Development of breeding lines with three pyramided resistance genes that confer broad-spectrum bacterial blight resistance and their molecular analysis in rice

Jung-Pil Suh, Ji-Ung Jeung, Tae-Hwan Noh, Young-Chan Cho, So-Hyun Park, Hyun-Su Park, Mun-Sik Shin, Chung-Kon Kim and Kshirod K Jena*

Abstract

Background: The development of resistant cultivars has been the most effective and economical strategy to control bacterial leaf blight (BB) disease of rice caused by Xanthomonas oryzae pv. oryzae (Xoo). Molecular markers have made it possible to identify and pyramid valuable genes of agronomic importance in resistance rice breeding. In this study, three resistance genes (Xa4 + xa5 + Xa21) were transferred from an indica donor (IRBB57), using a marker-assisted backcrossing (MAB) breeding strategy, into a BB-susceptible elite japonica rice cultivar, Mangeumbyeo, which is high yielding with good grain quality.

Results: Our analysis led to the development of three elite advanced backcross breeding lines (ABL) with three resistance genes by foreground and phenotypic selection in a japonica genetic background without linkage drag. The background genome recovery of the ABL expressed more than 92.1% using genome-wide SSR marker analysis. The pathogenicity assays of three resistance-gene-derived ABL were conducted under glasshouse conditions with the 18 isolates of Xoo prevalent in Korea. The ABL exhibited very small lesion lengths, indicating a hypersensitive reaction to all 18 isolates of Xoo, with agronomic and grain quality traits similar to those of the recurrent parent. Pyramiding the resistance genes Xa4, xa5 and Xa21 provided a higher resistance to Xoo than the introduction of the individual resistance genes. Additionally, the combination of two dominant and one recessive BB resistance gene did not express any negative effect on agronomic traits in the ABL.

Conclusions: The strategy of simultaneous foreground and phenotypic selection to introduce multiple R genes is very useful to reduce the cost and the time required for the isolation of desirable recombinants with target resistance genes in rice. The resistance-gene-derived ABL have practical breeding value without a yield penalty by providing broad-spectrum resistance against most of the existing isolates of BB in South Korea and will have a high impact on the yield stability and sustainability of rice productivity.

Keywords: Rice, Bacterial leaf blight, Gene pyramiding, Marker-assisted breeding, Xa4, xa5, Xa21
Background

Bacterial leaf blight (BB), caused by *Xanthomonas oryzae pv. oryzae* (Xoo), is a devastating disease in the rice-growing countries of Asia. Infection at maximum tillering stage results in blighting of leaves, which eventually causes significant yield losses in severely infected fields ranging from 20 to 30%, but this can reach as high as 80% (Mew et al. 1992; Noh et al. 2007; Shin et al. 1992). Korean BB isolates have been grouped into five races (K1 to K5) by using five rice cultivars as the *Xoo* differential system (Yun et al. 1985). Recent pathotyping results indicated that the Korean race K1 has shown a decreasing trend in infection by the spread of rice cultivars with *Xa1* and *Xa3* genes, whereas races K2 and K3 have increased their pathogenicity in Korea (Kim et al. 2009; Noh et al. 2007; Shin et al. 1992). Most of the japonica cultivars possess *Xa1* or *Xa3* or *Xa4* genes for BB resistance, but these genes are showing susceptibility to the new BB strains of Korea (Jeung et al. 2006; Kim et al. 2009; Shin et al. 2011). A new BB race, K3a, that evolved recently caused serious damage to rice production in the southwestern coastal areas of Korea in 2003 (Noh et al. 2003). Moreover, BB disease is spreading to all regions of Korea because of the effect of climate change and it is causing genetic vulnerability in modern cultivars. Therefore, rice yield has declined and grain quality has decreased by the infection of bacterial blight (Noh et al. 2007; Shin et al. 1992).

Breeding and the development of resistant cultivars carrying major resistance (*R*) genes have been the most effective and economical strategy to control BB disease to have a neutral effect on the environment (Huang et al. 1997; Jena and Mackill, 2008; Singh et al. 2001). Qualitative resistance, which confers major gene-specific resistance against some pathogen races, is the easiest to incorporate into breeding programs and is usually considered a gene-for-gene type of resistance. For many pathogens and insects, this type of qualitative resistance is not often durable because of rapid changes in the virulence in the pathogen or biotype of the population (Leach et al. 2007). As a result, increasing attention has focused on the accumulation of major disease resistance genes in crop plants. Pyramided lines carrying two, three or four bacterial blight resistance genes showed broad-spectrum and higher resistance than the lines with a single resistance gene (Gu et al. 2005; Jeung et al. 2006; Kim et al. 2009; Singh et al. 2001; Suh et al. 2009a). However, conventional breeding methods to improve rice cultivars for BB resistance have not found much success (Shin et al. 2011).

To date, at least 38 BB resistance genes conferring host resistance against various strains of *Xoo* have been identified (Bhasin et al. 2012; Natrajkumar et al. 2012). All these resistance genes follow a Mendelian pattern of major gene inheritance and express resistance to a diverse group of *Xoo* pathogens (Cheema et al. 2008; Gu et al. 2005; Korinsak et al. 2009; Lee et al. 2003; Sun et al. 2004). Several of these genes have already been incorporated into rice cultivars, which are now widely cultivated in many countries (Huang et al. 1997; Singh et al. 2001; Sundaram et al. 2008). Of the 38 *R* genes, six are physically mapped (*Xa2, Xa4, Xa7, Xa30, Xa33 and Xa38*) and six are cloned (*Xa1, xa5, xa13, Xa21, Xa26 = Xa3 and Xa27*) (Bhasin et al. 2012; Cheema et al. 2008; Gu et al. 2005; Liu et al. 2006; Natrajkumar et al. 2012; Song et al. 1997; Sun et al. 2003; Yang et al. 1998). BB resistance gene *Xa4* is one of the most widely exploited resistance genes in many rice breeding programs and it confers durable resistance in many commercial rice cultivars (Mew et al. 1992; Sun et al. 2003). The *Xa21* gene was identified in the wild species *Oryza longistaminata* and is highly effective against BB races of South and Southeast Asia (Khush et al. 1990). The *xa5* gene, which is naturally found only within the *Aussubpopulation of rice* (Garris et al. 2003), provides recessive resistance to several *Xoo* races of the Philippines.

