Effects of Pyramiding Quantitative Resistance Genes *pi21*, *Pi34*, and *Pi35* on Rice Leaf Blast Disease

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Abstract

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Development of resistant cultivars has been an effective method for controlling rice blast disease caused by *Magnaporthe oryzae*. Quantitative blast resistance genes may offer durable resistance because the selection pressure on *M. oryzae* to overcome resistance is low as a result of the genes' moderate susceptibility. Because the effects of individual resistance genes are relatively small, pyramiding these genes in rice cultivars is a promising strategy. Here, we used near-isogenic and backcross lines of rice cultivar Koshihikari with single- or two-gene combinations of blast resistance genes (*pi21*, *Pi34*, and *Pi35*) to evaluate the suppression

of leaf blast. The severity of the disease was assessed throughout the infection process. Resistance varied among the lines: *Pi35* conferred the strongest resistance, while *Pi34* showed the weakest effects. Two types of combined-gene interactions were observed, and they varied on the basis of gene combination and characteristic of the infection: (i) the combination of two resistance genes was more effective than either of the genes individually or (ii) the combination of two resistance genes was similar to the level of the most effective resistance gene in the pair. The most effective gene combination for the suppression of leaf blast was *pi21* + *Pi35*.

Rice blast caused by *Magnaporthe oryzae* B.C. Couch & L.M. Kohn (anamorph, *Pyricularia oryzae*) is one of the most economically devastating rice diseases worldwide. The use of resistant rice cultivars is an effective way to manage this disease. To date, more than 80 genes for blast resistance have been reported (18). Blast resistance in rice has generally been classified into two categories: qualitative (complete or true) and quantitative (partial or field) resistance (5,25). A majority of the reported blast resistance genes in rice are qualitative resistance genes, which prevent reproduction of the pathogen, and are often associated with a hypersensitive response.

Qualitative resistance genes are race-specific and follow a model of gene-for-gene interaction. Deployment of uniform rice cultivars with simply inherited resistance over vast areas has led to the emergence of new virulent races of blast fungus: this has resulted in the breakdown of cultivar resistance to the disease (3,16), presumably because of the high specificity of the blast resistance genes.

Resistance controlled by quantitative resistance genes allows the formation of sporulating lesions but suppresses lesion expansion or spore formation on rice cultivars, and consequently slows the spread of blast disease. Quantitative resistance genes presumably confer broad-spectrum and durable resistance because the selection pressure on blast fungus to overcome this resistance is low as a result of their moderate susceptibility (5,21,27). Therefore, improving the level of blast resistance in rice using quantitative resistance genes has been a major goal in rice breeding (15).

Recently, intensive genetic analyses of quantitative blast resistance genes including *pi21*, *Pi34*, *Pi35*, *Pi39*, and *Pb1* have been conducted (7,8,10,24,33,39), and DNA markers that are tightly

linked to these resistance genes have been developed (17). Marker-assisted selection (MAS) facilitates the selection of rice lines carrying quantitative resistance genes of interest in breeding programs. Since the effects of individual quantitative resistance genes are relatively small, pyramiding of these genes is a useful strategy for further improving resistance to rice blast. However, information on appropriate combinations of resistance genes is largely lacking for this disease.

The effects of pyramiding resistance genes have been investigated for several plant-microbe interactions. In some cases, pyramiding of bacterial blight quantitative resistance genes in rice provided a higher level of resistance than did the introduction of individual genes (1,29,30,38,40). In other cases, pyramiding provided a level of resistance that was comparable to that provided by introducing a single gene in the combination (29). In potatoes, a higher level of resistance against late blight was shown when the resistance genes *R_{Pi-mcd1}* and *R_{Pi-ber}* were pyramided (32). Barley lines containing pyramided quantitative trait loci (QTLs) involved in resistance against stripe rust were found to have increased resistance (26). In a study of three rice blast resistance QTLs (*qBR4-2a*, *qBR4-2b*, and *qBR4-2c*), increasing the number of QTLs led to reduced lesion area (6). However, detailed information about the effects of gene pyramiding on the infection process of blast fungus is limited.

