Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is undoubtedly a severe and broadly distributed disease of cultivated rice (*Oryza sativa* and *O. glaberrima*). BB affects rice production in both rain-fed and irrigated environments (Furuya et al. 2012). According to a review by Mew et al. (1993), reduction in rice yield might be as high as 50% in fields where the crop is infected severely. Infected at the tillering stage can engender total crop losses. More commonly, however, plants are affected at the maximum tillering stage. Yields are reduced by 10–20%. BB is prevalent in both tropical and temperate climates, and reportedly in all rice growing regions worldwide except North America (Niño-Liu et al. 2006, Ou 1985).

BB caused by *Xoo* has been ranked fourth among the ten most destructive bacterial plant pathogens (Mansfield et al. 2012) because of the great losses it causes to rice production. Many control measures have been assessed and practiced to combat BB disease. They encompass biological control, chemical control, cultural control, and physical control, as reviewed by Ezuka and Kaku (2000). However, varietal resistance is regarded as the most effective and economical means of controlling BB (Khan et al. 2014, Verdier et al. 2012).

About 40 known resistance genes (R genes) confer resistance to various strains of *Xoo* (http://www.shigen.nig.ac.jp/rice/oryzabase/locale/change?lang=en, Busungu et al. 2016, Khan et al. 2014, Xia et al. 2012). These R genes have been identified in both cultivated rice cultivars and wild relatives of rice, and some from mutation induction. However, rapid changes in the pathogenicity of *Xoo* and the continuous evolution of pathogenic races are causing the breakdown of resistance in many improved varieties (Khan et al. 2014, Xia et al. 2012). Consequently, novel BB R genes are still highly demanded for rice. More analyses of these R genes are needed to combat this destructive disease effectively in the future. A report of our previous study (Busungu et al. 2016) describes the identification of a recessive *R* gene from the ‘XM14’ line. This gene was named and registered as *xa42* at the *XA42* locus according to the gene nomenclature system for rice (McCouch and CGSNL 2008).

**Research Paper**

*High-resolution mapping and characterization of *xa42*, a resistance gene against multiple *Xanthomonas oryzae* pv. *oryzae* races in rice (*Oryza sativa* L.)*

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Improvement of resistance against rice bacterial blight (BB) disease is an important breeding strategy in breeding programs across the world, especially in Africa and southern Asia where BB is more prevalent. This report describes a high-resolution map and characterization of *xa42* at *XA42* locus, a rice BB resistance gene in XM14, a mutant line originating from IR24. The candidate gene region was narrowed down from 582 kb, which had been obtained in our previous study, to 57 kb. XM14 shows brown spots in its leaves like lesion mimic mutants. This line also shows a shorter stature than the original cultivar IR24. In *XA42* gene segregating populations, homozygotes of *xa42* allele were consistently resistant to the six Japanese *Xanthomonas oryzae* pv. *oryzae* races used for this study. They also showed brown spots and markedly short stature compared with the other genotypes, suggesting that *xa42* gene exhibits pleiotropic effects.

**Key Words:** DNA marker, resistance by a recessive gene, pleiotropic effect, lesion mimic mutant, brown spot, *Oryza sativa* L., bacterial blight resistance.

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High-resolution mapping and characterization of **xa42** in rice

**Materials and Methods**

**Bacterial races, inoculation and scoring**

_Xoo_ have been classified into many races according to virulence and origin. The present study used six Japanese races for inoculation tests: race I (strain T7174), race IIA (strain T7147), race IIB (strain H9387), race III (strain T7133), race IV (strain H75373) and race V (strain H75304). To conduct the inoculation tests, each _Xoo_ inoculum was prepared and cultured using potato semi-synthetic agar medium (Wakimoto 1954) and was incubated at 28°C for 48 hr. The inoculum was then diluted with distilled water. Subsequently, the absorbance was adjusted to \( A = 0.05 \) (620 nm) using a spectrophotometer. This value corresponds to the concentration of \( 10^8 \) colony-forming units per milliliter (cfu/ml), which is the optimum concentration required to cause BB disease. _Xoo_ was inoculated using clipping method, a procedure described by Kauffman _et al._ (1973), during booting to the flowering stage. BB severity was scored by measuring the lesion length (LL) of the inoculated leaves of rice plants with measurement using a ruler 18 days after _Xoo_ inoculation.

**Plant materials**

‘IR24’ is an elite Indica rice cultivar produced by the International Rice Research Institute. IR24 is highly susceptible to all Philippines and Japanese _Xoo_ races (Busungu _et al._ 2016, Taura _et al._ 1991). ‘XM14’ is a resistant mutant line produced by induction of MNU mutagen to IR24 (Busungu _et al._ 2016). ‘IAS16’ is a chromosome segment substitution line carrying segments from a Japonica cultivar ‘Asominori’ with the background of IR24 (Kubo _et al._ 2002). IAS16 carries the chromosome segment containing _XA42_ locus from Asominori. It is susceptible to the six Japanese _Xoo_ races described above (Busungu _et al._ 2016).