Molecular markers can be used to identify and pyramidal favorable (or deleterious) and multiple alleles for biotic and abiotic stress resistance in a collection of diverse genotypes (Jena and Mackill, 2008; Lee et al. 2003; Singh et al. 2001; Suh et al. 2009a). Marker-assisted selection (MAS) for pyramiding important genes along with rapid background recovery of the recurrent parent, while maintaining the exquisite quality characteristics of rice, could be an effective approach for rice improvement (Shanti et al. 2010; Singh et al. 2001; Suh et al. 2009a; Suh et al. 2011; Sundaram et al. 2008; Xu and Crouch, 2008; Ye 2010). Gene pyramiding is difficult using conventional breeding methods due to the dominance and epistasis effects of genes governing disease resistance. Moreover, genes with similar reactions to two or more races are difficult to identify and transfer through conventional approaches (Joseph et al. 2004; Rajpurohit et al. 2011; Sundaram et al. 2009). However, the availability of molecular markers closely linked to each of the resistance genes makes the identification of plants with two and three genes possible (Shanti et al. 2010; Singh et al. 2001; Sundaram et al. 2008). Three BB resistance genes (xa5, xa13 and Xa21) were pyramided in cultivar PR106 using MAS. Testing with 17 *Xanthomonas oryzae pv. oryzae* (Xoo) isolates under artificial inoculation and field conditions showed that the combination of genes provided a wider spectrum of resistance to the pathogen populations prevalent in the region (Singh et al. 2001). In a previous study, the IR24 NILs (IRBB lines) containing *Xa4, xa5, Xa7* and *Xa21* genes and their combinations conferred different degrees of resistance to K1, K2, K3 and K3a races in a field.
inoculation experiment in Korea (Jeung et al. 2006; Kim et al. 2009; Suh et al. 2009a). The resistance gene pyramid of $Xa4 + xa5 + Xa21$ would be the most effective strategy for improving Korean japonica cultivars for BB resistance (Jeung et al. 2006; Kim et al. 2009). The identification of closely linked markers has also enabled pyramiding of $Xa4$, $xa5$ and $Xa21$ using MAB.

This study reports a successful transfer of bacterial leaf blight resistance genes $Xa4$, $xa5$ and $Xa21$ from indica rice into an elite japonica rice cultivar using MAB and marker-assisted background analysis of selected BC progenies using SSR markers.

**Results**

**Transferring BB resistance genes by MAB**

$F_1$ plants with heterozygous alleles of the three BB resistance genes ($Xa4$, $xa5$ and $Xa21$) were obtained from the cross of Mangeumbyeo and IRBB57. They were confirmed for their heterozygosity by DNA analysis of markers linked with the three R genes and were backcrossed with Mangeumbyeo as the female parent. A total of 288 BC$_1$F$_1$ progenies were produced and individual plants heterozygous at the $Xa4$, $xa5$ and $Xa21$ loci were identified and used for further backcrossing with the recurrent parent. Of the 288 BC$_1$F$_1$ plants that were analyzed with three STS markers, 28 plants were selected as having an allele of three resistance genes on the basis of molecular marker analysis and phenotypic selection. The advanced backcross progenies of BC$_2$ and BC$_3$ were obtained from the crosses of selected resistant BC$_1$F$_1$ (28 plants from 288 plants), BC$_2$F$_1$ (32 plants from 536 plants) and BC$_3$F$_1$ (42 plants from 645 plants) plants based on the dual-selection procedure of the BB-resistant phenotype and foreground selection using the $Xa4$, $xa5$ and $Xa21$ gene-specific DNA markers (Figure 1). Progenies of the BC$_3$F$_1$ generation were advanced by dual-selection and selfing, and promising BB-resistant breeding lines were developed. Phenotypic selection at each backcross and selfing generation was conducted to eliminate plants with linkage drag traits such as high sterility, tall plant type and late flowering. Thus, the population size for MAS could be reduced as we removed the plants with an undesirable phenotype. We selected three ABL from BC$_4$F$_5$ progenies based on their reaction to selected BB

![Figure 1 Scheme for the development of $Xa4$, $xa5$ and $Xa21$ gene-pyramided backcross breeding lines using marker-assisted foreground and background selection.](http://www.thericejournal.com/content/6/1/5)
isolates and the presence of homozygous marker alleles for the BB resistance genes and desirable agronomic traits (Table 1 and Figure 2).

Evaluation of BB resistance
The ABL with three resistance genes were evaluated for their resistance to BB under glasshouse conditions with the 18 isolates of Xoo prevalent in Korea. One of these isolates, HB01009 belongs to race K3a, a widely distributed Xoo pathotype in the southwestern coastal areas of Korea (Noh et al. 2003). The lesion lengths obtained after inoculation with these isolates are shown in Table 2. Mangeumbyeo was highly susceptible to all isolates, with lesion length ranging from 9 to 18.2 cm, whereas donor line IRBB57 pyramided with the R genes Xa4, xa5 and Xa21 was highly resistant against all isolates, with lesion length of <0.5 cm. Compared to Mangeumbyeo, the leaves of the NILs with Xa4, xa5 and Xa21 genes showed susceptible, moderately resistant and resistant reactions to the BB strains. However, the ABL with x4 + xa5 + xa21 pyramided genes in the Mangeumbyeo background exhibited very small lesion lengths, indicating very high resistance to all 18 isolates of Xoo, with average lesion lengths being <0.3 cm. Our results indicated that the genes in combinations were more effective against the pathogen than a single gene (Table 2). Resistance genes xa5 and Xa21 were effective against 14 of the isolates from Korea used in this study, whereas resistance gene Xa4 was resistant to 8 isolates only. Based on this result, we infer that, individually, xa5 and Xa21 were more effective resistance genes than Xa4.