In this study, we focused on three quantitative blast resistance genes, *pi21*, *Pi34*, and *Pi35* in rice. The *pi21* gene is recessive and its dominant allele, *Pi21*, encodes a proline-rich protein that includes a putative heavy metal-binding domain and protein-protein interaction motifs (7). *Pi34* and *Pi35* are the two dominant genes and are located on chromosomes 11 and 1, respectively (8,24,39). While *Pi34* has not yet been isolated, *Pi35* was shown to encode a protein containing a nucleotide binding site (NBS) and leucine-rich repeats (LRRs) (8), which are common structures of resistance genes. We used near-isogenic lines (NILs) and backcross lines of the cultivar Koshihikari containing one or two resistance genes (*pi21*, *Pi34*, and *Pi35*), to evaluate the effectiveness of these genes against leaf blast by artificial inoculation. We compared the severity of blast disease in each rice line according to five disease characteristics: (i) penetration frequency of blast fungus; (ii) number of infected rice cells at

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40 h after inoculation; (iii) number of sporulating lesions; (iv) lesion length and width; and (v) percentage of diseased leaf area.

Materials and Methods

Plant materials. The materials used in this study included two NILs containing the blast resistance genes *pi21* and *Pi34* in the Koshihikari background, a breeding line Chugoku IL1 (23) (called *Pi34*-IL in this study), and a cultivar Chubu 125 (28) (called *pi21*-NIL in this study). Chugoku IL1 was developed from the Chugoku 40 breeding line, which carried *Pi34*. Chugoku 40 was backcrossed six times with Koshihikari, and a line carrying *Pi34* was selected by MAS (23). Chubu 125 was developed from a Sensho cultivar that carried the resistant *pi21* allele. Sensho was crossed four times with the cultivar Mineasahi, and was then backcrossed twice with Koshihikari, and a progeny carrying *pi21* was selected by MAS (7,28). The mother of Mineasahi is Kanto 79, which is a mutant line of Koshihikari (7,28). More than 300 polymorphic genetic markers between parents confirmed that most of the genomic regions of Chubu 125 originated from Koshihikari (7,28).

The rice cultivar Hokkai 188 was used as a donor of the blast resistance gene *Pi35* (24). Koshihikari was crossed with Hokkai 188: F₁ plants were backcrossed with Koshihikari to develop the BC₁F₁ and the resulting plants were self-pollinated. Individuals that were homozygous for the *Pi35* allele were selected from the BC₁F₂ populations by MAS. One selected BC₁F₂ plant was self-pollinated, and the BC₁F₃ plants were designated as *Pi35*-L. Although the genetic background of *Pi35*-L differed in part from that of Koshihikari, leaf blast resistance in *Pi35*-L was assumed to be mainly controlled by *Pi35*. *Pi35* is the only QTL from Hokkai 188 detected in an F₃ population derived from a cross between rice cultivar Danghang-Shali, which has poor resistance, and Hokkai188 (8,24). In addition, the susceptibility of Danghang-Shali to leaf blast was comparable to that of Koshihikari; in the field test, the percentage of diseased leaf area was approximately 80% in Danghang-Shali and Koshihikari and 7% in Hokkai 188. Thus, *Pi35*-L could be used to study the effect of *Pi35*.

Pyramided lines (called *pi21* + *Pi34*-NIL, *pi21* + *Pi35*-L, and *Pi34* + *Pi35*-L in this study) carrying two blast resistance genes were developed using *pi21*-NIL, *Pi34*-IL, and *Pi35*-L. F₁ plants carrying two resistance genes were self-pollinated and individuals with homozygous alleles at the resistance gene loci were selected from the F₂ population using MAS.