Progeny of the cross from the cross between XM14 and IAS16 was used for this study. All plant materials were grown and analyzed in the following manner, with some exceptional cases described in the next subsections. Germinated seeds were sown in seedling boxes in a greenhouse in May. About two weeks after sowing, seedlings were transferred out of the greenhouse. Then, after about three weeks, seedlings were transplanted to a paddy field in the experimental farm of the Faculty of Agriculture, Kagoshima University, Kagoshima, Japan. Fertilizers were applied one week after transplantation at the rate of 6 g of N, 3 g of K\(_2\)O, 3 g of P\(_2\)O\(_5\)/m\(^2\). Two weeks before _Xoo_ inoculation test, N was applied at a rate of 3 g/m\(^2\). The plant spacing was 15 × 30 cm.

**High-resolution mapping of XA42**

In 2015, about 1000 F\(_2\) plants and 20 plants from each parental line were subjected to high-resolution mapping of _XA42_. In 2016, 2950 F\(_2\) plants and 30 plants from each parental line were subjected to high-resolution mapping in the same manner as 2015. In addition to F\(_2\) plants, 17 F\(_3\) lines (30 plants per line with some exceptional cases) from recombinants obtained in 2015 were subjected to the progeny test for the genotype of _XA42_ locus. _Xoo_ race IIA (strain T7147) was inoculated at the booting stage. Some F\(_2\) lines were also inoculated with the other five _Xoo_ races for resistance against multiple races (see below). The LL of one representative leaf from each plant was measured after careful visual observation.

**Test for resistance of xa42 against multiple Xoo races**

In 2016, six F\(_3\) lines were selected so that _XA42_ gene segregation was expected and that recombination had occurred very close to _XA42_ gene (Table 2). About 150 plants per F\(_3\) line were transplanted to the paddy field. At the booting stage, the tillers of each plant from each line were
Test for pleiotropic effects of xa42 gene on brown spots and agronomic traits

The XM14 line exhibits brown spots in its leaves (Fig. 1). In all, 982 F2 plants for xa42 mapping in 2015, 2950 F2 plants for xa42 mapping and the six F3 lines for test for resistance of xa42 against multiple Xoo races in 2016 were evaluated for their brown spots immediately before Xoo inoculation test because the presence or absence of brown spots on leaves was most clearly recognizable at the stage. The ‘B’ score was given to plants with brown spots on their leaves; the ‘N’ score was given to those with normal leaves. At the maturity stage in 2016, three agronomic traits, culm length, plant height and number of tillers, were evaluated in F2 generation (250 plants), F3 generation (297 plants), parental lines (20 plants per line), and IR24 (20 plants per line). The F2 plants in this test were selected randomly in 2950 F2 plants. The F3 came from the progeny of recombinant plant Nos. 10 and 14 (Table 2), two of the six F3 lines used for multiple Xoo races test of xa42 gene. Statistical analyses were conducted using software (SPSS statistics 23; IBM Inc. New York, USA).

Molecular techniques

Two weeks before inoculation tests, leaf samples from each F2 and F3 plant were collected for DNA extraction. DNA was extracted from 50 mg leaf of each plant in segregating populations according to the method described by Dellaporta et al. (1983) with some modifications. The polymerase chain reaction (PCR) mixture (6 µL) consisted of 10 ng genomic DNA, 200 µM dNTPs, 0.2 µM of each primer, 0.25 U of Taq polymerase (AmpliTaq Gold; Applied BioSystems, CA, USA), and 10× buffer containing MgCl2. PCR was performed under the following conditions: 4 min at 94°C for initial denaturation, followed by 35 cycles of 94°C for 30 s, annealing temperature for 30 s, 72°C for 30 s, with final extension at 72°C for 7 min. The PCR products were separated in 10% (29 : 1) polyacrylamide gels, stained with ethidium bromide, and visualized using ultraviolet light (GelDoc-It® TS Imaging System; UVP, CA, USA). Before electrophoresis, the PCR products of CAPS and dCAPS markers were digested using the respective restriction enzymes.

