Development of introgression lines derived from *Oryza rufipogon* and *O. glumaepatula* in the genetic background of *japonica* cultivated rice (*O. sativa* L.) and evaluation of resistance to rice blast

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Wild relatives of cultivated rice (*Oryza sativa* L.) are useful sources of alleles with economic value for rice breeding programs. To effectively identify such alleles, three sets of introgression lines (ILs) carrying one or more chromosome segments derived from wild relatives with the A genome in the background of *japonica* rice cultivars Koshihikari and Itadaki were developed using marker-assisted backcrossing. The donor wild species include two accessions of *O. rufipogon* (IRGC Acc. 104814 and 104812) and one accession of *O. glumaepatula* (IRGC Acc. 100968) while 2 *japonica* rice cultivars, Koshihikari and Itadaki, were used as recurrent parents. The two sets of ILs from *O. rufipogon* in the Koshihikari background consisted of 40 and 47 lines, respectively. The average proportions of recurrent parental genome were 94.3% and 96.0% in the ILs derived from accessions 104814 and 104812, respectively. The ILs from *O. glumaepatula* in the Itadaki background consisted of 47 lines, with an average proportion of recurrent parental genome of 91.0%. To demonstrate the potential of these ILs in identifying useful alleles, the two sets of ILs were screened for resistance to rice blast (*Magnaporthe grisea*). By substitution mapping, two new loci for partial resistance to rice blast were detected on chromosomes 3 and 11 of *O. rufipogon* (104812).

**Key Words:** *Oryza rufipogon, Oryza glumaepatula, introgression lines (ILs), blast resistance, Magnaporthe grisea.*

**Introduction**

Rice is one of the most important staple food crops, feeding more than half of the world’s population. Rice (genus *Oryza*) has two cultivated species, *O. sativa* L. and *O. glaberrima* (Coffman and Herrera 1980), and 22 wild species containing high degree of genetic diversity (Brar and Khush 1997, Lu et al. 2002, Park et al. 2003, Ren et al. 2003, Sun et al. 2001). Wild rice species are generally inferior to that of cultivated rice in terms of agriculturally important traits. However, they carry many desirable genes that have been lost in cultivated rice. This explains why the wild rice species are adapted to unfavorable environments and show resistance to many pests and disease (Shan et al. 2009). It is important, therefore, to discover these useful genes hidden in wild rice species and use these in rice breeding programs.

To date, several resistance genes from wild rice have already been utilized to improve cultivated rice. These include genes for resistance to grassy stunt, bacterial blight, blast, and brown planthopper (Ishii et al. 1994, Jena and Khush 1990, Khush et al. 1977, 1990, Multani et al. 1994, Zhang et al. 2000). However, attempts to introduce genes controlling quantitative traits other than disease and insect resistance from wild rice relatives have been limited and unsuccessful due to cross incompatibilities between cultivated rice and its wild relatives. In addition, genes from wild rice which control good agronomic traits are often linked with genes governing undesirable traits.

 Tanksley et al. (1996) clearly explained that QTL mapping and alien gene introgression could be done simultaneously, and developed the advanced backcross quantitative trait loci (AB-QTL) strategy. Using the AB-QTL method, Brondani et al. (2002), Li et al. (2002), Moncada et al. (2001), Septiningsih et al. (2003), Thomson et al. (2003),...
Tian et al. (2006), and Xiao et al. (1996, 1998) identified QTLs for increased yield in *O. rufipogon* and *O. glumaepatula*. Thus, the method was proven effective not only in identifying genes that control qualitative traits such as disease and insect resistance but also genes for complex traits like grain yield.