***M. oryzae* inoculation and disease scoring.** *M. oryzae* isolate Ao 92-06-2, which is pathogenic to Koshihikari, was used to inoculate Koshihikari and the lines for analysis of resistance gene effects. The method for preparing spore suspensions was the same as described previously (36). Seeds were sown in cell trays containing soil: each cell was 3 × 3 × 4.5 cm and each tray contained 8 rows of 16 cells. Plants were grown in a greenhouse at 20 to 30°C under ambient lighting conditions.

The leaf sheath inoculation method (31) was used to investigate the penetration frequency of blast fungus and the number of infected rice cells at 40 h after inoculation. A sheath of the sixth leaf from the bottom was detached at the 6.5-leaf stage and filled internally with *M. oryzae* spores (1 × 10⁴ to 3 × 10⁶/ml suspended in distilled water) using a syringe. Inoculated leaf sheaths were placed in petri dishes (9 cm diameter) that were kept in a chamber at 25°C in darkness. Slices of tissue from the inner surface of the leaf sheaths were removed with a razor blade at 40 h after inoculation and used for observation by bright-field microscopy. The number of rice cells invaded by infection hyphae was counted on each appressorium. Two independent experiments were performed, each using five sheaths from five plants of each rice line, and 114 appressoria were observed for each sheath.

We used the previously described spray inoculation method (36) to measure the number of sporulating lesions and the percentage of diseased leaf area. A 400-ml sample of *M. oryzae* spore suspension (1 × 10⁵ to 3 × 10⁵/ml suspended in distilled water) was sprayed on plants at the 5.8-leaf stage. Three independent experiments were performed, each using 18 to 39 plants for each rice line. The number of sporulating lesions on each plant was counted 6 days after inoculation. At 7 days after inoculation, the sixth leaf from the bottom of each plant was picked and the percentage of diseased leaf area was quantified as previously described (7).

To measure lesion size, we modified the spot inoculation method described by Jia et al. (12). An intact rice plant at the 5.5-leaf stage with roots was placed horizontally in a plastic tray and the sixth leaf from the bottom was inoculated with a 5-μl droplet of spore suspension (5 × 10⁵ to 1 × 10⁶/ml suspended in distilled water containing 0.05% Tween 20 [v/v]). The plant trays were immediately placed in a dark chamber with a moisture-saturated atmosphere at 25°C for 20 h and were then transferred to a greenhouse at 25°C under ambient lighting conditions. At 20 days after inoculation, the length and width of each lesion were measured with a caliper (DIGIPA, Mitutoyo, Kawasaki, Japan). Three independent experiments were performed using 10 to 30 plants for each rice line.

Statistical analysis. All data were analyzed using JMP statistical software (version 8.0.1, SAS Institute, Cary, NC). The effect of the presence or absence of the three resistance genes, interactive effects of the genes, and date of inoculation (experimental trial as a random effect) were assessed for each parameter. Penetration status of the blast fungus was assessed using logistic regression analysis. Numbers of infected plant cells at 40 h after inoculation were compared using Poisson regression analysis to evaluate the effects mentioned above as well as differences among individual plants (nest); when necessary, pairwise comparisons were performed using the Mann-Whitney *U*-test weighted by the Bonferroni correction. Data on numbers, length, and width of lesions were compared using three-way mixed analysis of variance (ANOVA). To satisfy the requirement for normality and homoscedasticity of data, arcsine transformation was conducted on all data for percentage of diseased leaf area, and the transformed data were compared using three-way mixed ANOVA. Where necessary, pairwise comparisons of parametric data were performed using Tukey's honestly significant difference (HSD) test.

Results

Penetration frequency. The penetration frequency of *M. oryzae* into the epidermal cells of each rice line was evaluated at 40 h after inoculation and was >90% in all lines including Koshihikari. None of the resistance genes (*pi21*, *Pi34*, and *Pi35*) were effective in reducing the penetration frequency of *M. oryzae* (Table 1).

