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Pyramiding of Four Blast Resistance QTLs into Thai Rice Cultivar RD6 through Marker-assisted Selection

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Abstract

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Thai rice cultivar RD6 is well known for its cooking and eating qualities. However it is susceptible to blast disease, a major rice disease caused by the fungus *Magnaporthe oryzae*. This study focused on the pyramiding of four QTLs for blast resistance located on chromosomes 1, 2, 11 and 12, from two RD6 introgression lines. Marker-assisted selection was performed and facilitated the selection with 8 microsatellite flanking markers to enable the selection in $BC_2F_{2:3}$ lines. All possible combinations of the four QTL alleles were assessed for blast resistance by artificial inoculation using 8 diverse isolates in a greenhouse and under field conditions using the upland short row method. The results showed that the RD6 introgression lines carrying a high number of QTLs for blast resitance achieved from pyramiding have high levels of blast resistance and broad spectrum of resistance to the blast pathogens prevalent in the region. Only one of the *M. oryzae* isolates, THL185, was virulent to all the breeding lines, suggesting that the identification of new blast resistance genes or QTLs and pyramiding them into RD6 for durable blast resistance and no yield penalty should be the focus of further research.

Keywords: broad spectrum; introgression lines; *Magnaporthe oryzae*; *Oryza sativa*; severity index

The rice cultivar RD6 has high cooking and eating qualities and rich aroma. It is widely grown through the North and Northeastern regions of Thailand. However, this cultivar is usually susceptible to blast disease, one of the diseases of rice that is widespread in Asia and Africa (Liu *et al.* 2010). Rice blast is caused by the fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*). The severity of rice blast is dependent on many factors, including cultural practices, cultivars, climate and nitrogen fertilizer (Ou 1985). The disease can reduce grain yield by up to 90% (Khush & Jena 2009).

The utilization of host resistant rice cultivars is the most economical and efficient strategy to control this disease. Blast resistance in rice is controlled by multiple genetic loci (Saka 2006). Currently, more than 90 disease resistance genes and 347 QTLs have been identified for blast resistance (Liu *et al.* 2010) and used for rice variety improvement. However, the cultivation of varieties with single resistant genes on a large scale may enable the pathogen to overcome blast resistance in the longer term. This possibility can potentially be delayed by the introgression of multiple resistant genes through gene pyramiding in rice cultivars, to enable rice varieties to achieve broad-spectrum resistance (Servin *et al.* 2004).

Due to the genetic basis of blast disease resistance, phenotypic selection is compromised by many factors. The availability of molecular markers, along with marker-assisted selection (MAS) strategies, is essential for the development of rice varieties with durable blast resistance against different races of *M. oryzae* (Ashkani *et al.* 2012). The pyramiding of three blast R genes, *Pi1*, *Piz-5* and *Pita-2*, into cultivars

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was undertaken to provide broad-spectrum resistance to many isolates of *M. oryzae* (Hittalmani *et al.* 2000). Moreover, the pseudo-backcrossing approach is the method for increasing the recurrent genetic background of the pyramiding population (Rueng-phayak *et al.* 2015). The objective of this study was to determine the resistance level conferred by four QTLs in an introgression line by pyramiding of blast resistance QTLs into the rice cultivar RD6 to provide a variety with durable blast resistance in Thailand.

