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Evaluating Panicle Blast Progression in Six Rice (*Oryza sativa* L.) Cultivars/Lines Carrying Quantitative Resistance Genes

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Abstract

Breeding rice crops that are resistant to rice blast is a protective and viable strategy. The proper characterization of the phenotypes of resistance (*R*) genes to leaf and panicle blast contributes to effective resistance breeding. However, observer subjectivity complicates the evaluation of panicle blast-resistance phenotypes. Hence, we evaluated panicle blast-resistance phenotypes using the number of panicles classified by the location of damage to the panicle tissues. Six cultivars/lines carrying *Pi35*, *pi21*, *Pi34*, *Pi39(t)*, *Pb1*, and *qPbm11*, which conferred quantitative resistance, including *Pb1* and *qPbm11*, panicle resistance genes and loci, respectively, were evaluated. These cultivars/lines were planted in the field, and disease progression of panicle blast was investigated. Our data revealed that the severity of spikelet damage differed among the cultivars/lines. In addition, the panicle number of damaged spikelets approximately two weeks after heading was correlated with the panicle number of damaged rachises and necks approximately four weeks after heading. These results suggested that the severity of spikelet damage is crucial for assessing *R* gene-mediated panicle resistance. Our results provide essential information on panicle blast progression and provide insights into the role of *R* genes in panicle blast.

Discipline: Agricultural Environment
Additional key words: leaf blast, *Pyricularia oryzae*

Introduction

Rice blast is caused by the fungus *Pyricularia oryzae*. It is a highly destructive disease affecting rice crops worldwide. One of the most economical strategies to protect plants from this disease is to introduce resistance (*R*) genes into cultivars. Characterizing the phenotypes of these *R* genes is the first step toward breeding disease-resistant cultivars.

The *R* genes for rice blast have been classified into two primary categories based on the phenotype of the lesion on the leaf: qualitative (complete) and quantitative (partial or field) resistance genes (Ezuka 1972). Quantitative resistance reactions are milder than those of qualitative resistance, resulting in sporulating lesions. The moderately susceptible lesions produced by quantitative resistance are thought to exert low selection pressure on the rice blast pathogen (Ezuka 1972, Niks et al. 2015, Ning et al. 2020). Using this strategy, *R* genes such as *Pi35*, *pi21*, *Pi34*, *Pi39(t)*, *Pb1*, and *qPbm11* have been identified (Fukuoka & Okuno 2001, Nguyen et al. 2006, Fukuoka et al. 2009, Fukuoka et al. 2014, Terashima et al. 2008, Zenbayashi-Sawata et al. 2007). In particular, *Pb1* and *qPbm11* have been identified as panicle resistance genes and loci, respectively (Fujii et al. 2000, Hayashi et al. 2010, Ishihara et al. 2014).

Rice blast disease affects leaves (leaf blast) and panicles (panicle blast) (Ou 1985). *R* genes and quantitative trait loci (QTL) have been identified for panicle resistance because panicle blast is directly linked

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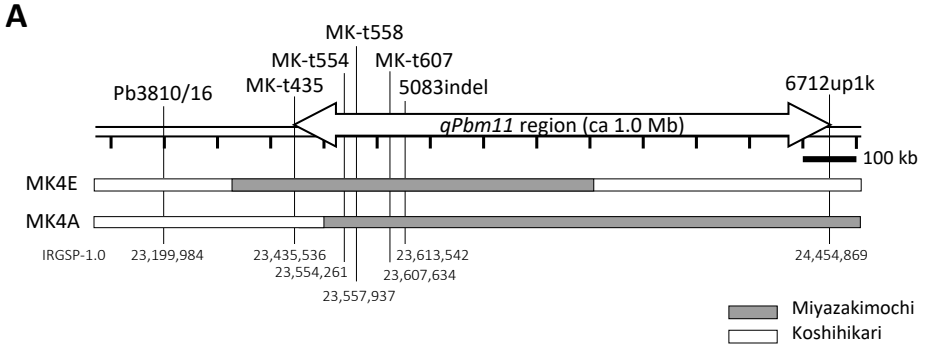
to yield loss (Kalia & Rathour 2019). Studying the properties of *R* genes in leaf and panicle blast is crucial, although evaluating the *R* genes in panicle blast is more challenging than evaluating those in leaf blast. The severity and progression of panicle blast are estimated by the damaged area on the panicle and/or yield loss via visual inspection. Several genes, such as *pi21*, *Pb1*, and *qPbm11*, have been assessed for their severity (Horo et al. 2016, Hayashi et al. 2010, Ishihara et al. 2014, Inoue & Hayashi 2019). However, field evaluation is difficult because it can vary because of the subjectivity of the observers. Thus, subjectivity can be an obstacle to acquiring the detailed properties of *R* genes for panicle blast.