Number of infected rice cells at 40 h after inoculation. In Koshihikari, approximately 3.9 rice cells were infected at 40 h after inoculation (Fig. 1), while the numbers of infected cells in lines containing the individual resistance genes *pi21*, *Pi34*, and *Pi35* were approximately 3.2, 1.8, and 2.3, respectively (Fig. 1). Poisson regression analysis indicated that *Pi34* and *Pi35* were significantly effective at reducing the numbers of infected cells at 40 h after inoculation (Table 2). *Pi34* was the most effective of the three genes at reducing the number of infected cells (Fig. 1).

The numbers of infected rice cells in the lines with *pi21* + *Pi34* was comparable to that in lines with only *Pi34*, which had fewer infected cells than lines with *pi21* (Fig. 1). Similarly, the number of infected cells in the rice lines with *pi21* + *Pi35* was comparable to that in lines with only *Pi35*, which had fewer infected cells than lines with only *pi21* (Fig. 1). In contrast, lines containing *Pi34* + *Pi35* showed significantly lower numbers of infected cells than lines containing either *Pi34* or *Pi35* alone (Fig. 1). Poisson regression analysis revealed a negative interaction between *Pi34* and *Pi35*, which meant that the combined ability of *Pi34* + *Pi35* to reduce the number of infected rice cells was weaker than the individual effects of these genes (Table 2).

Table 1. Effects of individual resistance genes (*pi21*, *Pi34*, and *Pi35*) and their interactions on the penetration of the *Magnaporthe oryzae* isolate in comparison with the cultivar Koshihikari, evaluated using logistic regression analysis

Sources	df	G-value	P value ^a
<i>pi21</i>	1	0.48	0.49
<i>Pi34</i>	1	0.08	0.78
<i>Pi35</i>	1	0.00	0.98
<i>pi21</i> × <i>Pi34</i>	1	0.05	0.82
<i>pi21</i> × <i>Pi35</i>	1	2.60	0.11
<i>Pi34</i> × <i>Pi35</i>	1	2.44	0.12
Random effect	3	21.45	<0.0001*

^a Asterisk (*) indicates significance (*P* < 0.05).

Number of sporulating lesions. Each Koshihikari plant contained approximately 85 sporulating lesions (Fig. 2), while the mean number of lesions in lines with *pi21*, *Pi34*, and *Pi35* were 22, 66, and 37, respectively (Fig. 2). Each of the three resistance genes led to a significant reduction in the number of sporulating lesions (Table 3) and their rank in order of effectiveness was *pi21* > *Pi35* > *Pi34* (Fig. 2).

Fig. 1. Number (mean $\pm$ standard error) of rice leaf sheath cells invaded by infection hyphae of *Magnaporthe oryzae* isolate at 40 h after inoculation. Values with the same letter are not significantly different (Mann-Whitney *U*-test with Bonferroni correction, $P > 0.05$). Note that Poisson regression may underestimate the standard error.

Table 2. Effects of individual resistance genes (*pi21*, *Pi34*, and *Pi35*) and their interactions on the number of rice cells infected by the *Magnaporthe oryzae* isolate in comparison with the cultivar Koshihikari, evaluated using Poisson regression analysis

Sources	df	G-value	P value ^a
<i>pi21</i>	1	2.17	0.1405
<i>Pi34</i>	1	891.42	<0.0001*
<i>Pi35</i>	1	175.60	<0.0001*
<i>pi21</i> $\times$ <i>Pi34</i>	1	58.31	<0.0001*
<i>pi21</i> $\times$ <i>Pi35</i>	1	92.36	<0.0001*
<i>Pi34</i> $\times$ <i>Pi35</i>	1	55.02	<0.0001*
Random effect	3	136.19	<0.0001*
Nest	28	134.72	<0.0001*

^a Asterisk (*) indicates significance ($P < 0.05$).

Fig. 2. Numbers of lesions per plant (mean $\pm$ standard error) produced by *Magnaporthe oryzae* isolate at 6 days after spray inoculation. Values with the same letter are not significantly different (Tukey's honestly significant difference test, $P > 0.05$).

