Development and Identification of Introgression Lines from the Cross of *Oryza sativa* and *Oryza minuta*

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**Abstract:** Introgression line population was effectively used in mapping quantitative trait loci (QTLs), identifying favorable genes, discovering hidden genetic variation, evaluating the action or interaction of QTLs in multiple conditions and providing the favorable experimental materials for plant breeding and genetic research. In this study, an advanced backcross and consecutive selfing strategy was used to develop introgression lines (ILs), which derived from an accession of *Oryza minuta* (accession No. 101133) with BBCC genome, as the donor, and an elite indica cultivar IR24 (*O. sativa*), as the recipient.

Introgression segments from *O. minuta* were screened using 164 polymorphic simple sequence repeat (SSR) markers in the genome of each IL. Introgressed segments carried by the ILs contained 131 ILs covering the whole *O. sativa* genome. The mean number of homozygous *O. minuta* segments per introgression line was about 9.99. The average length of introgressed segments was approximate 14.78 cM, and about 79.64% of these segments had sizes less than 20 cM. In the genome of each introgression line, the *O. minuta* chromosomal segments harbored chromosomal fragments of *O. sativa* ranging from 1.15% to 27.6%, with an overall average of 8.57%. At each locus, the ratio of substitution of *O. minuta* alleles had a range of 2−33, with an average of 10.9. Based on the evaluation of the phenotype of these ILs, a wide range of alterations in morphological and yield-related traits were found. After inoculation, 41, 11, 7 ILs showed high resistance to bacterial blight, brown planthopper, and whitebacked planthopper, respectively. These *O. minuta* and *O. sativa* ILs will serve as genetic materials for identifying and using favorable genes from *O. minuta*.

**Key words:** *Oryza sativa*; *Oryza minuta*; introgression lines; bacterial blight; brown planthopper; whitebacked planthopper

Rice is one of the most important crop plants and provides the staple food for more than 50% of the world’s population. Genetic variation in cultivated rice has been reduced tremendously during domestication and modern plant breeding. Currently, the narrow genetic base of breeding programs has resulted in a bottleneck effect in rice variety development (Tanksley and McCouch, 1997). Wild relatives of cultivated rice remain to be highly diversified and hold various genes conferring resistance to biotic and abiotic stress, thus unlocking the tremendous genetic potential from wild rice might break the genetic bottleneck and improve modern varieties (Zamir, 2001).

More and more attention has been paid to introgression of beneficial alleles from wild rice into elite breeding lines (Multani et al, 1994; Brar and Khush, 1997), and molecular tagging of new resistance genes from wild rice species has been greatly facilitated with advances in DNA marker technology (Gu et al, 2004). While it is relatively straightforward to identify and transfer major genes from unadapted germplasm into adapted germplasm, identification of useful quantitative trait loci (QTLs) from unadapted germplasm is difficult and requires specialized genetic design.

Exploitation and utilization of the favorable genes from wild rice could overcome the yield plateaus of cultivated rice improvement. An approach known as introgression line (IL) analysis (Eshed and Zamir, 1995) has been extensively used in rice and other crop species (Monforte and Tanksley 2000a; Li et al, 2004). ILs can be obtained through consecutive backcrossing and selfing to introgress small chromosomal segments from the donor into the recurrent parent, with marker-assisted selection (MAS) to identify the donor segment number and length.

Due to its simple genetic background, ILs have become a useful experimental material for genetic analysis and molecular breeding and could be used to evaluate the action and interaction of genes over multiple years and in multiple site experiments (Monforte and Tanksley, 2000b). Using a set of ILs...
carrying the whole donor genome, nearly isogenic lines (NILs) could be constructed rapidly through backcrossing and MAS after a target gene is found. In addition, for fine mapping and positional cloning of certain target genes or QTLs, an enormous secondary F2 population derived from an advanced backcross between selected introgression line and the recipient parent could also be rapidly developed (Tian et al, 2006b). Recently, several sets of ILs were constructed in crop species including rice (Li et al, 2005; Tian et al, 2006a; Tan et al, 2007), wheat (Pestova et al, 2001), barley (Matus et al, 2003), tomato (Eshed and Zamir 1995; Chetelat and Meglic, 2000; Monforte and Tanksley, 2000a), Brassica napus (Howell et al, 1996) and melon (Eduardo et al, 2005). These IL populations would accelerate molecular breeding and improve the traits of agronomic importance.