MATERIAL AND METHODS

Plant materials and marker-assisted selection for blast resistance QTLs

The two RD6 near isogenic lines (NILs), NIL RD6 (2, 12) and NIL RD6 (1, 11), were used for the development of the pyramided lines. Both NILs maintained the genetic background of the RD6 variety by MAS (Figure 1). NIL RD6 (2, 12) harbour the resistance QTLs on chromosomes 2 and 12 from the P0489 recombinant line (Theeraamphon, personal communication). P0489, a recombinant line derived from Azucena × IR64, had been identified as a blast resistant line due to carrying QTLs on chromosomes 2 and 12 (Silprakhon 2004). NIL RD6 (1, 11) contained the major resistance QTLs located on chromosomes 1 and 11 from the Jao Hom Nil (JHN) variety (Noenplab *et al.* 2006). Both of the RD6 NILs lines were crossed for the development of the pyramided lines. The F_1 plants (containing the QTLs located on chromosomes 1, 2, 11 and 12) were crossed with RD6 (the recurrent parent of both NILs) through $BC_2F_{2:3}$ and MAS (Figure 1) with flanking SSR markers was used, following Korinsak (2009) (Table 1). Selected lines containing all possible QTL combinations were classified into 16 groups and subsequently evaluated for blast resistance.

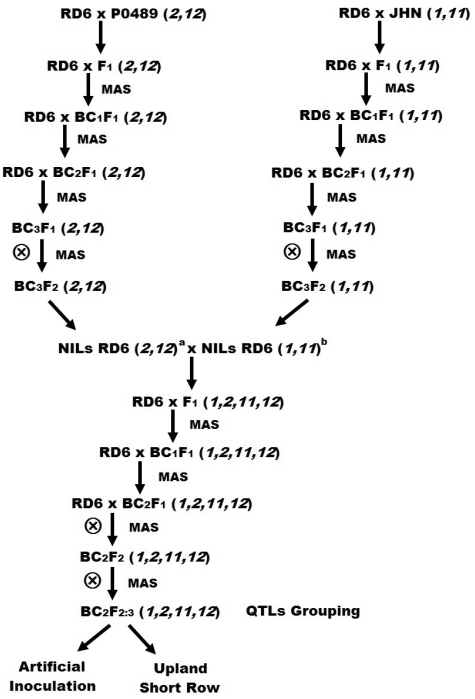

Figure 1. Breeding scheme of gene pyramiding for improved RD6 NILs resistant to rice blast disease through marker-assisted selection (MAS) with flanking markers located on chromosomes 2/12 and 1/11, respectively ([a]Theeraamphon, personal communication; [b]Noenplab *et al.* 2006)

Phenotypic evaluation of resistance to *M. oryzae*

Greenhouse conditions. Eight virulent *M. oryzae* isolates from diverse outbreak areas (Figure 2) (Sirithanya *et al.* 2008) were used for evaluation against 16 combination groups of resistance QTLs. Fungal isolates used in the pathogenicity test were grown on rice flour agar media (Korinsak 2009). The $BC_2F_{2:3}$ population, the parents and control varieties were laid out in a randomized complete block design (RCBD) with three replications, in the wet season (August to October) of 2010 at Khon Kaen University, Khon Kaen, Thailand. At 21 days after sowing (DAS), the plants

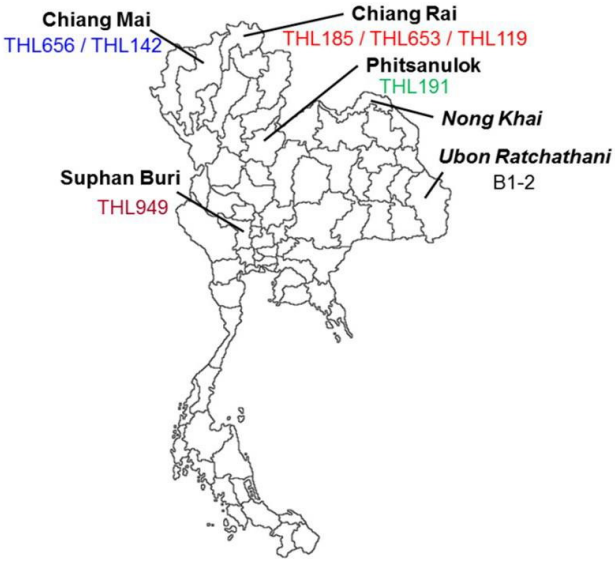

Figure 2. Distributions of 8 *Magnaporthe oryzae* isolates used for artificial inoculation in greenhouse conditions (the provinces in italics were the location for later upland short row tests)