The number of sporulating lesions in the rice line containing *Pi34* + *Pi35* was comparable to that in the line containing *Pi35* only (Fig. 2). The lines with *pi21* + *Pi34* or *pi21* + *Pi35* showed significantly fewer lesions than those with *pi21* only (Fig. 2). Negative interactions between resistance genes were detected by the Poisson analysis (Table 3) and indicated that the combined effects of two genes were comparatively smaller than the effects of the individual genes.

Lesion length and width. The shape of lesions on the leaf blades varied with the resistance genes in each rice line (Fig. 3). For example, plants with *pi21* formed narrower lesions compared with Koshihikari. Thus, we measured the length and width of sporulating lesions independently.

The mean lesion length in rice lines containing *pi21* and *Pi34* was comparable to that in Koshihikari (Fig. 4). Rice plants with *pi21* had slightly longer lesions than Koshihikari (Fig. 4). The small effect of *pi21* on lesion elongation was verified by ANOVA (Table 4). Lesions in rice plants with *Pi35* were significantly shorter than those in Koshihikari (Fig. 4). The length of lesions in the line with *pi21* + *Pi34* was comparable to that in the line with *pi21* only (Fig. 4), while lesions in lines containing *pi21* + *Pi35* or *Pi34* + *Pi35* were comparable in length to those in lines with *Pi35* alone (Fig. 4).

Mean lesion width in the rice line with *Pi34* was comparable to that in Koshihikari (Fig. 5), while *pi21* or *Pi35* corresponded to significantly narrower lesions (Table 5). The width of lesions in rice lines with *pi21* + *Pi34* or *Pi34* + *Pi35* were comparable to those in rice lines containing the single resistance gene associated with narrower lesions, *pi21* and *Pi35*, respectively (Fig. 5). However, the line with *pi21* + *Pi35* formed narrower lesions than that containing either *pi21* or *Pi35* alone (Fig. 5), despite detection of a negative interaction between *pi21* and *Pi35* by ANOVA (Table 5).

Percentage of diseased leaf area. The mean percentage of diseased leaf area in Koshihikari was approximately 60% (Fig. 6), while diseased leaf area in lines with only *pi21*, *Pi34*, or *Pi35* was approximately 29, 52, or 15%, respectively (Fig. 6) (all significantly lower than Koshihikari) (Fig. 6). The percentages of diseased leaf area in the rice lines with *pi21* + *Pi34* were significantly lower than that in the line with *pi21* only, which in turn had a lower percentage of diseased leaf area compared with the line with *Pi34* only (Fig. 6). Similarly, the percentage of diseased leaf area in the line with *pi21* + *Pi35* was significantly lower than that in the line with *Pi35* only, which showed less diseased area than the line with *pi21* only (Fig. 6). However, the percentage of diseased leaf area in the line with *Pi34* + *Pi35* was comparable to that in the line with *Pi35*, and these two lines had less diseased leaf area than the line containing *Pi34* (Fig. 6). Negative interactions between two of the combined resistance genes were detected, indicating that the effect of these genes in combination was smaller than the sum of the individual effects of the genes (Table 6).

Discussion

Quantitative blast resistance genes in rice permit the formation of sporulating lesions, but they have the potential to limit proliferation of the disease via specific components of partial resistance. Here, we

Table 3. Effects of individual resistance genes (*pi21*, *Pi34*, and *Pi35*) and their interactions on the number of lesions caused by the *Magnaporthe oryzae* isolate in comparison with the cultivar Koshihikari, evaluated using three-way mixed analysis of variance (ANOVA)

Sources	df	SS	F-value	P value ^a
<i>pi21</i>	1	139423.00	578.25	<0.0001*
<i>Pi34</i>	1	1102.08	4.57	0.0329*
<i>Pi35</i>	1	37518.70	155.61	<0.0001*
<i>pi21</i> $\times$ <i>Pi34</i>	1	2082.33	8.64	0.0034*
<i>pi21</i> $\times$ <i>Pi35</i>	1	18245.50	75.67	<0.0001*
<i>Pi34</i> $\times$ <i>Pi35</i>	1	9112.29	37.79	<0.0001*
Random effect	2	5780.79	11.99	<0.0001*
Error	566	136469.25		