SSR-based genetic background profiling of ABL
A total of 248 SSR markers were used for background selection of the three ABL along with the BB-resistant donor line IRBB57 and a genetic map covering a 1,446.6 cM region of the O. sativa genome was constructed (Figure 3). The marker polymorphisms between Mangeumbyeo and IRBB57 were 83%. Each ABL contains an SSR marker-defined chromosome segment from the donor in the genetic background of the recurrent parent, Mangeumbyeo. The average percentage of donor parent chromosome substitution in ABL4225, ABL4228 and ABL4242 was 7, 5.5 and 7.9%, respectively (Table 3). The substituted chromosome segments in ABL were distributed around the regions of xa5 located on chromosome 5 and Xa4 and Xa21 located on chromosome 11. In our study, ABL4228 inherited the smallest size (5.5%) of the substituted chromosome segments from the donor genotypes.

Agronomic traits and grain quality performance of ABL
The agronomic traits of ABL evaluated in the field and laboratory showed that most of the morphological traits, including plant type and grain quality, were similar to those of the recurrent parent, Mangeumbyeo (Table 4). Traits such as days to heading, panicle number, grain yield, 1,000-grain weight of brown rice, amylose content and alkali digestion value of milled rice, protein content of brown rice and alkali digestion value of the selected three ABL were almost the same as those of Mangeumbyeo. However, the DTH of ABL4225 and ABL4228 were 12–13 days less than those of Mangeumbyeo. The culm length of the three ABL was shorter by 4–8 cm than that of Mangeumbyeo. This is a desirable agronomic trait for lodging resistance, thus reducing yield loss. The grain yield of ABL did not show a significant difference from Mangeumbyeo even though the number of grains per panicle of the three ABL was more than that of the recurrent parent. This may be due to a reduction in spikelet fertility per se and number of grains per panicle. All of the ABL were recovered with japonica grain characteristics of the recurrent parent with a non-chalky appearance and similar values for AC, PC, ADV and grain shape (short grain type), having homozygous alleles of the Xa4, xa5 and Xa21 genes (Table 4).

Discussion
Most japonica rice cultivars exhibit high susceptibility to BB disease, except to race K1 in Korea, because of their narrow genetic diversity. It is imperative to develop new
BB-resistant rice cultivars with high yield potential and grain quality using modern tools of biotechnology. However, it is often difficult to introduce the BB resistance genes from indica germplasm sources into a japonica genetic background by conventional breeding methods due to the unexpected linkage drag. Pyramiding resistance genes is difficult to accomplish using conventional breeding because of the dominance and epistasis effects of the genes controlling disease resistance. Nevertheless, using the tools of biotechnology, it is possible to transfer or pyramid valuable genes of BB resistance into rice without linkage drag (Rajpurohit et al. 2011; Shanti et al. 2010; Singh et al. 2001; Sundaram et al. 2008). Mangeumbyeo is a japonica cultivar with good grain and cooking quality and high yield potential but it is highly susceptible to BB races. An IRBB57 NIL carrying Xa4, xa5 and Xa21 genes in an IR24 genetic background conferred strong resistance to all Korean BB races, including K3a (Jeung et al. 2006; Suh et al. 2009a). We have introduced the three BB resistance genes (Xa4 + xa5 + Xa21) from IRBB57 into Mangeumbyeo through simultaneous foreground and phenotypic selection. Eventually, it was possible to introduce three BB resistance genes with desirable agronomic traits using marker-assisted

Figure 2 PCR analysis of the parental lines and BC3F2 plants (A) and resistance gene confirmation of advanced backcross breeding lines (B). DNA amplified with primers MP1 + MP2, 10603.T10Dw (digested with RsaI) and U1/I1 was linked with resistance genes Xa4, xa5 and Xa21, respectively. P1: Mangeumbyeo, P2: IRBB57, M: DNA ladder marker.

Table 2 Average lesion length in centimeters of advanced backcross breeding lines, near-isogenic lines carrying single bacterial blight resistance genes and recurrent and donor parents against each of 18 Korean Xanthomonas oryzae pv. oryzae isolates

| Isolate | RP | DP | IRBB4 | IRBB5 | IRBB21 | ABL4225 | ABL4228 | ABL4242 |
|---------|----|----|-------|-------|--------|---------|---------|---------|
| HB01009 | 13.0 | 0.1 | 2.8   | 0.5   | 1.0    | 0.3     | 0.6     | 0.4     |
| HB02010 | 14.1 | 0.1 | 2.5   | 0.1   | 15.0   | 0.1     | 0.1     | 0.1     |
| HB02024 | 13.0 | 0.3 | 2.0   | 2.5   | 10.0   | 0.1     | 0.2     | 0.1     |
| HB02038 | 10.2 | 0.1 | 9.0   | 7.0   | 1.5    | 0.1     | 0.1     | 0.4     |
| HB03034 | 9.0  | 0.2 | 8.0   | 1.5   | 7.5    | 0.5     | 0.1     | 0.3     |
| HB03055 | 9.5  | 0.2 | 2.1   | 1.5   | 7.5    | 0.7     | 0.2     | 0.1     |
| HB04024 | 18.2 | 0.1 | 2.3   | 7.5   | 1.5    | 0.1     | 0.1     | 0.1     |
| HB04030 | 14.0 | 0.1 | 7.5   | 1.5   | 1.2    | 0.1     | 0.1     | 0.1     |
| HB04032 | 10.3 | 0.1 | 8.5   | 2.5   | 1.5    | 0.1     | 0.1     | 0.2     |
| HB04040 | 14.0 | 0.5 | 9.0   | 2.5   | 2.0    | 0.3     | 0.4     | 0.1     |
| HB04052 | 13.0 | 0.1 | 9.5   | 2.5   | 1.8    | 0.1     | 0.7     | 0.1     |
| HB04064 | 15.4 | 0.1 | 10.5  | 1.5   | 16.0   | 0.1     | 0.1     | 0.1     |
| HB04079 | 15.4 | 0.1 | 11.5  | 1.5   | 3.0    | 0.5     | 0.1     | 0.1     |
| HB04084 | 12.2 | 0.1 | 3.5   | 7.5   | 3.0    | 0.1     | 0.5     | 0.5     |
| HB04087 | 10.0 | 0.3 | 2.5   | 7.0   | 1.0    | 0.4     | 0.5     | 0.5     |
| HB05004 | 18.0 | 0.1 | 9.5   | 2.0   | 1.5    | 0.7     | 0.8     | 0.1     |
| HB05027 | 13.5 | 0.1 | 7.5   | 2.0   | 1.0    | 0.7     | 0.1     | 0.1     |
| HB05029 | 17.2 | 0.1 | 2.5   | 1.5   | 2.0    | 0.1     | 0.2     | 0.1     |