Recent advances in molecular marker assisted breeding have made it possible to transfer genes from wild rice to cultivated varieties through backcrossing and selection. Moreover, introgression lines (ILs) and chromosome segment substitution lines (CSSLs), both of which carry a particular chromosome segment from a donor parent in the genetic background of a recipient parent, have been proven as powerful tools for detecting and precise mapping of QTLs as well as in evaluating gene action (Chetelat et al. 1995, Chetelat and Meglic 2000, Eshed and Zamir 1994, 1995, 1996, Kubo et al. 2002, Yano 2001). In particular, these lines are useful to detect QTLs with smaller effects that are masked by the influence of other QTLs with larger effects in primary populations such as F$_2$ populations and recombinant inbred lines (Ebitani et al. 2005, reviewed by Fukuoka et al. 2010). Several sets of ILs (Ahn et al. 2002, Doi et al. 1997, Kurakazu et al. 2001, Sobrizal et al. 1999, Tian et al. 2006) and CSSLs (Aida et al. 1997, Ebitani et al. 2005, Fukuoka et al. 2010, Kubo et al. 2002, Mei et al. 2006, Takai et al. 2007, Zhu et al. 2009) have been developed in rice, and several QTLs have been detected using these genetic resources. Kubo et al. (2002) described the development of chromosome segment substitution lines (CSSLs) as a possible alternative for resolving the issue of precise mapping of QTLs. To facilitate genetic mapping and map-based cloning of QTLs, it will be necessary to develop such novel mapping populations for a wide range of cross combinations (Ebitani et al. 2005). Currently, the availability of ILs derived from wild rice is limited. Thus, we embarked on developing ILs from wild rice and evaluate the breeding materials to explore and utilize potential genetic variation in wild relatives of rice. In this paper, we report the development of three sets of ILs derived from two accessions of *O. rufipogon* and one accession of *O. glumaepatula* in the background of cultivated *japonica* cultivars Koshihikari and Itadaki. Koshihikari is a leading cultivar in Japan because of superior eating quality and cool-temperature tolerance at booting stage. However, it is susceptible to lodging and has low level of resistance to blast. Itadaki, on the other hand, is resistant to lodging due to short culm, high yielding potential, moderate level of resistance to blast, and superior eating quality (Uehara et al. 2000).

Resistance to rice blast of *O. rufipogon* ILs was also surveyed. Blast disease, caused by *M. grisea*, is one of the most devastating diseases of rice worldwide. Incorporating rice blast resistance genes into improved cultivars has been considered a cost-effective and environmentally beneficial method of minimizing crop losses from this disease. Complete resistance is defined as race-specific resistance through a hypersensitive reaction caused by single major genes. Although effective at first, complete resistance is rapidly overcome in the field due to the emergence of new and highly virulent isolates of the pathogen (Kiyosawa 1982). Japanese upland rice cultivars have a major gene such as *ps1* and several QTLs for partial resistance (Fukuoka and Okuno 2001). Resistance in these cultivars remains stable for several decades. By pyramiding several genes for partial resistance, cultivars with durable resistance can be developed. DNA markers can facilitate breeding of such pyramided lines, but so far only a few genes for partial resistance to blast have been identified (Fujii et al., Fukuoka and Okuno 2001, Nguyen et al. 2006, Terashima et al. 2008, Zenbayashi et al. 2007). Therefore, it is necessary to identify new partial resistance genes. Here, we evaluated the partial resistance of two sets of ILs and identified several novel loci for partial resistance to rice blast in wild relatives.

### Materials and Methods

#### Plant materials

To develop the ILs, two accessions of *O. rufipogon* (IRGC Acc. No. 104814 and 104812) and one accession of *O. glumaepatula* (IRGC Acc. No. 100968, origin: Surinam) were used as donor parents while two *japonica* cultivars, Koshihikari and Itadaki served as the recurrent parents. The two accessions of *O. rufipogon* which originated from Thailand were selected as donors because of their superior seed fertility in preliminary crosses with *O. sativa*. These accessions, however, showed considerable differences in major characteristics such as plant type, leaf shape, and culm height.