DNA markers/restriction enzyme

For this study, we used 19 DNA markers (Table 1), 3 of which have already been described by Busungu et al. (2016). The other 16 markers, that is, 1 SSR, 2 dCAPS, 2 CAPS markers and 11 Insertion/deletion (Indel), were designed according to procedure elaborated below. In Busungu et al. (2016), XA42 gene was located between DNA markers KGC3_16.1 and RM15189. Apparently, a single sequence repeat (SSR) located between them was available. We checked for uniqueness of the DNA sequences surrounding the SSR using BLAST with BLASTScope in Oryzabase (Yamazaki et al. 2010, http://www.shigen.nig.ac.jp/rice/oryzabaseV4/blast/search). Primer pairs were designed automatically using software (PRIMER 3 ver. 4; http://bioinfo.ut.ee/primer3-0.4.0/primer3/, Untergasser et al. 2012). We adopted the following primer design parameters: Primers were 20–35 nucleotides long, with optimum set at 25. Primer Tm was 55–65°C with optimum set at 60°C. The maximum Tm difference was 2°C. Primer GC% contents of 20–80.

For Indel markers, we used Indel information released by Xu et al. (2012) or looked for Indel polymorphism (5–50 bp difference) between a Japonica cultivar ‘Nipponbare’ and an Indica cultivar ‘93-11’ and/or an Indica cultivar HR12. Oryza sativa (rice) Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BlastScope&PROG_SPEC=OGP_4530_9512) was used for Indel information. Blastn search optimized for highly similar sequences was applied using one thousand to ten thousand base Nipponbare sequence (Os-Nipponbare-Reference-IRGSP-1.0) as query and 93-11 sequence (GCA_0000046551) or HR12 sequence (GCA_000725085) as subject. Uniqueness of the DNA sequences surrounding Indel was confirmed using BLAST with BLASTScope in Oryzabase (Yamazaki et al. 2010).

As for CAPS and dCAPS markers, we used SNP information from Huang et al. (2012) and the Rice SNP seeker database (Alexandrov et al. 2015). Using various SNP datasets available in a single interface (Mansueto et al. 2017), we were able to detect SNP which can distinguish Indica (IR24) and Japonica (Asominori). We confirmed the SNP information using the genome information of five rice cultivars: three Japonica cultivars, Nipponbare, Hitomebore (GCA_000321445.1), and Koshihikari (GCA_000164945.1), and two Indica cultivars, 93-11 and HR12 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP_4530_9512). The DNA sequences surrounding the SNP were checked for uniqueness using BLAST with BLASTScope in Oryzabase (Yamazaki et al. 2010). DNASIS Pro software (ver. 2.10; Hitachi Software Engineering Co. Ltd., Tokyo, Japan) was used to detect restriction enzymes that can recognize SNP.

Primers surrounding Indel and SNP were designed using Primer 3 (Untergasser et al. 2012) with the parameters above. When SNPs were not detected by restriction enzymes, dCAPS markers were designed using dCAPS finder software (Neff et al. 2002).
Mapping and gene annotation

F<sub>2</sub> plant recombinants were used to determine the exact position of the nearest recombination event to the target region on chromosome 3 centromeric region. The high-resolution mapping of the target XA42 gene was constructed according to the physical distance of the DNA markers used in the study (Table 1). In addition to F<sub>2</sub> plants, the progeny test of all recombinants obtained in 2015 was applied to confirm the recombination events, segregation or non-segregation reaction against Xoo. Candidate genes of XA42 were sought within the candidate chromosomal region using ‘the rice annotation project database (RAP-DB)’ (http://rapdb.dna.affrc.go.jp/) (Kawahara et al. 2013, Sakai et al. 2013) and ‘rice genome annotation project’ (rice.plantbiology.msu.edu/) (Kawahara et al. 2013).

Results

High-resolution mapping of XA42 gene

In 2015, 982 F<sub>2</sub> plants from the cross between IAS16 and XM14 were evaluated for LL inoculated with Xoo race IIA (strain T7147). The distribution of LL showed a bimodal distribution (Fig. 2). The dividing point was set at 5 cm LL because of the clear gap of LL at 5 cm. LL shorter than 5 cm was regarded as resistant, whereas LL longer than 5 cm was regarded as susceptible to BB. Based on that dividing point, the resistant and susceptible plants were, respectively, 288 and 694. In 2016, the 2950 F<sub>2</sub> plants from the same cross combination were evaluated for LL inoculated with Xoo race IIA (strain T7147). The segregation indicated a clear bimodal distribution of LL (Fig. 3). We observed a clear gap of LL around 4 cm and classified the 2950 F<sub>2</sub> plants into 885 resistant plants with LL shorter than 3 cm and 2065 susceptible plants with LL longer than 3 cm.
## Table 2
Genotypes of informative recombinants for the DNA marker loci linked with XA42 in the F2 population (XM14 × IAS16), brown spots, and reaction against Xoo Japanese race IIA (T7147) inoculation in the F2 and F3 generations