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were inoculated with single isolate spore suspensions (5×10^5 spore/ml). The inoculated seedlings were kept in an incubation chamber at 26 ± 1°C for 24 h, under conditions of saturated humidity; they were subsequently transferred to growth chambers until disease scores were recorded. The reactions to the disease were scored on a scale from 0 (no lesions) to 6 (coalescence of > 4 mm lesions or 50% of the leaves killed and without dark margins) using a standard evaluation method (Roumen *et al.* 1997).

Field conditions. The $BC_2F_{2:3}$ lines and control varieties were planted in RCBD with two replications during the wet season of 2010 in experimental fields in the provinces Nong Khai and Ubon Ratchathani in Northeast Thailand. In each experiment, seed of each genotype was sown in a single row 1 m long with a 30 cm inter-row spacing. Fourteen days prior to planting, the highly susceptible rice cultivar KDML105 was sown as a trap around and between the experimental plots. At 30 DAS the infection on the leaves of each plant was scored for the blast reaction on a scale of 0–9 (IRRI 1996).

Data analysis

Analysis of variance of the rice blast disease scores data was done. Mean and SD of the blast scores for each line and variety were calculated, and the means of all lines were compared by Tukey's HSD using the R program Version 2.10 (R Development Core Team 2008). Based on the blast score, the severity index (SI) was estimated using the formula reported by Sirithanya (1998). Then the broad-spectrum resistance (BSR) was calculated using the formula of Ahn (1994). In addition, the probability of orthogonal comparison between the groups of $BC_2F_{2:3}$ populations carrying different combinations of the four QTLs, including the control and control varieties, was examined using all isolates with the exception of B1-2, an *M. oryzae* isolate from Ubon Ratchathani province.

RESULTS

Reaction to blast disease. The $BC_2F_{2:3}$ progenies of each of the RD6 lines homozygous for each of the single QTLs and those having different combinations, together with the control varieties, were screened for their reaction to the eight *M. oryzae* isolates prevalent in Thailand. The ANOVA showed a significant difference between the introgression lines with differences among the segments of QTLs (data not shown). The mean comparisons are shown in Figure 3. The JHN cultivar showed more resistance to the *M. oryzae* isolates (with an average blast

Table 1. Specific PCR primers used for the identification of four different QTLs with resistance to *Magnaporthe oryzae*

Marker	Chromosome	Sequence	Specific alleles for individual QTLs (bp)		
			P0489	JHN	RD6
RM319	1	5'ATCAAGGTACCTAGACCACCAC 3' 3'TCCTGGTGCAGCTATGTCTG 5'	–	123	120
RM212	1	5'CCACTTTCAGCTACTACCAG3' 3'CACCCATTTGTCTCTCATTATG5'	105	–	110
RM48	2	5'TGTCCCACTGCTTTCAAGC3' 3'CGAGAATGAGGGACAAATAACC5'	225	–	220
RM207	2	5'CCATTCGTGAGAAGATCTGA3' 3'CACCTCATCCTCGTAACGCC5'	170	–	180
RM224	11	5'ATCGATCGATCTTCACGAGG3' 3'TGCTATAAAAGGCATTCGGG5'	–	85	90
RM144	11	5'TGCCCTGGCGCAAATTTGATCC3' 5'GCTAGAGGAGATCAGATGGTAGTGCATG3'	–	130	125
RM313	12	5'TGCTACAAGTGTTCTTCAGGAC3' 3'GCTCACCTTTTGTGTTCCAC5'	120	–	115
RM277	12	5'CGGTCAAATCATCACCTGAC3' 3'CAAGGCTTGCAAGGGAAG5'	120	–	130