#### Developing introgression lines of *O. rufipogon* and *O. glumaepatula*

The procedures used in developing the ILs of *O. rufipogon* (designated RTKILs for Acc. 104812 and KRLILs for Acc. 104814) are summarized in Fig. 1. Koshihikari (as the female parent) was crossed to the two accessions of *O. rufipogon*. F$_1$ plants (as female parent) were then backcrossed to Koshihikari to develop the BC$_3$F$_1$ progenies. The BC$_3$F$_1$ was backcrossed two more times to Koshihikari, without marker-assisted selection (MAS), to produce the BC$_5$F$_1$ generation. In the BC$_5$F$_1$ generation, whole-genome genotyping was performed by using simple sequence repeat (SSR) markers distributed across the 12 rice chromosomes. On the basis of the genotypes of the BC$_3$F$_1$ plants, appropriate BC$_4$F$_1$ plants for the development of the ILs were selected and backcrossed to Koshihikari to generate the BC$_5$F$_1$ generation. In the BC$_5$F$_1$ generation, plants heterozygous for *O. rufipogon* alleles in the target regions of each chromosome were selected. Finally, we selected plants homozygous for *O. rufipogon* alleles in the target regions and homozygous for *O. sativa* alleles in non-target regions of each chromosome in the BC$_5$F$_2$ and BC$_6$F$_2$ generations to establish the ILs.

The procedures for the development of ILs from *O. glumaepatula* (designated IGILs) are also shown in
Blast resistance of the ILs was evaluated in the upland nursery at the National Institute of Crop Science in Tsukuba, Ibaraki, Japan. The disease severity of 40 to 50-day-old seedlings was scored using a rating scale from 0 (highly resistant: no symptoms) to 10 (highly susceptible: leaves and stems totally dead) based on the diseased leaf area, in accordance with the methods of Asaga (1976). To increase the amount of inoculum in the nursery, the rice cultivar Inabawase, which has the donor genome of O. rufipogon, was used as a spreader. In addition to MAS, 41 BC\textsubscript{1}F\textsubscript{2} generation, 66 plants were backcrossed to Koshihikari, giving a total of 504 BC\textsubscript{1}F\textsubscript{1} seeds. These seeds produced 504 BC\textsubscript{1}F\textsubscript{2} plants, which were genotyped with SSR markers. Of these, 60 BC\textsubscript{1}F\textsubscript{2} plants confirmed to be heterozygous for chromosome regions from O. rufipogon were selected and successively backcrossed to Koshihikari.

Ninety-nine out of 505 BC\textsubscript{1}F\textsubscript{1} plants were selected on the basis of their genotypes and allowed to self to produce the BC\textsubscript{1}F\textsubscript{2} generation. Subsequently, 3,545 BC\textsubscript{1}F\textsubscript{2} plants were subjected to MAS, and 41 BC\textsubscript{2}F\textsubscript{2} plants were selected to establish the ILs (KRILs). Graphical genotypes of the 40 KRILs were determined by SSR markers across the 12 chromosomes, with gaps remaining on chromosomes 3, 4, 7, 8, 10, and 11. The percentage of substituted chromosome segments in each IL ranged from 0.8% to 89.9% of the 12 chromosomes, with gaps remaining on chromosomes 3, 4, 7, 8, 10, and 11. The percentage of substituted chromosome segments in each IL ranged from 0.8% to 12.6%, on the basis of the physical size of the rice chromosomes. The average proportion of recurrent parent in each IL was 94.3%. The average heading dates of the KRILs in 2006 and 2007 were distributed between 31 July and 3

**Results**

**Development of O. rufipogon (Acc.104814) introgression lines (KRILs)**

The F\textsubscript{1} plants derived from a cross between Koshihikari and O. rufipogon (104814) were backcrossed to Koshihikari to produce 63 BC\textsubscript{1}F\textsubscript{1} plants. These BC\textsubscript{1}F\textsubscript{1} plants were again backcrossed to Koshihikari to develop the BC\textsubscript{2}F\textsubscript{1} generation. From the BC\textsubscript{2}F\textsubscript{1} generation, 66 plants were backcrossed to Koshihikari, giving a total of 504 BC\textsubscript{1}F\textsubscript{1} seeds. These seeds produced 504 BC\textsubscript{1}F\textsubscript{2} plants, which were genotyped with SSR markers. Of these, 60 BC\textsubscript{1}F\textsubscript{2} plants confirmed to be heterozygous for chromosome regions from O. rufipogon were selected and successively backcrossed to Koshihikari.