This study evaluated leaf and panicle blast-resistance phenotypes in six cultivars/lines carrying *Pi35*, *pi21*, *Pi34*, *Pi39(t)*, *Pb1*, and *qPbm11*. To capture the disease progression of panicle blast in the cultivars/lines without observer variability, we assessed panicle blast severity by the number of panicles based on the position of the damage to the panicle tissues.

Materials and methods

1. Plant materials

"Koshihikari Aichi SBL" and "Mine-haruka" are rice cultivars, whereas Chugoku IL1, 10-4381B, 12-9367B, and MK4E are the experimental lines. "Koshihikari Aichi SBL," Chugoku IL1, 10-4381B, 12-9367B, and MK4E were developed from the leading Japanese rice cultivar "Koshihikari," which is a susceptible cultivar. "Koshihikari Aichi SBL" is a cultivar bred as a Koshihikari near-isogenic line developed from "Tukinohikari," which is a donor line of *Pb1* (Sugiura et al. 2004). "Mine-haruka (Chubu 111)" is a cultivar that retains *Pi39(t)* and *Pii* and has the same heading date as that of "Koshihikari" (Saka et al. 2007). Chugoku IL1 is a Koshihikari near-isogenic line developed from Chugoku 40, which is a donor line for *Pi34* (information regarding the line is given in the National Agriculture and Food Research Organization (NARO) website; https://www.naro.go.jp/patent/experiment/cropsystem/cropsystem_kind/rice/007082.html). 10-4381B is a BC4F5 line developed from the *Pi35* donor line Hokkai 188 by back-crossing with "Koshihikari" (Fukuoka et al. 2014). 12-9367B is a BC1F7 line developed from the Koshihikari back-crossed line of K14, which retains the resistant *pi21* allele in "Senshou"; K14 was developed by marker-assisted selection (Saka et al. 2010). MK4E (NARO Genebank: JP272234; available for users in Japan) is a BC4F4 line developed from the *qPbm11* donor line "Miyazakimochi" by back-crossing with "Koshihikari." The introgressed region of *qPbm11* (approximately 1 Mb) detected using the seven markers is shown in Figure 1. The markers were developed using the sequence data of the whole "Miyazakimochi" genome and MK4E. Sequence data



B

Marker	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Type
Pb3810/16	TCTACGAGAGTTACAGCTTCTCC	GAGTTGCATGGTACACCTAGCC	indel
MK-t435	AAAAGCTATTAGTTTCATACTTCC	TAAGCCTTGTGAGTCTCACAC	indel
MK-t554	ATTGGTTGGAAGAATGACAAAGC	ACCATATGTTGTGGTCACTGGTC	indel
MK-t558	ATCATTCATGATCTCACTATCCC	TTGTGACTTCTGGGATTGCTGC	indel
MK-t607	GTTACACGTCTACGGGTGGC	ATGCCAAAAGTTAGTGGGTCAC	indel
5083indel	TTCAGTCACCTCACAAGGCA	TGCCTCACCAATCACATACG	indel
6712up1k	GCCTGGTAAATTGCTTCAGGATA	TGCTGGACATGATTCCCAATCAT	indel

Fig. 1. Information on the introgressed region of "Miyazakimochi" and markers for MK4E
(A) Graphical representation of the MK4E genotype. The MK4A genotype was used as a reference line to identify the introgressed region corresponding to *qPbm11*. MK4A is the sister line of MK4E. (B) Primer sequences used for genotyping MK4E. Pb3810/16 and 5083indel are referred to Hayashi et al. (2010) and Ishihara et al. (2014), respectively.