– not amplified

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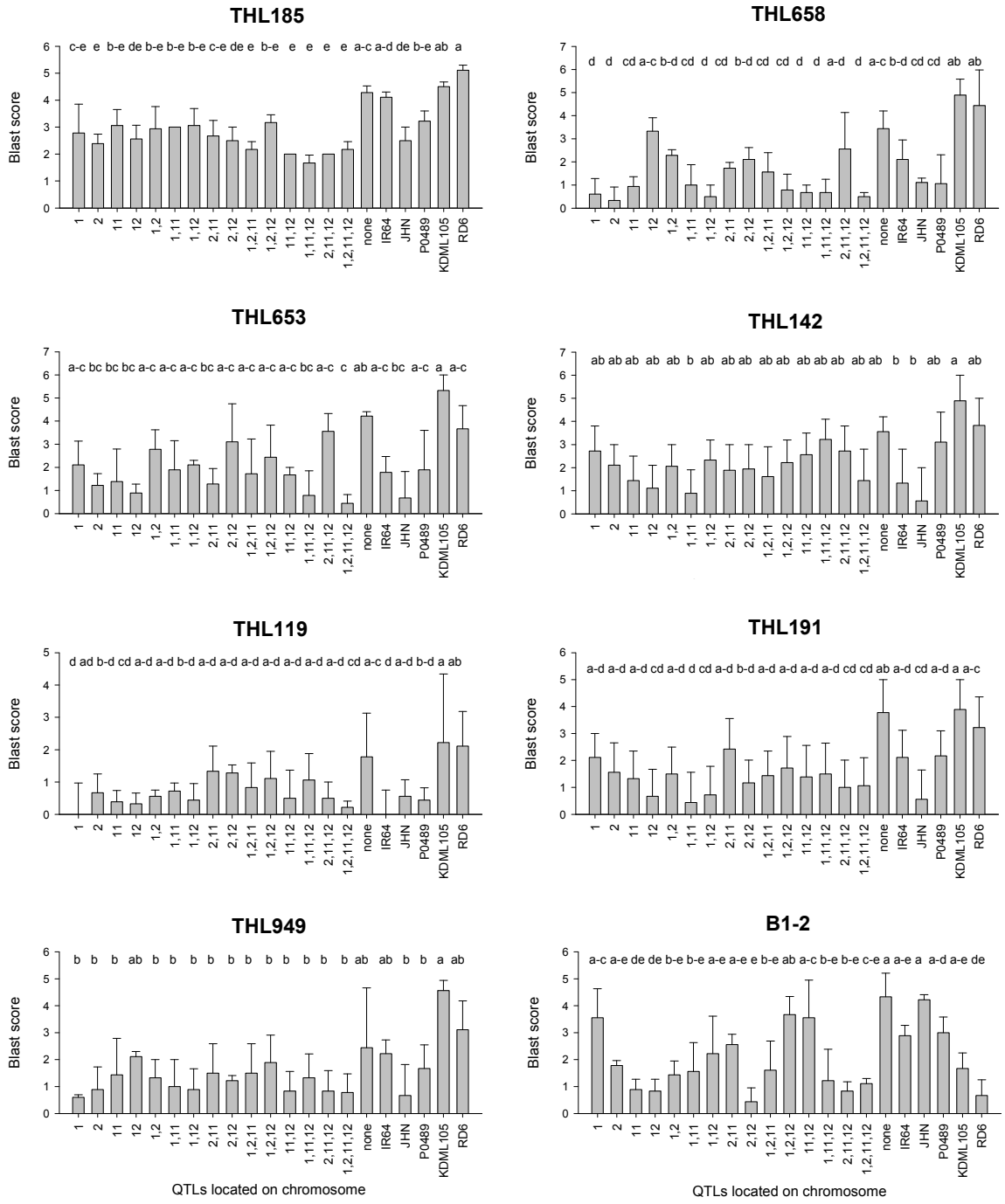