Ninety-nine out of 505 BC\textsubscript{1}F\textsubscript{1} plants were selected on the basis of their genotypes and allowed to self to produce the BC\textsubscript{1}F\textsubscript{2} generation. Subsequently, 3,545 BC\textsubscript{1}F\textsubscript{2} plants were subjected to MAS, and 41 BC\textsubscript{2}F\textsubscript{2} plants were selected to establish the ILs (KRILs). Graphical genotypes of the 40 KRILs were determined by SSR markers across the 12 chromosomes (Fig. 2). The substituted chromosome segments derived from O. rufipogon (104814) in the ILs covered 89.9% of the 12 chromosomes, with gaps remaining on chromosomes 3, 4, 7, 8, 10, and 11. The percentage of substituted chromosome segments in each IL ranged from 0.8% to 12.6%, on the basis of the physical size of the rice chromosomes. The average proportion of recurrent parent in each IL was 94.3%. The average heading dates of the KRILs in 2006 and 2007 were distributed between 31 July and 3
September, and 10 August for Koshihikari. The donor parent *O. rufipogon*, on the other hand, did not flower in both plantings. In the KRLs, average culm length, panicle length, and panicle number were distributed between 64 and 118 cm, 17.7 and 23.9 cm, and 8.2 and 22.9 panicles/plant, respectively. Koshihikari, on the other hand, had 96 cm, 19.9 cm and 12.3 panicles/plant, respectively.

Development of *O. rufipogon* (Acc. 104814) introgression lines (RTKILs)

The RTKILs were developed in the same way as KRLs (Fig. 1). The *F*₁ plants derived from Koshihikari × *O. rufipogon* (104812) were backcrossed to Koshihikari to produce BC₁F₁ plants. Seven BC₁F₁ and 23 BC₂F₁ plants were backcrossed to Koshihikari to develop the BC₂F₁ and then the BC₃F₁ generation. No marker selection was used until the BC₃F₁ generation. Twenty-three plants were selected from 47 BC₂F₁ lines based on SSR genotypes across the whole genome and backcrossed again to Koshihikari. Twenty-seven BC₃F₁ plants were selected out of 288 plants derived from BC₂F₁. A total of 3,656 BC₃F₂ plants were subjected to MAS, and 47 BC₄F₂ plants were selected to establish the ILs. Graphical genotypes of the 47 RTKILs were determined by using SSR markers across the 12 rice chromosomes (Fig. 4). The substituted chromosome segments in the ILs covered 87.9% of the 12 chromosomes, with gaps remaining on chromosomes 5, 6, 8, 9, and 12. The percentage of substituted chromosome segments in the ILs ranged from 0.3% to 7.8%, as determined on the basis of the physical map distance. The average proportion of recurrent parent in each IL was 96.0%. The heading dates of the RTKILs in 2007 were distributed between 6–22 August and 10 August for Koshihikari. Culm length, panicle length, and panicle number of the RTKILs were distributed between 72 and 133 cm, 17.5 and 22.4 cm, and 9.7 and 21.9 panicles/plant, respectively. Koshihikari values, on the other hand, were 90 cm, 19.8 cm and 12.5 panicles/plant. No flowering of *O. rufipogon* (104812) occurred during 2007.

Development of *O. glumaepatula* (IGILs)

The IGILs were developed by the method used for KRLs and RTKILs. The *F*₁ plants derived from a cross between Itadaki and *O. glumaepatula* were backcrossed with Itadaki to produce BC₁F₁ plants. Fifteen BC₁F₁ and 127 BC₂F₁ plants were successively backcrossed to Itadaki to develop the BC₃F₁ and BC₄F₁ generations, respectively. No marker selection was used until the BC₃F₁ generation. Sixty-six plants from 127 BC₂F₁ lines were selected on the basis of their genotypes (assessed across the whole genome) and backcrossed again to Itadaki. Sixty-six BC₂F₁ plants were identified by MAS, and all 66 were allowed to self to produce the BC₃F₂ populations. A total of 2,170 BC₃F₂ plants derived from the 66 BC₂F₁ plants were tested by MAS to develop candidate ILs lines. Forty-seven BC₃F₂ plants were also selected to establish the IGILs. Graphical genotypes of the 47 IGILs were determined by using SSR markers across the 12 rice chromosomes (Fig. 4). The substituted chromosome segments in the ILs covered 87.9% of the 12 chromosomes, with gaps remaining on chromosomes 1, 3, 5, 7, 8, 9, and 12. The percentage of substituted chromosome segments in the ILs ranged 0.6% to 9.3%, as determined by the physical map distance. The average proportion of recurrent parent in each IL was 91.0%. Heading dates of the IGILs in 2008 were distributed between 7 August and 4 September, whereas those of Itadaki and *O. glumaepatula* were 10 August and 26 August, respectively. Culm length, panicle length, and panicle numbers of the IGILs were distributed between 63 and 113 cm, 18.3 and 26.9 cm, and 9.0 and 15.0 panicles/plant, respectively, whereas those of Itadaki and *O. glumaepatula* were 79 cm and 116 cm, 19.8 cm, and 28.3 cm, and 11.5 and 10.0 panicles/plant, respectively.
Information about the markers used is available at the Web site of the National Institute of Crop Science, Japan, http://nics.naro.affrc.go.jp/.