Table 1. Heading dates and investigation days after heading of the seven cultivars/lines grown in the experimental fields in 2019 and 2020

Cultivar/line	Gene	Heading dates	Investigation days after heading			Heading dates	Investigation days after heading		
		2019	1st	2nd	3rd	2020	1st	2nd	3rd
Koshihikari	–	Aug. 14	12	18-19	25-26	Aug. 12 and 13	15-16	22-23	28-29
Koshihikari Aichi SBL	*Pb1*	Aug. 14 and 15	11-12	17-18	24-25	Aug. 13 and 14	14-15	21-22	27-28
MK4E	*qPbm11*	Aug. 14 and 15	11-12	17-19	25	Aug. 12 and 13	15-16	22-23	28-29
10-4381B	*Pi35*	Aug. 14 and 15	11-12	17-19	24-25	Aug. 13 and 14	14-15	21-22	27-28
12-9367B	*pi21*	Aug. 13 and 14	12-13	18-20	26	Aug. 10, 11 and 12	16-18	23-25	29-31
Chugoku IL1	*Pi34*	Aug. 14 and 15	11-12	17-19	25	Aug. 13	15	22	28
Mine-haruka	*Pi39(t)*	Aug. 15 and 17	9-11	15-18	23-24	Aug. 12 and 13	15-16	22-23	28-29

were submitted to DDBJ (BioProject accession: PRJDB17049; Accession numbers: DRR515525 and DRR515526). Whole-genome resequencing was performed using an Illumina HiSeq system (Macrogen, Kyoto, Japan). The heading date for the two years of investigation is shown in Table 1.

2. Assay of resistance to leaf blast with artificial inoculation

Plants were grown in a greenhouse and artificially inoculated with fungal spores by spraying as previously described (Hayashi et al. 2016). The sixth leaf was assessed for resistance using the *P. oryzae* isolate Ao92-06-2 (race number: 337.1). The percentage of diseased leaf area was scored using digital imaging 7 days postinoculation. The diseased leaf area was estimated using digital images as previously described (Hayashi et al. 2019). Digital images of infected leaves stained with blue cut-flower dye (Flower Fantasy; Palace Chemical, Yokohama, Japan) were obtained from five independent plants. Five leaf samples were pasted onto white tape, and images were captured using a digital camera (Tough TG-5; Olympus, Tokyo, Japan). The images were processed using a digital microscope software (VHX-5000; Keyence, Osaka, Japan) to extract the regions corresponding to damaged leaf tissues according to hue (47-75), saturation (13-108), and brightness (128-255). Whole leaf regions of the five leaves were extracted using hue (32-156) and saturation (21-255). The extracted areas were calculated using digital microscope software as follows: Diseased leaf area (%) = (Damaged leaf area)/(Whole leaf area) × 100.

3. Plant growth condition in paddy fields

Leaf and panicle blast data were collected in an experimental paddy field with high disease pressure at the Mountainous Region Agricultural Institute of the Aichi Agricultural Research Center (35°12.7′N, 137°30.4′E). Fifteen plants were transplanted by hand in 90-cm-long rows at 30-cm intervals. Diseased plants inoculated with a blast fungus population collected from a panicle blast experimental field in the previous year were planted around the edge of the field. Heading days were checked for each row.