| F2 Individual | Year | Lesion length | Reaction | Brown spots | Genotypes of the DNA marker loci | No. of F3 plants |
|---------------|------|---------------|----------|-------------|----------------------------------|-----------------|
|               |      |               |          |             | KGC3 16.1 | KGC3 16.180 | KGC3 16.209 | KGC3 16.255 | KGC3 16.278 | KGC3 16.3 | KGC3 16.342 | KGC3 16.370 | KGC3 16.371 | KGC3 16.399 | KGC3 16.407 | KGC3 16.421 | KGC3 16.514 | KGC3 16.594 | KGC3 16.613 | KGC3 16.636 | RM |
| 1             | 2015 | 18.8          | S        | N            | A       | A       | A       | A       | A       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | 7 : 22 |
| 2             | 2015 | 14.6          | S        | N            | A       | A       | A       | A       | A       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | 0 : 30 |
| 3             | 2015 | 19.4          | S        | N            | H       | H       | H       | H       | H       | A       | A       | A       | A       | A       | A       | A       | A       | A       | A       | A       | 0 : 30 |
| 4             | 2015 | 31.5          | S        | N            | H       | H       | H       | H       | H       | A       | A       | A       | A       | A       | A       | A       | A       | A       | A       | A       | 0 : 30 |
| 5             | 2015 | 18.3          | S        | N            | H       | H       | H       | H       | H       | A       | A       | A       | A       | A       | A       | A       | A       | A       | A       | A       | 0 : 30 |
| 6             | 2015 | 21.6          | S        | N            | H       | H       | H       | H       | H       | A       | A       | A       | A       | A       | A       | A       | A       | A       | A       | A       | 6 : 24 |
| 7             | 2015 | 0.1           | R        | B            | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | 30 : 0 |
| 8             | 2015 | 2.8           | R        | B            | H       | H       | H       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | 30 : 0 |
| 9             | 2015 | 12.7          | S        | N            | H       | H       | H       | H       | H       | H       | H       | H       | X       | X       | X       | X       | X       | X       | X       | X       | 31 : 28 |
| 10            | 2015 | 14.5          | S        | N            | X       | X       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | 31 : 109|
| 11            | 2015 | 22.1          | S        | N            | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | X       | X       | X       | X       | X       | 42 : 117|
| 12            | 2015 | 1.1           | R        | B            | X       | X       | X       | X       | X       | X       | X       | X       | X       | H       | H       | H       | H       | H       | H       | H       | 30 : 0 |
| 13            | 2015 | 0.3           | R        | B            | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | 30 : 0 |
| 14            | 2015 | 23.3          | S        | N            | X       | X       | X       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | 43 : 110|
| 15            | 2015 | 18.5          | S        | N            | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | 42 : 117|
| 16            | 2015 | 23.4          | S        | N            | X       | X       | X       | X       | X       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | 34 : 116|
| 17            | 2015 | 18.2          | S        | N            | X       | X       | X       | X       | X       | X       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | 5 : 24  |
| 18            | 2016 | 0.2           | R        | B            | H       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 19            | 2016 | 0.1           | R        | B            | H       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 20            | 2016 | 0.1           | R        | B            | H       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 21            | 2016 | 0.1           | R        | B            | H       | H       | H       | H       | H       | H       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 22            | 2016 | 0.1           | R        | B            | H       | H       | H       | H       | H       | H       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 23            | 2016 | 22.8          | S        | N            | H       | H       | H       | H       | H       | H       | H       | H       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 24            | 2016 | 0.1           | R        | B            | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 25            | 2016 | 0.2           | R        | B            | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 26            | 2016 | 13.0          | S        | N            | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | H       | H       | H       | H       | H       | H       | Not tested|
| 27            | 2016 | 26.5          | S        | N            | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | X       | Not tested|
| 28            | 2016 | 19.4          | S        | N            | X       | X       | X       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | Not tested|
| 29            | 2016 | 25.0          | S        | N            | X       | X       | X       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | Not tested|
| 30            | 2016 | 13.6          | S        | N            | X       | X       | X       | X       | X       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | H       | Not tested|

\(^{a}\)X, H, and A respectively denote homozygotes for XM14, heterozygotes, and homozygotes for IAS16.

\(^{b}\)Progeny of Plant Nos. 9, 10, 11, 14, 15, and 16 were subjected to test for resistance of xa42 against multiple Xoo races.

\(^{c}\)R and S respectively denote resistant and susceptible.

\(^{d}\)B and N respectively denote plants showing brown spots on their leaves and those showing normal leaves.

\(^{e}\)LL shorter than 3.0 cm were scored as R, whereas those longer than 5.0 cm were scored as S. As for the materials for resistance against multiple races, reactions to multiple races were summed up (see Fig. 5).
High-resolution mapping and characterization of xa42 in rice

For KGC3_16.370 as indicated: (strain T7147) inoculation. Three classified genotypes were assessed from the cross between XM14 and IAS16 after 2015, a total of 17 recombinants between them were selected. Then they were subjected to genotyping of internal DNA markers (Table 1). In addition to F2 tests, we performed F3 test of 17 recombinants. The results of genotyping of informative F2 plants and their F3 tests facilitated detailed mapping of the XA42 gene (Table 2).