**Evaluation for partial resistance to blast of O. rufipogon (104814) introgression lines (KRLs)**

Blast resistance scores of the 40 KRLs, Koshihikari, and O. rufipogon (104814) were evaluated in upland nursery
trials during 2006 to 2008 (Fig. 5). During 2006 and 2007 trials, the average disease scores of Koshihikari and \textit{O. rufipogon} (104814) were 4.86 and 1.29, respectively, indicating that blast resistance of \textit{O. rufipogon} (104814) was superior. KRIL3, 9, 15, 17 and 36, showed significantly lower disease scores than Koshihikari in all three years, and KRIL4, 5 and 33 showed lower disease scores in two of the three years. The lines that showed significantly lower disease scores in at least two years were considered to have higher stability of resistance to blast. Some KRILs showed significantly lower disease scores in a single year during the test period. KRIL15 and 36, which showed significantly lower disease scores than Koshihikari across all three years, both contained the same segment of \textit{O. rufipogon} from chromosome 4 (Fig. 2). The disease score of KRIL17 was very low. KRIL17 contained three substituted segments from chromosomes 3, 5 and 6 (Fig. 2). These results suggest that resistance genes to rice blast are located on segments of chromosome 3 and on the short arm of chromosome 11 of \textit{O. rufipogon}.\[\text{Evaluation of partial resistance to blast of introgression lines of} \text{\textit{O. rufipogon} (104812) (RTKILs)}\]

The blast resistance of RTKILs and Koshihikari was evaluated in both 2007 and 2008 (Fig. 6). The donor parent, \textit{O. rufipogon} (104812), was not tested for blast resistance because of insufficient seed supply. RTKIL13 and 43 showed significantly lower disease scores than Koshihikari across all three years, both contained the same segment of \textit{O. rufipogon} from chromosome 4 (Fig. 2). The disease score of KRIL17 was very low. KRIL17 contained three substituted segments from chromosomes 3, 5 and 6 (Fig. 2). These results suggest that resistance genes to rice blast are located on segments of chromosome 3 and on the short arm of chromosome 11 of \textit{O. rufipogon}.\[\text{Evaluation of partial resistance to blast of} \text{\textit{O. glumaepatula} (IGILs) introgression lines}\]

The disease scores of \textit{O. glumaepatula} and Itadaki were 7.67 and 5.44, respectively, in 2009. As the difference between both parents showed inferior resistance of \textit{O. glumaepatula} to blast, evaluation for blast resistance in the IGILs was no longer pursued.\[\text{Discussion}\]

Wild rice generally shows tolerance to both biotic and abiotic stresses. Though inferior to those of cultivated species in terms of morphological and physiological characteristics, wild species carry many useful genes that are not found in cultivated rice. It is difficult to identify such genes (e.g., for yield and grain quality) directly in wild rice, because these useful genes are generally hidden by many other genes in the wild rice genome. To identify effectively such agronomically useful genes, a molecular genetics approach is necessary.
In recent years, molecular markers and sequence information in rice have been accumulating very rapidly. However, plant materials for genetic analysis are being developed at a much slower rate because it is not only tedious but usually requires a long period of time. Lack of these genetic materials limits our understanding of the genes associated with quantitative traits (Ebitani et al. 2005).

Advanced mapping populations such as ILs and CSSLs can be used to locate QTLs and evaluate the action of each QTL as a single Mendelian factor. Any difference between an IL or CSSL and its parents must be due to the presence of a QTL in the introgressed region. In this study, we developed three sets of ILs derived from wild rice to clarify the genetic basis of complex traits, analyze the genetics of traits special to O. rufipogon and O. glumaepatula, and explore the genetic potential of the species.