RP (Recurrent parent): Mangeumbyeo, DP (Donor parent): IRBB57.
ABL: advanced backcross breeding lines having Xa4 + xa5 + Xa21 genes.
backcrossing. All three co-dominant molecular markers linked to the target genes (Xa4, xa5 and Xa21) were used for MAB and the markers were polymorphic between the donor parent IRBB57 and recurrent parent Mangeumbyeo. The validated markers could thus be used successfully to pyramid and confirm the three resistance genes in advanced backcross lines. Finally, we also analyzed the genetic background of the three selected ABL (BC₃ progenies) with high background genome recovery. Conventional backcross breeding has difficulty in confirming the several resistance genes combined in breeding lines using phenotypic selection with Xoo inoculation (Rajpurohit et al. 2011; Shanti et al. 2010; Sundaram et al. 2008). The best strategy to pyramid or introduce multiple genes and recover a maximum recurrent parent background effect in the shortest time will be to take up the transfer of genes simultaneously, generate a large backcross population and select the target genes through foreground selection and flanking marker analysis to reduce the persistent linkage drag.
However, if we select backcross lines with target genes using molecular markers, linkage drag often occurs in indica/japonica. So, we selected the backcross progenies in each backcross and segregating generation through foreground and phenotypic selection simultaneously to reduce the linkage drag. This expensive, cumbersome and time-consuming background selection can be avoided and substituted by another backcross with the recurrent parent, if necessary. Final backcross progenies could be confirmed with the substituted chromosome segments by background analysis using genome-wide molecular markers. On the basis of comprehensive foreground selection, phenotypic selection for morphological and quality traits, and background genotyping, three BC3F5 gene-pyramid lines with pyramided genes homozygous at all three target loci were derived from the donor parent. The three R-gene-derived ABL exhibited high resistance upon inoculation with Xoo strains and had nearly the average expected 93.75% background genome recovery.

In an earlier study, it was reported that the favorable characteristics of Pusa Basmati 1 with two BB resistance genes could be recovered using MAS just in BC1 because of stringent phenotypic selection without any background selection only in segregating generations (Joseph et al. 2004). Similarly, BC4 pyramided lines of Sambha Mahsuri with three BB resistance genes (xa5, xa13 and Xa21) were developed by simultaneous foreground and background selection and the selected lines recovered 97% recurrent parent background, exhibiting a broad-spectrum resistance against multiple Xoo isolates (Sundaram et al. 2008). In this study, we selected elite ABL with three BB resistance genes in the BC3 generation because BC 1 and BC2 progenies were having

### Table 3
Simple sequence repeat markers with polymorphism between the recurrent parent and the donor parent and substituted chromosome segments from donor parent in advanced backcross breeding lines of rice

| Chr. no. | No. of markers | Chr. length (cM) | Interval (cM) | PM (%) of RP/DP | Chromosome segments of DP (%) |
|----------|----------------|-----------------|--------------|-----------------|-------------------------------|
| 1        | 32             | 181.5           | 5.7          | 84.4            | ABL4225 2.0 2.8 10.5         |
| 2        | 27             | 151.6           | 5.6          | 85.2            | ABL4228 1.2 1.2 1.2          |
| 3        | 25             | 157.9           | 6.3          | 88.0            | ABL4242 0.0 5.4 5.4          |
| 4        | 21             | 126.5           | 6.0          | 76.2            |                               |
| 5        | 21             | 118.0           | 5.6          | 76.2            |                               |
| 6        | 21             | 122.7           | 5.8          | 85.7            |                               |
| 7        | 19             | 94.1            | 5.0          | 94.7            |                               |
| 8        | 17             | 103.2           | 6.1          | 76.5            |                               |
| 9        | 14             | 91.3            | 6.5          | 85.7            |                               |
| 10       | 15             | 83.8            | 5.6          | 86.7            |                               |
| 11       | 20             | 112.9           | 5.6          | 75.0            |                               |
| 12       | 16             | 103.1           | 6.4          | 81.3            |                               |
| Average (total) | (248) | (1446.6) | 5.9 | 83.0 | 7.0 | 5.5 | 7.9 |

### Table 4
Performance of principal agronomic and grain quality traits of three ABL, which were selected as the most promising lines

| Variety     | DTH | CL (cm) | PL (cm) | PN | NGP | FER (%) | GY (t/ha) | GW (g) | L/W | AC (%) | PC (%) | ADV (1–7) |
|-------------|-----|---------|---------|----|-----|---------|-----------|--------|-----|-------|-------|-----------|
| Mangeumbyeo | 115b| 81 cd   | 19a     | 15a| 108b| 96b     | 7.86ab    | 19.9a  | 1.85| 20.8a| 6.3a  | 6.8b      |
| IRBB57      | 115b| 67a     | 23b     | 14a| 128c| 92a     | 7.57a     | 22.5b  | 3.04| 24.5b| 7.3b  | 2.0a      |
| ABL4225     | 102a| 73b     | 21ab    | 14a| 118b| 94ab    | 7.70ab    | 18.7a  | 1.74| 19.9a| 6.8ab | 6.7b      |
| ABL4228     | 103a| 75bc    | 19a     | 15a| 117b| 93a     | 7.81ab    | 18.2a  | 1.78| 19.1a| 6.7ab | 6.6b      |
| ABL4242     | 114b| 77bcd   | 20a     | 15a| 120bc| 95ab    | 7.98b     | 19.1a  | 1.74| 20.5a| 6.5ab | 6.8b      |

DTH: days to heading, CL: culm length (cm), PL: panicle length (cm), PN: panicle number, NGP: number of grains per panicle, FER: fertility of spikelets (%), GY: grain yield (t/ha), GW: 1,000-grain weight of brown rice (g), L/W: ratio of seed length/width, AC: amylose content of milled rice (%), PC: protein content of brown rice (%), ADV: alkali digestion value (1–7), and a higher value indicates better quality. Means followed by the same letter are not significant at the 5% significance level by the least significant difference test (LSD = 0.05).
some undesirable phenotypic traits such as awns, shattering and spikelet sterility. It is possible to recover the recurrent parent phenotype in one or two backcrosses if we introduce multiple resistance genes from indica to indica cultivars (Joseph et al. 2004; Rajpurohit et al. 2011; Singh et al. 2001) and we may also need at least two backcrosses to introduce one resistance gene from indica to japonica cultivars (Suh et al. 2009b; Suh et al. 2011). However, our results suggest that at least three backcrosses are essential to recover the phenotype of the recurrent parent if multiple resistance genes such as *Xa4*, *xa5* and *Xa21* are transferred from an indica cultivar into a japonica cultivar for broad-spectrum BB resistance.