Genetic information from each informative recombinant was mutually consistent (Table 2). The discontinuous LL distribution like Fig. 3 was observed in F3 lines in Table 2 in 2016: LL shorter than 3.0 cm were scored as resistant (R), whereas those longer than 5.0 cm were scored as susceptible (S). Important recombination events occurred between DNA markers KGC3_16.3 and KGC3_16.407. Plant Nos. 12 and 13 both show that the XA42 gene is located on the left side of DNA marker KGC3_16.399 because the F3 lines of the two plants were fixed for resistant plants with brown spots. Plant No. 17 showed that the gene was located on the right side of DNA marker KGC3_16.341 because the F3 line of the plant is composed of 5 resistant plants with brown spots and 24 susceptible plants with no brown spots. The result for Plant No. 22 was consistent with that, although its progeny test was not performed. Therefore, XA42 gene was located in the 57 kb-chromosomal region between DNA markers KGC3_16.341 and KGC3_16.399. Results for other plants in Table 2 all support this idea. A linkage map comparing results obtained from our previous study (Busungu et al. 2016) and this study is portrayed in Fig. 4.

The search for candidate gene using RAP-DB (http://rapdb.dna.affrc.go.jp/) found five ORFs in the candidate region of XA42 (Table 3). The rice genome annotation project (rice.plantbiology.msu.edu/) found seven ORFs, three of which might correspond to those found in RAP-DB, and two of which encoded retrotransposon proteins (data not shown). The other two are LOC_Os03g28430 described as encoding hypothetical protein and LOC_Os03g28420 described as 3-oxoacyl-synthase. Its first exon corresponds to Os03g40220.

**Resistance of xa42 to multiple Xoo races**

Inoculation of Xoo to parental lines showed that IAS16 line was susceptible, whereas the XM14 line was resistant to the six Japanese Xoo races used for this study. All F2 lines showed segregation in the reaction against the six races (Fig. 5). For this study, we inoculated two races to one plant. The reaction against one race was almost identical to that against the other race. When a plant showed short LL against one race, it showed short LL against the other race. A plant showing long LL against one race showed long LL against the other race. The parental F2 plants of F3 lines used in this experiment were all heterozygous at KGC3_16.370 locus, which proved to co-segregate with XA42 in the

### Table 1

| Plant | Genotype | KGC3_16.370 | KGC3_16.594 |
|-------|----------|-------------|-------------|
| No. 1 | 1/2      | R           | R           |
| No. 2 | 1/2      | S           | S           |
| No. 3 | 1/2      | R           | S           |

### Table 2

| Plant | Genotype | KGC3_16.370 | KGC3_16.594 |
|-------|----------|-------------|-------------|
| No. 4 | 1/2      | R           | R           |
| No. 5 | 1/2      | S           | S           |
| No. 6 | 1/2      | R           | S           |

### Table 3

| ORF     | Description                  |
|---------|------------------------------|
| LOC_Os03g28430 | encoding hypothetical protein |
| LOC_Os03g28420 | 3-oxoacyl-synthase           |
DNA marker KGC3_16.370 locus showed that these plants were homozygotes of XM14 allele. Therefore, they proved to be homozygotes of \( xa42 \) allele. In contrast, all the F\(_2\) and F\(_3\) with normal leaves exhibited susceptible reactions to the \( Xoo \) races used for this study and were either homozygotes of IAS16 allele or heterozygote at KGC3_16.370 locus. These results suggest strongly that brown spots are caused by \( xa42 \).

### Pleiotropic effect of \( xa42 \) gene on agronomic traits

Table 6 presents the culm length, plant height and number of tillers of parental lines (XM14, IR24, IAS16), the F\(_2\) population and sum of the two F\(_3\) lines derived from the cross between XM14 and IAS16. Analysis of variance (ANOVA) revealed significant difference among parental lines, and among genotypes at KGC3_16.370 locus in the segregating populations for the entire three traits (data not shown). According to the multiple mean comparisons, XM14 showed significantly smaller value than IAS16 and previous subsection. Homozygotes of XM14 allele at KGC3_16.370 locus showed very short LL (shorter than 1 cm in most cases), indicating that they were resistant against inoculated \( Xoo \) races. Heterozygotes and homozygotes of IAS16 allele at the locus showed long LL (longer than 5 cm in most cases), indicating that they were susceptible to inoculated \( Xoo \) races (Fig. 5). When the six F\(_3\) lines were summed up, the segregating chromosomal region around \( XA42 \) gene was limited to 121 kb (Tables 1, 2).