Figure 3. Rice blast disease scores of RD6 introgression NILs containing 15 combinations of rice blast resistance QTLs and their parents and control varieties; different small letters indicate that are significantly different at the 95% confidence level using Tukey's HSD

score of 1.35) than P0489 (with an average blast score of 2.07), while RD6 was very susceptible to the *M. oryzae* isolates (with an average blast score of 3.27). The RD6 introgression lines with single QTLs on chromosome 11 provided more effective resistance against the predominant *M. oryzae* isolates in Thailand, as reflected in the resistant reaction (with blast scores < 2.00) to 7 isolates, whereas lines with a single QTL on chromosomes 1, 2 and 12 showed a resistant reaction to 3, 6 and 5 isolates, respectively.

In this study, the RD6 introgression lines with both single QTL and combinations of resistance QTLs showed resistance to the *M. oryzae* isolates. However, the THL185 isolate produced the high blast

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Table 2. Severity index (SI) and broad-spectrum resistance (BSR) of NILs of RD6 introgression lines containing 15 combinations of rice blast resistance QTLs, their parents and control varieties

Lines, varieties	QTLs located on chromosome	SI and reaction to rice blast disease[a]								BSR
		THL185	THL658	THL653	THL142	THL119	THL191	THL949	B1-2	
BC_2F_2 9-1/15-1-33	1	39.46 (MR)	8.03 (R)	33.74 (MR)	26.60 (MR)	0.54 (R)	29.46 (MR)	8.03 (R)	50.83 (MS)	0.38
BC_2F_2 16-1/15-38	2	46.60 (MS)	30.89 (MR)	22.32 (MR)	20.89 (MR)	9.46 (R)	32.32 (MR)	13.75 (R)	48.03 (MS)	0.25
BC_2F_2 6-1/15-1-50	11	43.73 (MS)	12.32 (R)	9.46 (R)	19.46 (R)	5.17 (R)	12.32 (R)	15.17 (R)	12.32 (R)	0.88
BC_2F_2 6-1/15-1-43	12	36.60 (MR)	46.60 (MS)	12.32 (R)	15.17 (R)	3.75 (R)	9.46 (R)	28.03 (MR)	10.89 (R)	0.63
BC_2F_2 19-1/15-1-34	1, 2	40.89 (MS)	32.32 (MR)	39.46 (MR)	30.89 (MR)	8.03 (R)	20.89 (MR)	18.03 (R)	19.46 (R)	0.38
BC_2F_2 6-1/15-1-20	1, 11	42.32 (MS)	13.75 (R)	15.17 (R)	12.32 (R)	9.46 (R)	5.17 (R)	13.75 (R)	22.32 (MR)	0.75
BC_2F_2 6-1/15-1/16	1, 12	43.74 (MS)	6.60 (R)	23.74 (MR)	18.03 (R)	5.17 (R)	9.46 (R)	12.32 (R)	30.89 (MR)	0.63
BC_2F_2 9-1/15-1-31	2, 11	38.03 (MR)	23.74 (MR)	18.03 (R)	12.32 (R)	10.89 (R)	39.46 (MR)	20.89 (MR)	36.60 (MR)	0.38
BC_2F_2 6-1/15-2-24	2, 12	35.17 (MR)	29.46 (MR)	29.46 (MR)	26.6 (MR)	18.03 (R)	23.74 (MR)	16.60 (R)	5.17 (R)	0.38
BC_2F_2 6-1/15-1-22	1, 2, 11	30.89 (MR)	22.32 (MR)	12.32 (R)	10.89 (R)	9.46 (R)	18.03 (R)	16.60 (R)	22.32 (MR)	0.63
BC_2F_22 6-1/15-1-41	1, 2, 12	45.17 (MS)	10.89 (R)	19.46 (R)	15.17 (R)	10.89 (R)	23.74 (MR)	16.60 (R)	52.31 (MS)	0.63
BC_2F_2 6-1/15-1-13	11, 12	28.03 (MR)	9.46 (R)	23.74 (MR)	36.6 (MR)	6.60 (R)	19.46 (R)	10.89 (R)	50.89 (MS)	0.50
BC_2F_2 16-1/15-1-9	1, 11, 12	23.74 (MR)	9.46 (R)	5.17 (R)	26.60 (MR)	15.17 (R)	16.60 (R)	18.03 (R)	16.60 (R)	0.75
BC_2F_2 19-1/15-2-4	2, 11, 12	28.03 (MR)	36.60 (MR)	30.89 (MR)	19.46 (R)	6.60 (R)	18.03 (R)	10.89 (R)	16.60 (R)	0.63
BC_2F_2 9-1/15-1-27	1, 2, 11, 12	30.89 (MR)	6.60 (R)	5.17 (R)	19.46 (R)	2.32 (R)	16.60 (R)	10.8 (R)	15.17 (R)	0.88
Control BC_2F_2	none	60.89 (S)	48.03 (MS)	59.46 (MS)	59.46 (MS)	25.17 (MR)	59.46 (MS)	52.31 (MS)	55.17 (MS)	0.00
IR64	R control	58.03 (MS)	19.46 (R)	20.17 (MR)	10.89 (R)	0.54 (R)	19.46 (R)	30.89 (MR)	40.89 (MS)	0.50
JHN	donor (1, 11)	35.17 (MS)	15.17 (R)	3.75 (R)	8.03 (R)	8.03 (R)	8.03 (R)	9.46 (R)	59.46 (MS)	0.75
KDML105	S control	63.74 (S)	69.46 (S)	76.60 (S)	69.46 (S)	30.89 (MR)	59.46 (MS)	65.17 (S)	28.03 (MR)	0.00
P0489	donor (2, 12)	45.17 (MS)	8.03 (R)	36.60 (MR)	32.32 (MR)	5.17 (R)	20.89 (MR)	19.74 (R)	39.46 (MR)	0.38
RD6	recurrent	72.31 (S)	73.74 (S)	56.60 (MS)	62.31 (S)	29.46 (MR)	50.89 (MS)	43.74 (MS)	13.75 (R)	0.13