^a Asterisk (*) indicates significance ($P < 0.05$).

found that the development of resistance varied among rice lines containing individual resistance genes. The *pi21* gene did not suppress elongation of infection hyphae 40 h after inoculation but did contribute to a reduction in lesion formation. Fukuoka et al. (7) observed that the rate of invasion of hyphae grown from penetrated cells into adjacent cells 48 h after inoculation was significantly lower in rice containing *pi21*. These results suggested that the growth of infection hyphae was reduced later than 40 h after inoculation, resulting in a decrease in the number of lesions in rice lines with *pi21*. After the formation of sporulating lesions on rice lines containing *pi21*, narrow lesions formed that were similar in length to the lesions on Koshihikari. In contrast to *pi21*, plants containing *Pi34* showed strongly reduced elongation of early infection hyphae and slightly suppressed lesion formation. Following the formation of sporulating lesions in rice lines containing *Pi34*, the length and width of lesions increased to a size that was comparable to those in Koshihikari. Unlike *pi21* and *Pi34*, rice containing *Pi35* showed consistently reduced numbers and expansion of lesions. None of the resistance genes had an effect on penetration frequency of the blast fungus.

Two types of combined gene interactions occurred and varied according to the specific gene combination and characteristic of the infection: (i) the combination of two resistance genes was more effective than either of the genes individually; or (ii) the combination of two resistance genes and the most effective of the two resistance genes were similarly effective.

In most cases, rice lines carrying pairs of resistance genes showed disease suppression comparable to that observed in lines containing the gene with the stronger suppressive effect. For example, the number of infected rice cells at 40 h after inoculation in lines with *pi21* + *Pi34* was comparable to that in the line with only *Pi34* alone, and the number of infected cells in the rice line with *pi21* + *Pi35* was comparable to that in the line with only *Pi35*. Moreover, the lesion number and percentage of diseased leaf area in the line with *Pi34* + *Pi35* were comparable to those in the line with *Pi35*. In these cases, the behavior of the quantitative resistance genes was similar to that of the qualitative resistance genes reported by Yasuda et al. (37) in that the stronger (or faster-acting) resistance genes were epistatic to the genes with weaker (or slower-acting) effects in rice cultivars containing two resistance genes.

On the other hand, the number of lesions and percentage of diseased leaf area in lines with *pi21* + *Pi34* and *pi21* + *Pi35* were significantly lower than those in the lines with the individual resistance genes. The number of infected rice cells at 40 h after inoculation in the rice line containing *Pi34* + *Pi35* was less than that in the line with *Pi34* alone. We propose the following explanations for the improved suppression of blast disease by pairs of resistance genes compared with the individual genes: (i) in a given tissue, rice plants with two resistance genes may express similar resistance to that shown in

plants with one resistance gene; (ii) the member of the gene pair with the stronger effect may be epistatic; (iii) the identity of the epistatic gene may vary according to the type of rice tissue; (iv) the ability of a plant to suppress blast is represented by the total resistance of rice tissues invaded by the fungus; and (v) rice lines carrying pairs of resistance genes may suppress blast disease more effectively than lines carrying a single resistance gene when the epistatic gene varies throughout the infection process. For example, in the rice line

Fig. 4. Lesion length (mean $\pm$ standard error) produced by *Magnaporthe oryzae* isolate at 20 days after modified spot inoculation. Values with the same letter are not significantly different (Tukey's honestly significant difference test, $P > 0.05$).

Table 4. Effects of individual resistance genes (*pi21*, *Pi34*, and *Pi35*) and their interactions on the length of lesions caused by the *Magnaporthe oryzae* isolate in comparison with the cultivar Koshihikari, evaluated using three-way mixed analysis of variance (ANOVA)

Sources	df	SS	F-value	P value ^a
<i>pi21</i>	1	478.04	6.12	0.0138*
<i>Pi34</i>	1	122.87	1.57	0.2105
<i>Pi35</i>	1	161802.00	2071.22	<0.0001*
<i>pi21</i> $\times$ <i>Pi34</i>	1	456.83	5.85	0.016*
<i>pi21</i> $\times$ <i>Pi35</i>	1	48.27	0.62	0.4323
<i>Pi34</i> $\times$ <i>Pi35</i>	1	33.69	0.43	0.5117
Random effect	2	93.86	0.60	0.5489
Error	424	33122.59		

^a Asterisk (*) indicates significance ($P < 0.05$).