### Relation between resistance to \( Xoo \) and brown spots

In 2015, 982 F\(_2\) plants were examined for the presence of brown spots on their leaves. All 288 resistant plants were found to exhibit brown spots, although the 694 susceptible plants showed no signs of brown spots. In 2016, we used 2950 F\(_2\) and 920 F\(_3\) plants for the presence of brown spots (Tables 4, 5). Results showed that all the F\(_2\) and F\(_3\) plants which had brown spots consistently exhibited a resistant reaction to \( Xoo \) races used for this study. Genetic analysis at DNA marker KGC3_16.370 locus showed that these plants were homozygotes of XM14 allele. Therefore, they proved to be homozygotes of \( xa42 \) allele. In contrast, all the F\(_2\) and F\(_3\) with normal leaves exhibited susceptible reactions to the \( Xoo \) races used for this study and were either homozygotes of IAS16 allele or heterozygote at KGC3_16.370 locus. These results suggest strongly that brown spots are caused by \( xa42 \).

### Table 3. Annotation data by RAP-DB of putative ORFs in the candidate chromosomal region of \( XA42 \)

| ORF in RAP-DB     | Location on IRGSP 1.0 pseudomolecule of chromosome 3 | Description in RAP-DB                                                                 | Predicted length (bp) |
|-------------------|------------------------------------------------------|-------------------------------------------------------------------------------------|-----------------------|
| Os03g0401951      | 16358834-16359470 (+ strand)                         | Hypothetical gene                                                                  | 637                   |
| Os03g0402000      | 16359486-16362281 (– strand)                         | TRAPP I complex, Bet3 domain containing protein                                     | 993                   |
| Os03g0402200      | 16379079-16378724 (– strand)                         | Hypothetical protein                                                               | 274                   |
| Os03g0402400      | 16384695-16386794 (– strand)                         | Similar to ribosome-associated protein p40-like                                      | 1146                  |
| Os03g0402600      | 16397246-16397680 (– strand)                         | Predicted gene                                                                     | 435                   |
Fig. 5. Lesion length distribution of the F$_3$ plants from the cross between XM14 and IAS16 after six Japanese Xoo races. Each F$_3$ line, the progeny of recombinants in Table 2, was divided into three sublines comprising ca. 50 plants. Sublines were inoculated with two Xoo races shown along axes of subfigures. X, solid circle and open triangle respectively denote homozygotes for XM14, heterozygotes and homozygotes of IAS16 at the KGC3_16.370 locus. Dotted lines denote the dividing point between resistant and susceptible plants.
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Table 4. Relations between genotypes at KGC_16.370 locus, brown spots and reaction against Xoo Japanese race IIA (strain T7147) inoculation in the F2 population from the cross between IAS16 and XM14

| Year | Genotype at KGC3_16.370 locus | Reaction against Xoo race IIA | Brown spots |
|------|-------------------------------|-----------------------------|-------------|
|      |                               | Resistant | Susceptible | B | N |
| 2015 | Homozygote for XM14 allele     | 288       | 0           | 288 | 0  |
|      | Heterozygote                  | 0         | 506         | 0  | 506|
|      | Homozygote for IAS16 allele   | 0         | 188         | 0  | 188|
| 2016 | Homozygote for XM14 allele     | 885       | 0           | 885 | 0  |
|      | Heterozygote                  | 0         | 1529        | 0  | 1529|
|      | Homozygote for IAS16 allele   | 0         | 536         | 0  | 536|

* B and N respectively denote plants showing brown spots on their leaves and those showing normal leaves.

Table 5. Relations between brown spots and genotypes at KGC_16.370 locus in the F3 lines used for resistance against multiple Xoo strains

| Genotype at KGC3_16.370 | Brown spots in F3 linesa derived from recombinant plant No. |
|-------------------------|-------------------------------------------------------------|
|                         | B   | N   |   | B   | N   |   | B   | N   |   | B   | N   |   |
| Homozygote for XM14 allele | 31   | 31  | 42 | 43  | 42  | 34 |
| Heterozygote            | 80   | 73  | 80 | 80  | 87  | 79 |
| Homozygote for IAS16 allele | 48   | 36  | 37 | 30  | 30  | 37 |
| Total                   | 159  | 140 | 159| 153 | 159 | 150|

* B and N respectively denote plants showing brown spots on their leaves and those showing normal leaves.