[a]HR – highly resistant (SI = 0); R – resistant (0 < SI ≤ 20); MR – moderately resistant (20 < SI ≤ 40); MS – moderately susceptible (40 < SI ≤ 60); S = susceptible (60 < SI ≤ 80), S – susceptible (80 < SI ≤ 100)

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score in all lines, indicating that the THL185 isolate was virulent to all four resistance QTLs (Figure 3). Surprisingly, B1-2 isolate, the *M. oryzae* isolate from Ubon Ratchathani province, produced blast in all check varieties contrary to the expectations. The resistant control varieties and donor parents had high blast scores, while the susceptible control varieties and recurrent parent had low blast scores. However, most of the backcross lines showed resistance to the B1-2 isolate. The results indicate that RD6 might have transferred the resistance genes against *M. oryzae* isolate B1-2 into the backcross lines.

Based on the severity index (SI) and BSR values, JHN and P0489, the donor parents, had a low average SI (18.39 and 25.92) but a high BSR value (0.75 and 0.38). In contrast, RD6 had a high average SI (50.35) and low BSR value (0.13) (Table 2). The RD6 introgression lines with single QTLs on chromosome 11 showed the highest resistance reaction, followed by lines with a single QTL on chromosomes 12, 1 and 2, respectively. Therefore, the RD6 introgression lines with combinations of QTLs on chromosome 11 showed higher values than lines carrying single QTLs. The results indicate that the lines with a combination of QTLs located on chromosomes 11 and 12 were more effective in providing resistance against the pathogen than single QTLs. However, the BSR of lines with combinations of 4 resistance QTLs did not have greater BSR than the line with a single QTL on chromosome 11. These results indicate that the BSR cannot be extended beyond the genetic background of the parents. The BSR can be confirmed by the orthogonal comparison between lines with different combinations of blast QTLs (Table 3). The results also support the fact that the QTL effects on chromosome 11 are greater than on the other chromosomes. However, based on blast scores and SI values, the results suggest that the lines with combination of QTLs had greater resistance against the pathogen than a single QTL (Figure 3 and Tables 2, 3 and 4).