Three BB resistance-gene-derived ABL were evaluated for their resistance to BB under glasshouse conditions with the 18 isolates of *Xoo* prevalent in Korea. One of these isolates, called HB01009, belongs to the new race K3a (Noh et al. 2003). The *Xa21* and *xa5* genes and their combinations conferred strong resistance to the K3a isolate (Suh et al. 2009a, 2009b). Variable reactions of the *Xoo* isolates to *Xa4*, *xa5* and *Xa21* suggest that *xa5* and *Xa21* are more effective in resistance to 14 isolates than *Xa4* because *Xa4* showed resistance to 8 isolates only. However, the cumulative effect of the three resistance genes (*Xa4* + *xa5* + *Xa21*) in the ABL in the Mangeumbyeo genetic background exhibited very high resistance to all 18 isolates of *Xoo*, including the most virulent isolate of race K3a. The results indicated that the genes in combinations were more effective against the pathogen strains than a single resistance gene alone. The resistance appears to be more durable if different resistance genes are combined (Jeung et al. 2006; Kim et al. 2009; Singh et al. 2001; Suh et al. 2009a). This indicates that there is some kind of quantitative complementation with the presence of multiple resistance genes having an additive effect on the overall level of resistance. Accumulating major genes for resistance in an elite genotype by conventional breeding is laborious, time-consuming and very difficult when two or more of the resistance genes are pyramided into an elite cultivar. However, marker-assisted backcrossing with accurate phenotypic selection is the most effective method for a selective transfer or pyramiding of resistance genes into elite rice cultivars free from linkage drag, eventually restoring the recurrent parent genotype (Joseph et al. 2004; Shanti et al. 2010; Singh et al. 2001; Suh et al. 2011). The ABL with the three resistance genes in combination have a practical breeding value by providing a wider spectrum of resistance against most of the existing BB isolates in the region and will have a high impact on the yield stability and sustainability of the rice crop in the region. The grain quality characteristics of the three resistance-gene-derived ABL are not significantly different from those of the parent Mangeumbyeo. This indicates that the BB resistance-gene combinations are not closely linked with any negative allele controlling grain quality. It is also reported that *Xa1*, *Xa2* and *Xa3* genes have no negative effect for the traits associated with grain quality and the taste of cooked rice (Shin et al. 2006). The recurrent parent greatly influenced the determination of grain quality, milling characteristics and cooking and eating qualities. Therefore, the choice of the recurrent parent plays a critical role in backcross breeding programs (Shin et al. 2006; Ye 2010). The yield and agronomic traits of the ABL in this study are also similar to those of Mangeumbyeo, indicating that there is no apparent agronomic trait penalty associated with the presence of the resistance genes.

In our study, an additional backcross with the recurrent parent was required to recover the desirable phenotype in the BC3 progenies. Three BC3F5 progenies were mostly homozygous for the target traits based on MAS with agronomic traits similar to those of the recurrent parent, Mangeumbyeo, with high resistance to bacterial blight. The background genotype recovery varied from 92.1 to 94.5%. Even though the three ABL showed highly recovered chromosome segments, they could not exhibit a similar phenotype with the recurrent parent because the insertion of small chromosome segments also affected phenotype. Theoretically, with three backcrosses, the average background genotype recovery should be 93.75%, a background recovery rate similar to that of the selected ABL in this study. On the contrary, the background recovery of the recurrent wheat parent during the introgression of stripe rust resistance without marker-assisted background selection was only 82% in BC3F7 progenies (Randhawa et al. 2009). However, 97% of the background genotype was obtained in BC3F2,3 progenies by using foreground selection of the target traits, background selection for flanking markers, non-carrier chromosome markers and whole-marker screens during two successive backcrosses in a large backcross population. A high rate of background genotype recovery of the recurrent parent was 86.72% in the BC1F3 generation using MAS and phenotypic selection during the introgression of two BB resistance genes in indica/indica crosses (Joseph et al. 2004). In our study, a similar strategy of simultaneous foreground and phenotypic selection was followed for higher background genotype recovery in the japonica/indica cross in three backcrosses. This approach is very useful to reduce the cost and time required for the recovery of desirable recombinants to a considerable extent with target resistance genes in japonica/indica crosses. Therefore, it can be directly developed in a commercial variety. Introgression of resistance with a penalty in yield and grain quality characters would be a futile exercise, as...
the developed lines would not be accepted by farmers. The three-gene pyramided ABL developed in our study without a penalty in yield and grain quality would be of great advantage to rice farmers in BB-endemic areas.

Conclusions
Host-plant resistance is a cost-effective and environmentally safe approach to reduce yield loss caused by BB disease of rice. Several BB resistance genes identified to date are either race specific or express susceptibility to the emerging races of the pathogen. Our study provides some clues to a successful pyramiding of three BB resistance genes into an elite japonica cultivar to control BB disease caused by a new race, K3a. We used a dual-selection strategy of phenotypic and genotypic selection along with background genotyping to isolate improved breeding lines with three pyramided genes conferring strong resistance to BB. Furthermore, our study on the evaluation of agronomic traits revealed that the accumulation of three-gene pyramids did not show a yield penalty. Future studies on the transfer of these pyramided genes into other genetic backgrounds may help in controlling BB disease caused by different races of the pathogen.