Table 6. Tukey HSD mean comparisons of culm length (cm), plant height (cm) and the number of tillers of parental lines (XM14, IR24, and IAS16), and F2 and segregating F3 lines at KGC3_16.370 locus derived from the cross between XM14 and IAS16

| Group | Subgroup | Number of plants | Culm length (cm) | Plant height (cm) | Number of tillers |
|-------|----------|------------------|------------------|------------------|------------------|
|       |          |                  |                  |                  |                  |
| Parental lines | XM14 | 20 | 60.5 a | 79.1 a | 9.5 a |
|       | IR24 | 20 | 63.1 b | 84.0 b | 10.6 ab |
|       | IAS16 | 20 | 64.2 b | 86.2 b | 11.6 b |
| F2    | Homozygote for XM14 allele | 73 | 59.7 a | 81.3 a | 10.1 a |
|       | Homozygote for IAS16 allele | 45 | 67.5 b | 91.0 b | 12.3 ab |
|       | Heterozygote | 132 | 69.5 b | 92.6 b | 14.0 b |
| F3    | Homozygote for XM14 allele | 74 | 60.7 a | 81.2 a | 10.2 a |
|       | Homozygote for IAS16 allele | 66 | 65.5 b | 86.0 b | 11.6 ab |
|       | Heterozygote | 153 | 67.2 b | 88.0 b | 13.0 b |

* Values followed by the same letter in each trait in the same group are not significantly different at P = 0.05 according to Tukey’s HSD test.

IR24 for culm length and plant height. In the segregating population, homozygotes of XM14 showed a much smaller value than heterozygotes and homozygotes of IAS16 allele for culm length and plant height. We compared homozygotes of XM14 allele and the combination of the other genotypes, which corresponded respectively to resistant plants and susceptible plants in F2 and F3 populations, by applying t-tests: resistant plants showed significantly smaller values than susceptible plants for all three traits (data not shown).

Discussion

In this study, we first performed high-resolution mapping of XA42, narrowing the area of interest to 57 kb located between the two DNA markers, KGC3_16.342 and KGC3_16.399. This gene cosegregated with two DNA markers, KGC3_16.370 and KGC3_16.371. Five candidate genes were found using RAP-DB. With the aid of this DNA-marker assisted analysis, the recessive allele xa42 was thought to be resistant to the entire Japanese Xoo races used for this study, and to cause brown spots on leaves and shorter stature.

The XA42 gene candidate region was narrowed down from 582 kb (between KGC3_16.1 and RM15189) (Busungu et al. 2016) to 57 kb (KGC3_16.342 and KGC3_16.399) (Table 2). The high-resolution map depicted in this study (Fig. 4) established the foundation for XA42 gene map-based cloning and function characterization. As discussed in our previous study (Busungu et al. 2016), based on IRGSP 1.0 genome and RAP-DB, the candidate chromosomal region of XA42 does not contain genes encoded by three isolated recessive BB R genes: xa5, xa13 and xa25. No isolated plant disease R gene encodes 3-oxoacyl-synthase or TRAPP I complex, Bet3 domain containing protein, or ribosome-associated protein (p40-like), which was annotated in XA42 candidate region in the present study (Table 3). Similarly, no isolated plant lesion mimic mutant (see below) gene
encoded these proteins (for a review, Bruggeman et al. 2015). These data suggest that \textit{xa42} is a new R gene. However, we downloaded the chromosome 3 sequences of nine cultivars (‘Nipponbare’ (IRGS 1.0, NC_029258.1), ‘Nipponbare’ (assembled by Beijing Genomics Institute, CM000140.1), ‘HEG4’ (CM003066.1), ‘A123’ (CM003078.1), ‘Hitomebore’ (DG000055.1), ‘Koshihikari’ (DG000027.1), ‘RP Bio-226’ (CP012611.1), ‘93-11’ (CM000128.1), ‘IR8’ (CM007598.1), ‘Kasalath’ (no accession name, downloaded from http://rapdb.dna.affrc.go.jp/download/irgsp1.html)), and found that the DNA length surrounded by the two DNA markers KGC3_16.342 and KGC3_16.399 ranged from 57 kb (NC_029258.1) to 143 kb (CM003066.1). Both NC_029258.1 and CM000140.1 are Nipponbare chromosome 3 sequences, but the \textit{XA42} candidate region length of CM000140.1 is 132 kb. Our preliminary analysis of these genome alignments suggests that most of the sequences not covered by IRGS 1.0 (NC_029258.1) are repetitive sequences scattered in rice genome (data not shown). However, some of these sequences could be unique, and contain genes not found in IRGS 1.0 (data not shown). These data suggest that there might have been other candidate genes for \textit{XA42} than listed in Table 3. To identify \textit{XA42} gene, DNA sequencing of the candidate \textit{XA42} region of both XM14 and IR24 is necessary.