4. Assay of resistance to leaf blast in paddy fields

Leaf blast severity in the row was evaluated using the disease severity index (Asaga 1981) for 73 days (in 2018) and 54 days (in 2019) after planting in the field. The scores ranged from 0 (no symptoms on the leaves) to 10 (damage to the whole plant). Scores of 1-4 were assigned based on the extent of leaf damage, and scores of 5-9 were assigned depending on the severity of leaf withering.

5. Assay of resistance to panicle blast based on the position of the panicle tissue damage

In 2019 and 2020, we assessed panicle blast symptoms according to a classification based on the location of the damage to the panicle tissues, which resulted in white head symptoms (Fig. 2). A white head symptom caused by damage to the panicle tissue distal to the area of hyphal growth was used as a phenotypic marker to indicate the extent of the damage (Hayashi et al. 2019, Koga 1994). The scoring positions of the panicle tissues were selected according to Asaga's method (1981) for damage to key tissues: spikelet, branch, rachis, and neck. No damage to the panicle was scored as 0. The damage to one spikelet and its pedicel was scored as 1. The damage to the primary or secondary branch that affected adjacent two spikelets was scored as 2, and damage to the primary or secondary branch that affected more than three adjacent spikelets was scored as 3. The damage to the entire primary branch was scored as 4, whereas damage to the rachis node, internode of the rachis, and neck node was scored as 5, 6, and 7,

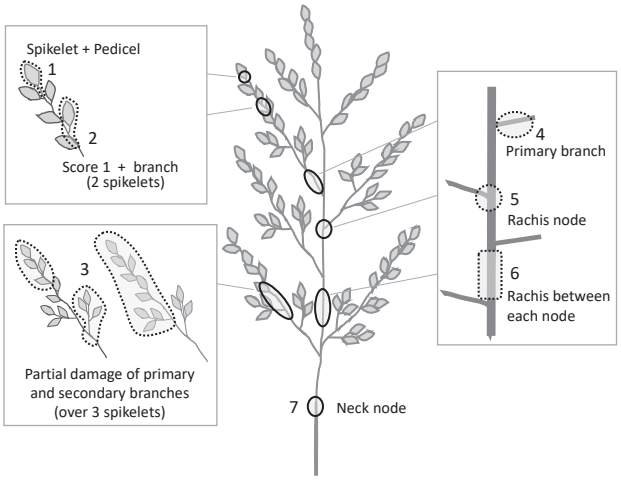


Fig. 2. Scoring positions based on damage by rice blast in the scoring rule
Examples of each score (1-7) are provided. Striped lines indicate the target area and position.

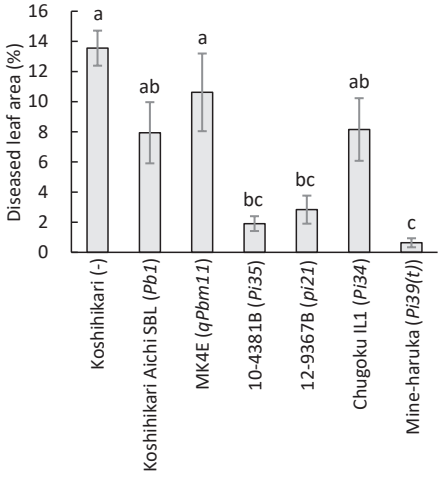


Fig. 3. Diseased leaf area 7 days after the artificial inoculation of *P. oryzae* isolates (race number, 337.1)
The error bars indicate the standard error (n = 5). Different letters above the bars represent significant differences according to Tukey's test at $P < 0.05$.

respectively. In this study, we defined spikelet damage as a score of 1; branch damage as scores of 2, 3, and 4; rachis damage as scores of 5 and 6; and neck damage as score of 7. If several scores were found in the panicle, the maximum score was assigned.

Data from 39 samples in each row were used in the analyses. The data for the time-dependent scoring change were obtained as follows: an average of 2-3 primary panicles (selected from panicles showing the largest size in a plant) on each plant were selected and labeled with a number on the rachis under the neck node in the first investigation, and panicle damage was scored at three time points (Table 1). Three rows were used as the experimental replicates. Samples with broken rachises caused by physical damage during the investigation were excluded from analysis.