Methods
Plant materials used
IRBB57, a near-isogenic line in the background of IR24 possessing a combination of three genes (Xa4 + xa5 + Xa21), was used as the donor parent for transferring BB resistance genes into japonica rice cultivars. Mangeumbyeo, a BB-susceptible elite japonica cultivar with good grain quality, was used as the recurrent parent. A cross was made between Mangeumbyeo and IRBB57, which carries three BB resistance genes. F1 plants were backcrossed with the recurrent parent. Advanced backcross breeding lines (ABL) in a japonica genetic background were developed by the marker-assisted backcross (MAB) breeding strategy. Among the BC1F1 plants, polymerase chain reaction (PCR)-based molecular markers linked to Xa4, xa5 and Xa21 were used to select plants with resistance alleles. A similar strategy was used in the BC2-3 F1 to obtain BC3F2 populations from which the introduced R genes were selected. The BC3F2 plants were selfed and advanced generation progenies were produced on the basis of marker-assisted selection (MAS) and were inoculated with BB isolates/races, including the K3a isolate. The selected and confirmed ABL were used for overall resistance evaluation and background profiling (Table 1).

Bacterial blight inoculation and evaluation
The parents and segregating ABL materials were grown in the glasshouse of the National Institute of Crop Science (NICS). At the maximum tillering stage, the plants were inoculated with the K3a isolate (HB01009) of Xanthomonas oryzae pv. oryzae (Xoo) using the leaf clipping method (Kauffman et al. 1973). Plant reaction to the disease was scored 14 days after inoculation by measuring lesion length (cm). The reaction of resistance was expressed in lesion length (resistant: < 3 cm, moderately resistant: 3–5 cm, susceptible: > 5 cm) (Jeung et al. 2006). The selected ABL confirming three resistance genes were inoculated with 18 predominant Xoo isolates from Korea (Table 2).

Resistance gene confirmation by DNA markers
Genomic DNA was extracted from fresh frozen leaves of rice plants using the CTAB method with little modification (Murray and Thompson 1980). Three gene-specific PCR markers, MP1 + MP2, 10603.T10Dw and U1/I1, tightly linked to the resistance genes Xa4, xa5 and Xa21, respectively, were used to confirm the presence of the R genes in each backcross generation (Table 5). PCR was performed in a total volume of 20 μl containing 40 ng of DNA template, 10 pmole of each primer, 0.2 mM of dNTP and 1U of Taq polymerase (Suh et al. 2009a). The PCR amplification condition was with one cycle at 95°C for 4 min, followed by 35 cycles at 95°C for 30 s, at 56°C (MP1 + MP2 and U1/I1) or 65°C (10603.T10Dw) for 30 s and at 72°C for 1 min, with a final extension at 72°C for 10 min (Bio-Rad, PTC-200 Thermocycler; Germany). Marker allele types of the genotypes were determined based on the unique band sizes as well as the banding patterns derived from PCR products (MP1 + MP2 and U1/I1) or from cleaved PCR products (10603.T10Dw) by RsaI enzyme, for which 4 μl of the PCR product was digested by 2.5 U of restriction

| Resistance gene | Chr. no. | Marker name | Primer sequences used for gene detection | Expected size (bp) | Band type | Reference |
|-----------------|----------|-------------|------------------------------------------|-------------------|----------|-----------|
|                 |          |             | Forward (5'-3')                           | Reverse (5'-3')   |          |           |
| xa5             | 5        | 10603. T10Dw | GCACACGCGAACCATTCAAGAATCT                | CCTAGGAGAAAATAGCCGTTCCA | 280 | Co-dominant | Jeung et al. (Unpublished) |
| Xa4             | 11       | MP1 + MP2   | ATCGATCGATCTTCCAGG                      | TGCTATAAAAAGGCATTGCG | 150 | Co-dominant | Sun et al. 2003 |
| Xa21            | 11       | U1/I1       | CGATCGGTATAACAGCAAAAC                   | ATAGCAACTGATTGCTTG | 1,400 | Co-dominant | Wang et al. 1996 |
endonuclease in a 20 μl reaction volume at 37°C for 3 hours. Agarose gel (1.5%, 0.5×TBE, 150 V) and natural polyacrylamide gel (8% polyacrylamide, 0.5×TBE, 200 V) electrophoresis were used for the PCR products from 10603.T10Dw (treated by RsaI) and U1/I1 primers, and the PCR products from MP1 + MP2, respectively, and stained by ethidium bromide to visualize the DNA.

Background profiling by SSR marker analysis
A total of 248 SSR markers of known chromosomal positions distributed evenly on the 12 chromosomes with an average marker interval of 5.9 cM were used in a genome-wide survey to identify the chromosome segment substitution locations in the three ABL compared with the donor line. The SSR markers polymorphic between the two parents were used for background genotyping to recover the recipient parent genome. The lengths of substituted chromosome segments in ABL were estimated based on the graphical genotyping procedure (Suh et al. 2009b; Xi et al. 2006). A chromosome segment flanked by homozygous marker alleles of the donor parent was considered a 100% donor type, a chromosome segment flanked by homozygous marker alleles of the recipient parent was considered a 0% donor type and a chromosome segment flanked by one marker allele of the donor parent and another marker allele of the recipient parent was considered a 50% donor type. The linkage and orientation of SSR markers on chromosomes were assigned following the SSR map constructed by McCouch et al. (2002) and as depicted in Gramene (http://www.gramene.org/).

Agronomic and grain quality evaluation of the ABL
The parents and the three ABL were planted in a four-row plot with 35 plants per row by 30×15-cm spacing in a randomized complete block design with three replications and were evaluated for agronomic traits in the rice experimental plot of NICS, Suwon, Korea, using the standard evaluation method of rice (RDA Rural Development Administration 2003). The amount of standard fertilizer application in the experimental field was N-P₂O₅-K₂O = 90-45-57 kg/ha. Commercial pesticides were applied for the protection of plant materials. For each plot, five plants in the middle rows were used to determine days to heading (DTH), culm length (CL), panicle number (PN), panicle length (PL), number of grains per panicle (NGP), fertility of spikelets (FER), 1,000-grain weight of the brown rice (GW), ratio of seed length/width (L/W) and grain yield (GY; t/ha). DTH was evaluated as the number of days from sowing in the field until 50% heading of the panicles in the plants. CL was calculated as the average number in centimeters from the ground to the neck of the tallest panicle. PL was measured as the average number in centimeters from the panicle neck to the panicle tip based on an evaluation of all the panicles from the plants. PN was the average number of panicles on the plants. NGP was calculated by counting the total number of filled spikelets from the plants. FER was calculated as a percentage: the number of filled spikelets divided by the number of spikelets per panicle. GW was measured in grams as the average weight of 1,000 fully filled brown rice grain from each plant.