Fig. 3. Lesions produced by *Magnaporthe oryzae* isolate at 20 days after modified spot inoculation on A, Koshihikari; B, *pi21*-NIL; C, *Pi34*-IL; D, *Pi35*-L; E, *pi21* + *Pi34*-NIL; F, *pi21* + *Pi35*-L; and G, *Pi34* + *Pi35*-L. Scale bars = 1 cm.

containing *pi21* + *Pi34*, the spread of infection hyphae may be suppressed by *Pi34* in epidermal cells, and subsequent elongation of hyphae may be suppressed by *pi21* in mesophyll cells; as a result, these plants had fewer lesions than plants containing *pi21* or *Pi34* alone. We suggest that *Pi34* was epistatic to *pi21* in epidermal cells, and that *pi21* was epistatic to *Pi34* in mesophyll cells.

Fig. 5. Lesion width (mean $\pm$ standard error) produced by *Magnaporthe oryzae* isolate at 20 days after modified spot inoculation. Values with the same letter are not significantly different (Tukey's honestly significant difference test, $P > 0.05$).

Table 5. Effects of individual resistance genes (*pi21*, *Pi34*, and *Pi35*) and their interactions on the width of lesions caused by the *Magnaporthe oryzae* isolate in comparison with the cultivar Koshihikari, evaluated using three-way mixed analysis of variance (ANOVA)

Sources	df	SS	F-value	P value ^a
<i>pi21</i>	1	10.11	88.60	<0.0001*
<i>Pi34</i>	1	0.21	1.88	0.1707
<i>Pi35</i>	1	12.89	112.99	<0.0001*
<i>pi21</i> $\times$ <i>Pi34</i>	1	0.20	1.75	0.1866
<i>pi21</i> $\times$ <i>Pi35</i>	1	1.90	16.64	<0.0001*
<i>Pi34</i> $\times$ <i>Pi35</i>	1	0.13	1.13	0.2892
Random effect	2	9.92	43.46	<0.0001*
Error	424	48.38		

^a Asterisk (*) indicates significance ($P < 0.05$).

Fig. 6. Percentage of diseased leaf area (mean $\pm$ standard error) infected by *Magnaporthe oryzae* isolate at 7 days after spray inoculation. Values with the same letter are not significantly different (Tukey's honestly significant difference test, $P > 0.05$).

In addition to the lesion number, lesion size of the *pi21* + *Pi35* rice line was smaller than that of the *Pi35* line. This is because lesion width of the *pi21* + *Pi35* line was narrower than that of the *pi21* line, and lesion length was comparable to that of *Pi35*. The most effective gene combination to decrease the percentage of diseased leaf area was *pi21* + *Pi35*.

The *pi21* is a recessive gene conferring resistance to blast disease, and its structure differs from those of common resistance genes with NBS-LRR structure, such as *Pi35* (8). Nakao et al. (22) demonstrated that the susceptible *Pi21* allele of rice enhanced the movement of infection hyphae from penetrated epidermal cells to adjacent mesophyll cells in nonhost *Arabidopsis*. Thus, the resistance mechanism of *pi21* is thought to differ from that of NBS-LRR-type resistance genes. Our results suggested that a combination of genes having different resistance mechanisms, such as *pi21* + *Pi35*, would confer strong resistance effects.