Genome coverage of the ILs

Most parts of the IL genomes were covered by the introgressed segments from O. rufipogon and O. glumaepatula, but within each set of ILs gaps remained in several chromosome regions. Regions that were not covered included large regions of chromosomes 3 and 4 in the KRILs (Fig. 2) and a large region of chromosome 9 in the RTKILs (Fig. 3). In IGILs, large regions of chromosome 5 and 8 were not covered by introgressed segments, while a region at the middle of chromosome 6 was covered only by heterozygous alleles (i.e., was not fixed) (Fig. 4). These uncovered regions in the three sets of ILs might have occurred because MAS was not performed until the BC3 generation. It may be also caused by some biological factors such as hybrid sterility and gametophyte and heading-date genes (Doi et al. 1997, Kubo et al. 2002, Tian et al. 2006).

QTLs for partial resistance to blast

We evaluated the two sets of ILs to identify QTLs for partial resistance to blast. Among the KRILs, KRIL 3, 4 and 5, which carry a segment of the long arm of chromosome 1, and KRILs 15, 14 and 36, carrying a segment of the long arm of chromosome 4, were more resistant to blast than Koshihikari (Fig. 5). These results suggest the presence of QTLs on the long arms of chromosomes 1 and 4 that confer blast resistance. Nguyen et al. (2006) and Terashima et al. (2008) reported that Pi35(t) and Pi39(t) were located around RM1216 (32.01 Mb) and RM3843 and RM5473 (31.7 Mb) of chromosomes 1 and 4, respectively. We speculate that the two QTLs detected on chromosomes 1 and 4 in this study may be the same genes as Pi35(t) and Pi39(t), respectively (Fig. 2). The disease score of KRIL17 was very low, and this line had resistance against all the four blast races (007.0, 033.1, 035.1 and 037.1) tested. It is possible that KRIL17 carries a gene for complete resistance that is different from Pia, Pii, Pik, or Pik-m. KRIL17 carries three regions of O. rufipogon (104814) from chromosomes 3, 5 and 6. Several blast resistance genes on chromosomes 5 and 6 were previously reported by Koide et al. (2009). Thus, further test must be conducted to determine whether the presently identified QTLs on chromosomes 5 and 6 represent these previously reported genes.

Among the RTKILs, RTKIL13 and 43 with O. rufipogon
(104812) alleles on chromosomes 3 and 11, respectively, had enhanced partial resistance to blast (Fig. 6). These substitution mapping indicate the presence of QTLs around RM7117 (28.6 Mb) on the long arm of chromosome 3 and RM3225 (0.9 Mb) on the short arm of chromosome 11 (Fig. 3). These QTLs were considered as new genes, because there are no previous reports of QTLs in these regions. We will continue to map these QTLs by using detailed substitution lines for these two regions.

The results of the present study clearly demonstrate the potential of ILs in identifying QTLs of agronomic interest from wild rice species. Further evaluation of these ILs may, therefore, provide additional opportunities to identify QTLs for disease and insect resistance, tolerance to abiotic stresses, yield-related traits, and other useful genes from the two wild rice species. Once favorable alleles for different traits are detected by ILs, several alleles can be easily combined (pyramided) in the genetic background of the recurrent parent through MAS. Previously, several series of ILs in Taichung 65 (T65) have been developed using O. glaberrima, O. glumaepatula, and O. meridionalis as donors (Doi et al. 1997, Kurakuzu et al. 2001, Sobrizal et al. 1999). These ILs are very useful for genetic analysis, but those materials cannot be directly used in rice breeding programs because the genetic background of the recurrent parent, T65, is less desirable than current rice cultivars cultivated in Japan. Here, we used two elite cultivars (Koshihikari and Itadaki) as recurrent parent; hence, it would be easy to develop near-isogenic lines of the two varieties with pyramided useful genes controlling desirable agronomic traits.

The ILs developed in this study are available for research use and can be obtained at the Web site of the National Institute of Crop Science, Japan (http://nics.naro.affrc.go.jp/).

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