Grain yield per plot was evaluated based on a grain harvest of 100 plants in the central row of each plot. Grain quality was estimated for alkali digestion value (ADV), amylose content of milled rice (AC), protein content of brown rice (PC) and chalkiness of brown rice (CK: 0: non-chalkiness, 3: high chalkiness). ADV was evaluated based on the procedure of Little et al. (1998). AC was determined by the relative absorbency of starch-iodine color in a digested solution of 100-mesh rice flour by Juliano’s (Juliano 1973) modified method. PC was calculated by total nitrogen multiplied by 5.95 after determining the nitrogen content of rice material using the Micro-Kjeldahl method (Foss: 2300 Kjeltec Analyzer). The least significant difference (LSD) and Duncan’s multiple range test (DMRT) were used for multiple mean comparisons using the SAS statistical analysis software (version 8.2; SAS Institute, Cary, NC).

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JPS carried out the experiments and did foreground and background analysis of advanced backcross lines. JUG and SHP designed new primers for the xa5 gene, THN prepared BB inocula and did BB evaluation, YCC, HSP, MSS and CKK conducted marker-assisted breeding for BB resistance. KKJ conceptualized the study and participated in the preparation of the manuscript. All authors read and approved the final manuscript.

Acknowledgments
This research was supported by a grant from the Rural Development Administration (RDA), Republic of Korea. We are grateful to Bill Hardy (senior science editor, IRRI) for carefully editing the manuscript. All authors read and approved the final manuscript.

References
Bhasin H, Bhatia D, Raghuvanshi S, Lores JS, Sahi GK, Kaur B, Vikal Y, Singh K (2012) New PCR-based sequence-tagged site marker for bacterial blight resistance gene Xa38 of rice. Mol Breed 30:607–611
Cheema K, Grewal N, Vikal Y, Sharma R, Lores JS, Das A, Bhatia D, Mahajan R, Gupta V, Bharaj TS, Singh K (2008) A novel bacterial blight resistance gene from Oryza nivara mapped to 38 kb region on chromosome 4 L and transferred to Oryza sativa L. Genet Res 90:397–407
Garris AJ, McCouch SR, Kresovich S (2003) Population structure and its effect on haplotype diversity linkage disequilibrium surrounding the xa5 locus of rice (Oryza sativa L.). Genetics 165:759–769
Gu K, Yang B, Tian D, Wu L, Wang D, Seokkai C, Yang F, Chu Z, Wang GL, White FF, Yin Z (2005) R gene expression induced by a type-III effector triggers disease resistance in rice. Nature 435:1122–1125

Huang N, Angeles ER, Domingo J, Magpantay G, Singh S, Zhang Q, Kumaranavadi N, Bennett J, Khush GS (1997) Pyramiding of bacterial resistance genes in rice: marker aided selection using RFLP and PCR. Theor Appl Genet 95:313–320

Jena KK, MacMill DJ (2008) Molecular markers and their use in marker-assisted selection in rice. Crop Sci 48:1266–1276

Jeung JJ, Heu SG, Shin MS, Vera Cruz CM, Jena KK (2006) Dynamics of Xanthomonas oryzae pv. oryzae populations in Korea and their relationship to known bacterial blight resistance genes. Phytopathology 96:867–875

Joseph M, Gopalakrishnan S, Sharma RK, Singh VP, Singh AK, Singh NK, Mohapatra T (2004) Combining bacterial blight resistance and basmati quality characteristics by phenotypic and molecular marker-assisted selection in rice. Mol Breed 13:377–387

Juliano BO (1973) A simple assay for milled rice amylose. Cereal Sci Today 16:334–336

Kauffman HE, Reddy APK, Hsien SPY, Merda SD (1973) An improved technique for evaluating resistance of rice varieties to Xanthomonas oryzae: Plant Dis Rep 57:537–541

Khush GS, Balaangco E, Oyoga T (1990) A new gene for resistance to bacterial blight from O. longistaminata. Rice News 7:212–122

Kim KY, Shin MS, Kim WJ, Nam JK, Noh TH, Kim BK, Ko JK (2009) Effective combination of resistance genes against rice bacterial blight pathogen. Korean J Breed Sci 41(3):244–251

Korinsak S, Sripakhan S, Srithunya P, Janin J, Korinak S, Vanavichit A, Toojinda T (2009) Identification of microsatellite markers (SSR) linked to a new bacterial blight resistance gene xao3(A) in rice cultivar ‘Bat7’. Maejo Int J Sci Technol 3:235–247

Leach JE, Davidson R, Liu B, Manosalva P, Singh AK, Bruce M, Little RR, Hilder GB, Dawson EH (1998) Differential effect of dilute alkali on 25 genes on rice grain quality. J Crop Sci 6:122-126

Lee KS, Rasabandith S, Angeles ER, Khush GS (2003) Inheritance of resistance to bacterial leaf blight in 21 cultivars of rice. Phytopathology 93:147–152

Little RR, Hilder GB, Dawson EH (1998) Differential effect of dilute alkali on 25 varieties of milled white rice. Cereal Chem 35:111–126

Liu DJ, Ronald PC, Bogdanove AJ (2006) Xanthomonas oryzae pathovars: model pathogens of a model crop. Mol Plant Pathol 7:244–251

McCouch SR, Teytelman L, Xu Y, Lobos KB, Clare K, Walton M, Fu BY, Maghirang R, Li ZK, Xing YZ, Zhang QF, Kono I, Yano M, Fjellstrom R, Declercq G, Scheider D, Carinhour S, Ware D, Stein L (2002) Development and mapping of 2240 new SSR markers of rice (Oryza sativa L.). DNA Res 9:199–207

Mew TW, Vera Cruz CM, Medalla ES (1992) Changes in race frequency of Xanthomonas oryzae pv. oryzae in response to rice cultivars planted in the Philippines. Plant Dis 76:1029–1032