6. Statistical analysis

Statistical analyses were performed using the Bell Curve for Excel software developed by the Social Survey Research Information Co., Ltd.

Results

1. Resistance level of leaf blast in "Koshihikari" and six cultivars and lines

We evaluated the responses of six cultivars/lines carrying *Pb1*, *qPbm11*, *Pi35*, *pi21*, *Pi34*, and *Pi39(t)* to a *P. oryzae* isolate (race number: 337.1) in a greenhouse (Fig. 3). Although all cultivars/lines showed a lower diseased leaf area than the susceptible "Koshihikari" cultivar, only the resistant reaction of three cultivars/lines carrying *Pi35*, *pi21*, and *Pi39(t)* had a significantly lower diseased leaf area. In addition, the areas affected by the disease in the three cultivars/lines carrying *Pb1*, *qPbm11*, and *Pi34* were not significantly different from those in the susceptible lines. The same cultivar/line characteristics for leaf blast were observed in the experimental field for 2 years (Table 2). Three cultivars/lines carrying *Pi35*, *pi21*, and *Pi39(t)* had the lowest values (average score, 0.2-0.5 in 2018 and 0-0.3 in 2019), whereas three cultivars/lines carrying *Pb1*, *qPbm11*, and *Pi34* showed lower score levels (average score, 0.8-2.7 in 2018 and 2.0-2.7 in 2019) than those of "Koshihikari" (average score, 4.3 in 2018 and 3.3 in 2019).

2. Disease progression of panicle blast

In each cultivar/line, the severity of panicle blast of 117 panicles (the sum of 39 samples from three rows), which were labeled to track the damage process, was assessed at three time points (Table 1). We scored the severity of panicle blast on a scale of 0-7 based on the position of the damage to the panicle tissues (Fig. 2). A total of 819 samples (the sum of 117 samples from seven cultivars/lines) were investigated each year. Of the 1638 samples (the sum of 819 samples from two years of investigation), 1596 were transferred to a higher score (directed as score 0 to score 7) during the three investigations (Fig. 4), and 42 samples (4 samples in 2019 and 38 samples in 2020) were transferred to lower scores. Of the 42 panicles, 36 showed a difference of only one score. Statistical analyses included the results of 42 panicles, which were deducted due to human error.

Table 2. Leaf blast severity index of the seven cultivars/lines grown in the experimental field in 2018 and 2019

Cultivar/line	Gene	Years	The disease severity index				
			rep.1	rep.2	rep.3	Average	(±SD)
Koshihikari	–	2018	4.0	5.0	4.0	4.3	(±0.58)
		2019	3.0	4.0	3.0	3.3	(±0.58)
Koshihikari Aichi SBL	*Pb1*	2018	3.5	3.0	0.5	2.3	(±1.61)
		2019	3.0	3.0	2.0	2.7	(±0.58)
MK4E	*qPbm11*	2018	2.5	2.5	3.0	2.7	(±0.29)
		2019	2.0	3.0	2.0	2.3	(±0.58)
10-4381B	*Pi35*	2018	0.1	0.1	0.3	0.2	(±0.12)
		2019	0.0	1.0	0.0	0.3	(±0.58)
12-9367B	*pi21*	2018	0.5	0.5	0.5	0.5	(±0.00)
		2019	0.0	0.0	0.0	0.0	(±0.00)
Chugoku IL1	*Pi34*	2018	1.0	1.0	0.5	0.8	(±0.29)
		2019	2.0	2.0	2.0	2.0	(±0.00)
Mine-haruka	*Pi39(t)*	2018	0.5	0.5	0.5	0.5	(±0.00)
		2019	0.0	1.0	0.0	0.3	(±0.58)

3. Resistance level of panicle blast in "Koshihikari" and six cultivars and lines

The score distributions for no damage, slight damage (spikelet damage: score 1), and severe damage (rachis and neck damage: scores 5, 6, and 7) showed significant changes during the three investigations (Fig. 4). Therefore, we focused on the score distribution in the two categories of no/slight damage (sum of scores 0 and 1) and severe damage (sum of scores 5, 6, and 7) and compared them for seven cultivars/lines (Fig. 5).