Field evaluation of resistance to *M. oryzae*. The $BC_2F_{2:3}$ progenies were evaluated under field conditions by using the upland short row method. Introgression lines carrying single QTLs and pyramid combinations of the QTLs were grown in two rows in two locations (in Nong Khai and Ubon Ratchathani provinces) (Table 4). Based on the results, the virulence of blast disease is greater in Ubon Ratchathani province than in Nong Khai province. The QTL on chromosome 11 was the most effective single locus against the naturally prevalent pathogen in both regions. The blast score at different sites for all the tested lines showed the same response, indicating that the QTLs on chromosomes 1, 2, 11 and 12 are broadly effective against blast disease in both regions

Table 3. Orthogonal comparison of introgression lines carrying different numbers of QTLs

Class comparisons	Rice blast score	Probability
1QTL : 2QTLs	1.466 : 1.620	0.236
1QTL : 3QTLs	1.466 : 1.744	0.052
1QTL : 4QTLs	1.466 : 0.944	0.022
2QTLs : 3QTLs	1.620 : 1.744	0.334
2QTLs : 4QTLs	1.620 : 0.944	0.003
3QTLs : QTLs	1.744 : 0.944	< 0.001
JHN : 4QTLs	0.944 : 0.944	1.000
P0489 : 4QTLs	1.937 : 0.944	< 0.001
KDML105 : 4QTLs	4.325 : 0.944	< 0.001
RD6 : 4QTLs	3.643 : 0.944	< 0.001
IR64 : 4QTLs	1.918 : 0.944	0.001

Table 4. Rice blast disease scores of resistant RD6 introgression QTLs and control varieties tested under field conditions in Nong Khai and Ubon Ratchathani provinces

Lines, varieties	QTLs located on chromosome	Rice blast severity score (0–9)	
		Nong Khai	Ubon Ratchathani
BC2F2 9-1/15-1-33	1	3	5
BC2F2 16-1/15-38	2	3	4.5
BC_2F_2 6-1/15-1-50	11	2	5
BC2F2 6-1/15-1-43	12	2.5	5.5
BC_2F_2 19-1/15-1-34	1, 2	8	6
BC_2F_2 6-1/15-1-20	1, 11	5	4
BC_2F_2 9-1/15-1-31	2, 11	3	6.5
BC_2F_2 6-1/15-2-24	2, 12	8	7
BC_2F_2 6-1/15-1-22	1, 2, 11	4.5	4.5
$BC_2F_2$2 6-1/15-1-41	1, 2, 12	4	5.5
BC_2F_2 9-1/15-1-27	1, 2, 11, 12	3	5.5
Hangyi71	R control	3	3
KDML105	S control	9	9
RD23	R control	1	9
Supanburi60	R control	1	1
RD7	R control	1	9

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(Table 4). Again, surprisingly, in Ubon Ratchathani province, the two resistant control varieties (RD23 and RD7) showed susceptibility to blast disease, whereas in Nong Khai province they showed resistance (Table 4).

DISCUSSION

Although, work on improving the blast resistance of rice varieties in Thailand has been undertaken for several decades, to date no commercial cultivars with high stability of resistance over years and seasons have become available. In this study, success was demonstrated in improving blast resistance through pyramiding of respective QTLs in the cultivar RD6, using marker-assisted pseudo-backcrossing. The introgression lines showed a high level of resistance when they possessed a high number of QTLs for resistance.

