± 14                  | 0.50 ± 0.25             | 35 ± 4                  | 74                                        |           |

a Number of feeding marks/number of stylet sheaths × 100. Data were arcsine-transformed before the analyses.

b Percentage rate compared with Aichinokaori SBL.

* and ** indicate significant difference by t-test with 5% and 1% confidence levels, respectively.

The number represents the means ± standard deviation of one panicle observed by the binocular dissection microscope within two panicles on each plant examined after individual feeding treatment.
that the resistance mechanism of Milyang 44 is related to the difficulty the rice stink bug faces in inserting or piercing its stylet into the spikelet.

Our detailed investigations of the stylet sheaths showed that *L. chinensis* tended to insert its stylet into the gap of the hooking part (Fig. 2), indicating that the resistance of Milyang 44 to this species may be associated with difficulty in inserting or piercing the palea inside the hooking part, even when the stylet successfully reaches this area. By contrast, *C. punctiger* perforations and feeding marks were found under the stylet sheaths over the entire spikelet area (Fig. 1), indicating that the resistance of Milyang 44 to this species was caused by difficulty in penetrating the rice husks. Since all of the stylet sheaths and perforations were formed in the cleavage between the lines of the tubercles, any tissue from the upper epidermis to the lower epidermis of this cleavage portion is presumably difficult to pierce.

**Relationship between the interior structure of the rice husk and the resistance mechanism**

Histological analysis showed that the sclerenchymatous cell wall containing lignin was thicker in Milyang 44 than in Aichinokaori SBL (Fig. 4, 5). Seo and Ota (1983) considered that lignin formation in the rice husk contributes to the physical integrity of the fertile spikelets, and Nakamura et al. (2017) demonstrated that the spikelets of Milyang 44 are harder than those of Aichinokaori SBL by an average of 282% at 15 DAH and 14% at 25 DAH. Although Nakamura et al. (2017) could not distinguish the hardness of the grain from that of the rice husk, our results suggest that the hardness of the rice husk in Milyang 44 may contribute in interpreting lower degree of damage. Supporting this, although the number of spikelets sampled was small, we found that the exposed part of the Milyang 44 grain was extensively damaged by the rice stink bugs to a similar level as occurred in Aichinokaori SBL (Table 3). However, the resistance of Milyang 44 to sucking was unstable at 10 DAH (Nakamura et al. 2017). Our results suggest that the hardness of the rice husk in Milyang 44 at 10 DAH may be insufficient to suppress damage. This is because the sclerenchymatous cell wall in Milyang 44 at 10 DAH was significantly thicker than that in Aichinokaori SBL at 10 DAH, but was almost same as that in Aichinokaori SBL at 20 DAH (Fig. 4C–4F, 5B). After 10 DAH, the rice husks of Milyang 44 may become harder than Aichinokaori SBL by 20 DAH because of the thicker sclerenchymatous cell walls comparing with Aichinokaori SBL; it is likely that this acted as a resistance mechanism to...
rice stink bug damage, making it difficult for rice stink bugs to insert or pierce their stylets into the spikelets. This resulted in a low sucking success rate for both rice stink bug species. Further studies are required to clarify whether this is a common factor that suppresses the degree of damage caused by *L. chinensis* and *C. punctiger*, as well as a more detailed investigation of the structure of the corresponding part of the spikelet.

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