The two categories of cultivars/lines exhibited similar distribution patterns in the 2019 and 2020 populations. Figure 5A shows the number of panicles with no/slight damage in the first investigation. The line carrying *pi21* showed a similar level of damage to "Koshihikari" (showing the smallest number of panicles in Fig. 5A), whereas the line carrying *Pi35* showed the slightest damage (showing the largest number of panicles), and the others showed an intermediate level of damage between the lines carrying *pi21* or *Pi35*. Figure 5B shows the number of panicles with severe damage in the third investigation. Among them, the line carrying *pi21* had almost a similar level of damage to that of "Koshihikari" (showing the largest number of panicles in Figure 5B), whereas the line carrying *Pi35* showed the least damage (showing the smallest number of panicles), and the others showed an intermediate level of damage between them in both the 2019 and 2020 populations (Fig. 5B).

The scatterplot showed high negative correlation ($r = -0.86$) between the number of panicles with no/slight damage in the first investigation and that of severely damaged panicles in the third investigation among "Koshihikari" and six *R* gene-carrying cultivars/lines (Fig. 6).

Discussion

The severity of panicle blast is influenced by environmental conditions and is linked to spore production and dissemination (Ou 1985). Thus, visual assessment of disease progression is challenging, even with experimental replication. In this study, we assessed panicle blast resistance by tracking the severity of each panicle for which the damaged position of the panicles was scored (Fig. 2).

Analysis of a large number of panicle samples using this method revealed that the disease progressed similarly in susceptible and resistant cultivars/lines: panicles with no damage or only spikelet damage (scores 0 and 1) progressed to branch damage (scores 2, 3, and 4), followed by rachis damage (scores 5 and 6), and neck damage (score 7) (Fig. 4). In addition, our data revealed that the severity of spikelet damage approximately two weeks after heading was strongly correlated with the severity of rachises and neck damage approximately four weeks after heading (Table 1 and Fig. 6).

Infection during heading results in severe damage to the panicles (Katsube & Koshimizu 1970, Shindo & Asaga 1989). The results give rise to a presumption that the panicles are most susceptible to the fungus at this stage. The panicle branches of Japanese rice cultivars, including "Koshihikari," typically remain attached to the