Historically, several major blast resistance genes *Pib*, *Pita*, *Pia*, *Pi1*, *Pikh*, *Pi2* and *Pi4* have been introduced into rice varieties for blast resistance, through conventional breeding programs (Koizumi 2007). The rice cultivar Koshihikari, which is widely cultivated in Japan, was developed for blast resistance by crossing with several resistance genes including *Pia*, *Pii*, *Pita-2*, *Piz*, *Pik*, *Pik-m*, *Piz-t* and *Pib* (Ishizaki *et al*. 2005). Various strategies in conventional breeding programs have been used in rice variety improvement, including the combining of several genes to provide durable resistance. However, a major disadvantage of the conventional breeding methods is that it is time consuming, leading to a considerable time lag between the emergence of virulent pathotypes of the causal pathogen and the development of new resistant cultivars (Miah *et al*. 2013). In addition, the pyramiding of blast resistance genes with similar reactions to more than one race or isolate, using conventional breeding methods, is difficult due to the dominance and epistasis effects of genes. Currently, molecular markers closely linked to blast resistance genes/QTLs have been widely used for MAS to improve the resistance of rice varieties (Arunakanthi *et al*. 2008).

In this study, success was achieved in the pyramiding of four blast resistance QTLs with similar reactions to more than one isolate. This is difficult to achieve, using conventional breeding methods. The reaction of the pyramided lines to almost all isolates can be explained by the additive effect of QTLs on chromosomes 1, 2, 11 and 12 (Figure 3, Tables 2 and 3). Similarly to the results reported by Koide *et al*. (2009), it was found that there was no reduction in the resistance caused by combining the resistance QTLs. This suggests that the pyramiding of QTLs is potentially useful for improving the blast resistance of RD6. Based on the disease resistance reaction, if the blast resistance genes have similar reactions to more than one race or dominant isolate, the epistatic effect of the genes could affect the disease resistance (Korinsak 2009) that is shown in Figure 3 and Tables 2, 3 and 4, in which some single QTLs are more resistant than the QTL combinations. However, the use of a single QTL as a source of resistance may be more easily overcome by the pathogen, which can be explained by the gene-for-gene theory (Flor 1971). Moreover, the high genetic variation in the blast fungus may be the main reason for numerous resistance genes having evolved and been identified in rice (Koide *et al.* 2009). In this study, there were no parents or breeding lines with resistance to the THL185 isolate. Therefore, the identification of new sources of blast resistance would be necessary for the further variety improvement against this *M. oryzae* isolate.

Interestingly, B1-2 isolate, the *M. oryzae* isolate from Ubon Ratchathani province, showed virulence to all resistant parents, while it was avirulent to varieties RD6 and KDML105. According to the pedigree of RD6, this variety was derived from KDML105 by gamma radiation, with selection in the M_2 population for glutinous and blast-resistant mutants (Khambanonda 1978). The gene with resistance to the *M. oryzae* isolate B1-2 of RD6 and KDML105 is governed by an isolate specific QTL on chromosome 8 (Korinsak 2009).

The evaluation of the RD6 introgression lines with pyramided genes for resistance to the blast pathogen under field conditions was accomplished by using the upland short row method. Natural infection occurred in both locations. However, the pathogens in Ubon Ratchathani province are more virulent than in Nong Khai province, which indicates the prevalence of a highly virulent isolate in the former province (Table 4). Unfortunately, the parental lines P0489, RD6 and JHN were not included in this evaluation. However, the KDML105 variety, which has a very close genetic background to the RD6 recurrent parent, was used as the susceptible standard control. Broad-spectrum and durable resistance suggested in this case that most of the RD6 introgression lines are resistant to blast disease, especially the lines containing the QTLs on chromosome 11 which showed stable resistance in both locations (Table 4), confirming that the QTL on chromosome 11 has broad-spectrum resistance to the blast pathogen in Thailand. In addition, only one of the *M. oryzae* isolates, THL 185, was virulent to all of

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the breeding lines, suggesting that the identification of a new blast resistance gene or QTLs, and pyramiding them into RD6 for durable blast resistance and no yield penalty should be the focus of further research.

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