Murray MG, Thompson WF (1980) Rapid isolation of high molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Natraj Kumar P, Sujatha K, Laha GS, Srinivasarao K, Mishra B, Vinkathram BC, Hari Y, Reddy CS, Balachandran SM, Ram T, Sheshumadhav M, Sholharani N, Neeraja CN, Ashokreddy G, Shaik H, Sundaram RM (2012) Identification and fine-mapping of xao21, a novel gene for resistance to Xanthomonas oryzae pv. oryzae. Phytopathology 102:222–228

Noh TH, Lee DK, Kang MH, Shin MS, Na SY (2003) Identification of new race of Xanthomonas oryzae pv. oryzae (Xoo) in Korea. (Abstr.) Phytopathology 93(supp)S66

Noh TH, Lee DK, Park JC, Shin HK, Choi MY, Kang MH, Kim JD (2007) Effect of bacterial leaf blight occurrence on rice yield and grain quality in different rice growth stage. Res Plant Dis 13:20–23

Rajpurush D, Kumar R, Kumar M, Paul P, Awasthi AA, Basho PO, Puri A, Jhang T, Singh K, Dhaliwal HS (2011) Pyramiding of two bacterial blight resistance and a semi dwarving gene in Type 3 Basmati using marker-assisted selection. Euphytica 178:111–126

Randhawa HS, Mutti JS, Kidwell K, Morris CF, Chen X, Gill KS (2009) Rapid and targeted introgression of genes into popular wheat cultivars using marker-assisted backcross selection. PLoS One 4(6):e5752

RDA (Rural Development Administration) (2003) Manual for standard evaluation method in agricultural experiment and research. RDA, Suwon (Korea), p 358

Shanti ML, Shenoy VV, Devi GL, Kumar VM, Premalatha P, Kumar GN, Shashidhar HE, Zehr UB, Freeman WH (2010) Marker-assisted breeding for resistance to bacterial leaf blight in popular cultivars and parental lines of hybrid rice. J Plant Pathol 92(2):495–501

Shin MS, Choi YH, Kim KY, Shin SH, Ko JK, Lee JK (2006) Effect of recurrent parents and introduced xao1, xao2, and xao3 genes on rice grain quality. Korean J Breed 38(3):161–166

Shin MS, Kim KY, Park HS, Ko JK (2011) Breeding for resistance to bacterial blight in rice. Korean J Breed 43:251–261

Shin MS, Shin HT, Jun BT, Choi BS (1992) Effect of inoculation of compatible and incompatible bacterial blight races on grain yield and quality of two rice cultivars. Korean J Breed 24(3):264–267

Singh S, Siddhu S, Huang N, Vikal Y, Li Z, Bar D, Dhaliwal HS, Khush GS (2001) Pyramiding three bacterial blight resistance genes (xao2, xao3 and xao21) using marker-assisted selection into indica rice cultivar PR106. Theor Appl Genet 102:1001–1015

Song WP, Pi LY, Wang GL, Gardner J, Holston T, Ronald PC (1997) Evolution of the rice xao21 disease resistance gene family. Plant Cell 9:1279–1287

Suh JP, Noh TH, Kim KY, Kim JJ, Kim YG, Jena KK (2006a) Expression levels of three bacterial blight resistance genes against K3a race of Korea by molecular and phenotype analysis in japonica rice (O. sativa L.). J Crop Sci Biotechnol 12:103–108

Suh JP, Roh JH, Cho YC, Han SS, Kim YG, Jena KK (2006b) The Pi40 gene for durable resistance to rice blast and molecular analysis of Pi40-advanced backcross breeding lines. Phytopathology 96:243–250

Suh JP, Yang SJ, Jeung JJ, Pamplona A, Kim JJ, Lee JH, Hong HC, Yang CI, Kim YG, Jena KK (2011) Development of elite breeding lines conferring Xa21 gene-derived resistance to brown planthopper (BP4) by marker-assisted selection and genome-wide background analysis in japonica rice (Oryza sativa L.). Field Crops Res 120:215–222

Sun X, Cao Y, Yang Z, Xu C, Li X, Wang S, Zhang Q (2004) Xa26, a gene conferring resistance to Xanthomonas oryzae pv. oryzae in rice, encodes an LRR receptor kinase-like protein. Plant J 37:157–167

Sun X, Yang Z, Wang S, Zhang Q (2003) Identification of a 47 kb DNA fragment containing xao4, a locus for bacterial blight resistance in rice. Theor Appl Genet 106:683–687

Sundaram RM, Vishnupriya MR, Biradar SK, Laha GS, Reddy GA, Narayana PS, Sonti RV (2000) Marker assisted introgression of bacterial blight resistance in Samba Mahsuri, an elite indica rice variety. Euphytica 106:411–422

Sundaram RM, Vishnupriya MR, Laha GS, Narayana PS, Balachandran SM, Reddy GA, Sambha NP, Sonti RV (2009) Introduction of bacterial blight resistance into Triguna, a high yielding, mid-early duration rice variety. Biotechnol J 4:400–407

Xi ZY, He FH, Zeng RZ, Zhang ZM, Ding XH, Li WT, Zhang GQ (2006) Development of a wide population of chromosome single-segment substitution lines in the genetic background of an elite cultivar of rice (Oryza sativa L.). Genome 49:476–484

Xu Y, Crouch JH (2008) Marker-assisted selection in plant breeding: from publication to practice. Crops Sci 48:391–407

Xu Y, D. Sanchez A, Khush GS, Zhu Y, Huang N (1998) Construction of a BAC contig containing the xao5 locus in rice. Theor Appl Genet 97:1120–1124

Ye G (2010) Marker-assisted gene pyramiding for cultivar development. Plant Breed Rev 33:219–256

Yun MS, Lee EJ, Cho YS (1985) Pathogenic specialization of the rice bacterial leaf blight pathogen, Xanthomonas oryzae pv. oryzae: race classification based on reactions of Korean differential varieties. Korean J Plant Prot 24:97–101

Cite this article as: Suh et al. Development of breeding lines with three pyramided resistance genes that confer broad-spectrum bacterial blight resistance and their molecular analysis in rice. Rice 2013 6:5.