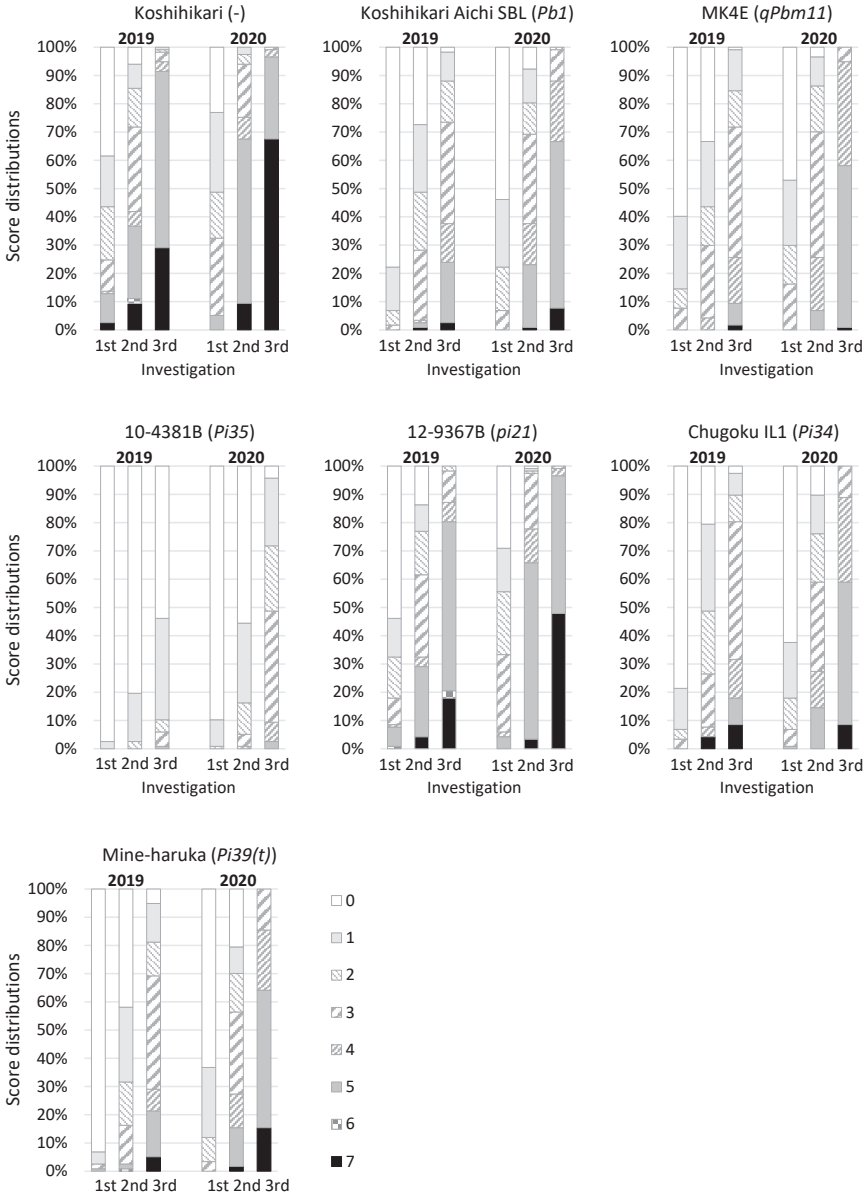


Fig. 4. Score distributions among 117 panicles of the rice cultivars "Koshihikari" and six *R* gene-carrying cultivars/lines

The ratio of scores 0-7 in the sample population in three investigations (first, second, and third investigation) is presented in a stacked column chart.

rachis until flowering (Hoshikawa 1989). Therefore, the severity of spikelet damage is crucial for panicle resistance because spores are believed to attach primarily to spikelets.

Under field conditions at the earliest investigated stage, 9-13 days after heading in 2019, the percentages of spikelet damage (percentage of scores 0 and 1 in Fig. 4) were 56.4% in "Koshihikari," 67.5% in the line carrying *pi21*, and > 85.0% in the lines carrying *qPbm11*, *Pi34*, *Pb1*, *Pi39(t)*, and *Pi35*. *Pi35* had the highest scores of 0 and 1 (100%) and showed the most stable resistance to both leaf and panicle blast. Two cultivars/lines, *Pi39(t)* and *Pi34*, with the same resistance level to panicle blast showed relatively high scores of 97.4% and 93.2% respectively, although *Pi34* had no strong effect on leaf blast (Fig. 3 and Table 2). *Pb1* and *qPbm11*, known as panicle resistance genes and loci, respectively, had a weak effect on leaf blast, with relatively high scores of 85.4% and 93.2%, respectively. Interestingly, *pi21*, which showed a strong effect on leaf blast, had a low score of 67.5% and a weak effect on panicle blast (Fig. 3, Table 2, Fig. 4, and Fig. 5A, 5B). These results suggest that a score of 0 or 1 might be more indicative of the reaction to panicle blast than the severity level of leaf blast.