Epistatic effects of resistance gene pairs have been reported in other plant-pathogen interactions. The effector Avr1, from *Fusarium oxysporum* f. sp. *lycopersici*, triggers disease resistance in host tomato plants that carry its resistance gene (*I* or *I-1*) in a gene-for-gene manner. However, Avr1 suppresses the effects of other resistance genes (*I-2* and *I-3*) (11). The *avrPphC* gene of *Pseudomonas syringae* pv. *phaseolicola* blocked the avirulence activity of *avrPphF* in the Red Mexican bean cultivar (34). A detailed scenario of epistasis was presented in the *Arabidopsis*-*Pseudomonas* interactions. The effector AvrRpt2 from *P. syringae*, which corresponds to the resistance gene *RPS2*, was found to interfere with AvrB- and AvrRpm1-mediated activation of another resistance gene, *RPM1*, in *Arabidopsis* because AvrRpt2 cleaves RIN4, the target protein of AvrB and AvrRpm1 (2,19,20).

Even if there is little interaction among the resistance and effector genes, the highly overlapping signaling network that characterizes resistance might help to explain the epistasis. For example, three resistance genes (*RPP4*, *RPP7*, and *RPP8*) against *Peronospora parasitica* in *Arabidopsis* triggered upregulation of common sets of genes, although these three genes differ in their signaling shortly after the recognition event (4). In addition to resistance gene-mediated responses, generally called effector-triggered immunity (ETI), plants express a basal defense of pattern-triggered immunity (PTI) (13). A recent study demonstrated that at least some cases of PTI and ETI depend on common elements of the signaling machinery (9). Katagiri and Tsuda (14) proposed that PTI and ETI result not from differences in the nature of the signaling machinery, but from differences in how the common signaling machinery is used.

In rice-*M. oryzae* interactions, the transcriptional profile at 24 h after inoculation of *Pi1*-mediated ETI was similar to that of *Pi9*-mediated ETI (35). Moreover, the transcriptional profile of PTI was similar to those of *Pi1*- and *Pi9*-mediated ETI but the amplitude of changes in gene expression were lower overall (35). These results may suggest PTI and ETI to rice blast also use common signaling machinery in different ways. Katagiri and Tsuda (14) proposed that after perception of a pathogen attack, plants use the common network differently and that different input signals can lead to different mechanisms of signal processing in the network. The epistatic output of the resistance response triggered by resistance gene pairs shown here might be produced

Table 6. Effects of individual resistance genes (*pi21*, *Pi34*, and *Pi35*) and their interactions on the percentage of diseased leaf area caused by the *Magnaporthe oryzae* isolate in comparison with the cultivar Koshihikari, evaluated using three-way mixed analysis of variance (ANOVA)

Sources	df	SS	F-value	P value ^a
<i>pi21</i>	1	5.51	305.76	<0.0001*
<i>Pi34</i>	1	0.71	39.29	<0.0001*
<i>Pi35</i>	1	8.27	458.69	<0.0001*
<i>pi21</i> $\times$ <i>Pi34</i>	1	0.20	11.09	0.0009*
<i>pi21</i> $\times$ <i>Pi35</i>	1	1.05	58.03	<0.0001*
<i>Pi34</i> $\times$ <i>Pi35</i>	1	0.17	9.57	0.0021*
Random effect	2	0.52	14.34	<0.0001*
Error	550	9.92		

^a Asterisk (*) indicates significance ($P < 0.05$).

through signal processing in the common network. Further studies on the molecular mechanisms of individual blast resistance genes will help to clarify positive and negative combinations of resistance genes.

For application to the development of blast-resistant cultivars, the most effective gene combination for reducing the percentage of diseased leaf area was *pi21 + Pi35*. The effectiveness of the combination of *pi21 + Pi34 + Pi35* in suppressing blast disease would presumably be comparable to that of *pi21 + Pi35*, because the extent of diseased leaf area in the *Pi34 + Pi35* line was comparable to that in the line containing *Pi35*. However, various factors, including plant age, temperature, insolation, humidity, and agronomic conditions, might influence the effectiveness of resistance genes. Further quantitative studies, implemented under different environmental conditions, should be performed to clarify the mechanisms and effectiveness of gene combinations that confer resistance to rice blast.

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