Because the probability of identifying \textit{Xoo} resistant mutant is small (Taura et al. 1991), the existence of simultaneous plural resistance mutations on one mutant line seems improbable. Therefore, these results strongly support the idea that \textit{xa42} is resistant to the six Japanese \textit{Xoo} races. Suzuki et al. (2008) induced mutation in a Japonica cultivar Taichung 65 using 1 mM of MNU, the same method of obtaining XM14 (Busungu et al. 2016), and estimated the mutation frequency as $3.2 \times 10^5$ nucleotide changes in a $4.3 \times 10^8$ rice genome corresponding to one mutation in every 135 kb. If the estimate is applied to our experimental results and the above rice genome information, there is high possibility that only one mutation occurred in the \textit{XA42} candidate chromosomal region. Therefore, these findings strongly support the idea that one mutation in \textit{XA42} locus induced resistance to the six Japanese \textit{Xoo} races, brown spots on leaves and shorter stature.

Results show a strong and significant correlation between BB resistance and brown spots, which suggests that \textit{xa42} exhibits a pleiotropic effect. Brown spot mutants are generally called spotted leaf (\textit{spl}) in rice. They are regarded as lesion mimic mutants. Yin at al. (2000) reported that four mutants (\textit{spl1}, \textit{spl5}, \textit{spl9}, and \textit{splII}) show enhanced resistance to blast, and that \textit{splII} shows resistance to four Philippine \textit{Xoo} races. Lesion mimic mutants have been studied extensively in light of programmed cell death leading to resistance to pathogens in many plants such as wheat (Li and Bai 2009) and \textit{Arabidopsis}, as reviewed by Lorrain et al. (2003). Therefore, \textit{xa42} might be a kind of lesion mimic mutant. However, the brown spots on its leaves are not as dense and thick as those of typical \textit{spl} mutants (Fig. 1). According to Oryzabase, two \textit{spl} genes, SPL3 and SPL30, were registered as located on chromosome 3. A comparative linkage map of chromosome 3 (https://shigen.nig.ac.jp/rice/oryzabase/marker/mapCirn/3) based on findings reported by Harushima et al. (1998), Tsunematsu et al. (1996), and Yoshimura et al. (1997) shows that SPL3 is located on the short arm, not close to the centromeric region. Therefore, SPL3 and \textit{XA42} differ. SPL30 was found to cosegregate with a DNA marker RM15380 in the segregating population comprising 2890 plants (Huang et al. 2011). RM15380 is located in 18,632 kb region in Rice_IRGSP_Ver1.0, which is more than 2000 kb from the \textit{XA42} candidate region (Fig. 4). Therefore, \textit{XA42} differs from two reported \textit{spl} genes on chromosome 3. Of about 40 reported rice BB resistance genes (Busungu et al. 2016, Khan et al. 2014), only \textit{Xa3} gene was reported to exhibit brown spots resembling disease symptoms (Kaku and Hori 1977). Cultivars carrying the \textit{Xa3} resistance gene develop the brown spots as a necrotic resistant response after inoculating the plant with \textit{Xoo} inoculums (Kaku and Hori 1977, Kaku and Ogawa 2001). The positive relation between brown spots and BB resistance reported in \textit{Xa3} seems similar to our observation in \textit{xa42}. However, the brown spots in \textit{Xa3} appear after inoculation in contrast to those of \textit{xa42} with brown spots appearing even without inoculation of \textit{Xoo}.

The pleiotropic effect of \textit{xa42} on agronomic traits can sometimes be negative in rice production when \textit{xa42} is used for breeding rice cultivars with multiple \textit{Xoo} resistance. Therefore, the combination of \textit{xa42} and genes masking the negative effect of \textit{xa42}, for example, allele conditioning tall stature on QTL controlling plant height, represents a possible solution.

\textit{XA42} gene segregation in F2 generation was distorted. However, when reactions to multiple strains were summed up for each F2 line, the ratio of resistant: susceptible all fit to 1 : 3 ($0.10 < P < 0.70$ for $\chi^2$ (1 : 3)), was expected from one-gene segregation (Table 2, the progeny of Plant Nos. 9, 10, 11, 14, 15, and 16). According to Fukuta et al. (2000), segregation of chromosomal regions of both the short arm and long arm on chromosome 3 were skewed in favor of Indica allele. Some possibility exists that genes on one arm are insufficient to distort segregation, and that the F2 lines were fixed for one arm, not expressing segregation distortion.

Repeated R gene failure and breakdown upon pressure from new strains and favorable environment to \textit{Xoo} have been reported many times, as reviewed by Khan et al. (2014). Planting of rice varieties with broad-spectrum disease resistance is the most sustainable strategy to protect rice from diseases and to ensure stable rice production. Results from this study suggest strongly that XM14 line, which has \textit{xa42}, falls in the category of race-non-specific or broad-spectrum resistance. To prove that \textit{xa42} is truly a broad spectrum R gene, it should be tested with international \textit{Xoo} races, especially those from south Asian and African
countries where putative new Xoo races have been reported (Gonzalez et al. 2007, Mishra et al. 2013, Verdier et al. 2012). If proven, the xa42 gene will be very useful in resistance breeding programs.

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