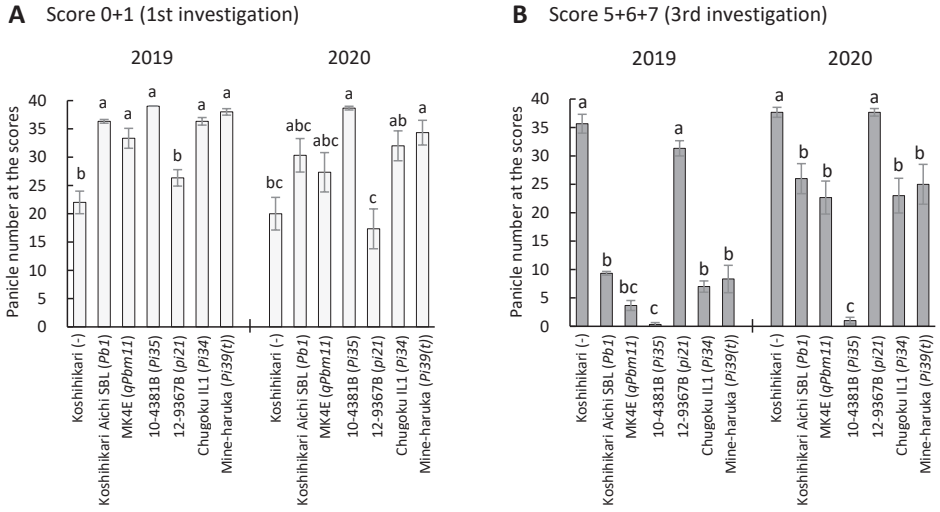


Fig. 5. Reanalysis of score distributions of "Koshihikari" and six *R* gene-carrying cultivars/lines

The data score distributions in Figure 4 were reanalyzed based on the panicle numbers of each row, with three replicated rows under investigation—each containing 39 panicles. (A) Distribution of scores 0 and 1 in the first investigation. (B) Distribution of scores 5, 6, and 7 in the third investigation. The bars represent the standard error (n = 3). Different letters above the bars indicate significant differences according to Tukey's test at $P < 0.05$. The Tukey's tests were performed independently in 2019 and 2020.

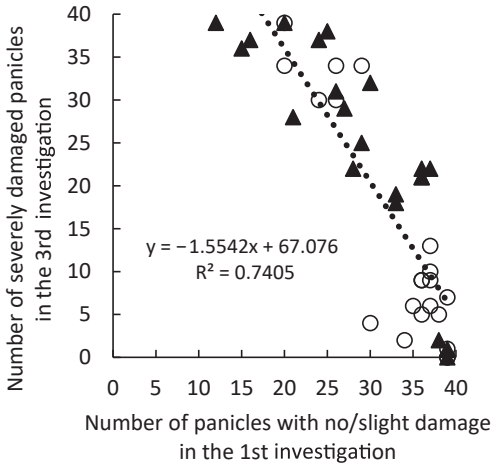


Fig. 6. Scatterplot between the number of panicles with no/slight damage in the first investigation (Fig. 5A) and that of severely damaged panicles in the third investigation (Fig. 5B) among "Koshihikari" and six *R* gene-carrying cultivars/lines

The score numbers in each cultivar/line were based on 39 panicles from three rows in 2019 (open circle) and 2020 (triangle). The correlation coefficient is −0.86, which is significant at $P < 0.01$.

The panicle blast estimation method described by Asaga (1981) is commonly used in rice blast-resistant breeding programs and phytopathological experiments in Japan. Therefore, we scored the damaged panicles using this method. The severity of panicle blast using the number of panicles can be classified by the location of damage to the panicle tissues. The scoring data were used for statistical analyses, as shown in Figure 6. Statistical analyses may provide credible information for studying panicle blast, such as QTL and factor analyses, under various environmental conditions. Our strategy to score the severity of panicle blast supports Asaga's method.

Breeding crops resistant to rice blast typically relies on the correlation between resistance to leaf blast and resistance to panicle blast (Bonman 1992, Ou 1985). However, several *R* genes do not follow this simple rule as various types of *R* genes, including panicle blast-resistance genes, have been identified. Thus, panicle blast evaluation that eliminates observer variability will enable the collection of unbiased data and contribute to the progress of genetic studies and breeding strategies for panicle blast resistance.

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