Oryza minuta, a tetraploid wild relative of cultivated rice with BBCC genome, is rich in gene sources, such as resistance to blast blight, bacterial blight (BB), brown planthopper (BPH), whitebacked planthopper (WBPH). And a number of resistant genes had been successfully transferred into cultivated rice from O. minuta (Amante-Bordeos et al, 1992; Rahman et al, 2009). In order to identify and use more desired genes from this wild species, it is still very important to construct O. minuta - O. sativa introgression lines.

In this study, a set of 131 ILs derived from a backcross between an accession of O. minuta, as the donor, and an elite indica cultivar IR24, as the recurrent parent, were constructed by MAS. The characteristics of introgressed segments, including the introgression ratio in various chromosomal regions, and the length and number of introgressed segments in each individual were assessed. Based on the evaluation of the phenotype of these ILs, BB, BPH and WBPH resistance were also identified.

MATERIALS AND METHODS

Population development

An elite indica cultivar IR24 (O. sativa ssp. indica) was used as a recipient parent in a backcross program and crossed with an accession of the tetraploid species O. minuta (accession No. 101133) with BBCC genome, which was kindly provided by the International Rice Germplasm Centre of the International Rice Research Institute in 2004. The F1 plants were backcrossed four times consecutively with IR24 until a BC4 population consisting of 351 plants was obtained. Then BC4F1 plants were self pollinated for three generations, and 351 BC4F3 families were obtained. On the basis of the results of genotypic analysis, 192 individuals containing specific different introgression segments were selected from 351 BC4F3 families. The 192 plants of BC4F3 were self pollinated for three generations consecutively. Finally, 131 ILs containing the chromosomal segments from O. minuta covering the whole O. sativa genome were developed. There were few artificial selections to any traits during the course of development of BC4F1 population, but there were strong phenotypic selections in selfing process from BC4 F2 to BC4 F6.

DNA extraction and SSR analysis

Genomic DNAs were extracted from young rice leaves by using CTAB method (Murray and Thompson, 1980). Totally, 208 rice SSR primers, which were distributed across the 12 chromosomes of cultivated rice, were retrieved from the publicly available website (http://www.gramene.org). Marker orders for the SSR were identical to the published map (Temnykh et al, 2000). PCR was performed in 25 μL reaction-solution containing 20 ng genomic DNA, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.8), 0.001% gelatin, 2.5 mmol/L MgCl2, 0.2 mmol/L each of dNTPs, 0.2 mmol/L each primer, and one unit of Taq polymerase. DNA amplification was carried out in a PTC-100 Programmable Thermal Controller, using a program consisting of an initial denaturation for 5 min at 94 °C, followed by 35 cycles of 50 s under 94 °C, 45 s under 55 °C, 1 min under 72 °C and a final extension for 5 min under 72 °C. PCR products were separated on 6% polyacrylamide denaturing gels (PAGE), and the allelic similarity and diversity of the SSRs were determined according to the bands in the gel revealed by silver staining (Panaud et al, 1996).

Percentage of the O. minuta genome in each introgression line

Graphic genotype from the GGT program (van Berloo, 1999) was carried out to determine the percentage of the total genome in each IL that came from each parent. Basically, if two consecutive loci had alleles coming from the same progenitor, the marker interval between them was considered to have the genome of that progenitor. If one locus had alleles from one parent and the consecutive locus had alleles from the other parent, then half of the marker interval between them was considered to have the genome of one progenitor and the other half from the other one (Young and Tanksley, 1989).
Field cultivation and trait evaluation

One hundred and thirty-one ILs and the recurrent parent IR24 were planted at the experiment farm of Rice Research Institute, Guangxi Academy of Agricultural Sciences, Nanning City (23° N, 108° E), China, in the spring of 2010, following a complete randomized block design, with two replications, five rows per plot, 10 plants per row, 15 cm between plants within each row and 25 cm between rows. The field management followed essentially the normal agricultural practice. At the harvest time, eight plants in the middle of each plot were selected and nine yield-related traits including days to heading (DTH), plant height (PHT), panicle number per plant (PPL), panicle length (PLH), grain number per panicle (GPP), spikelet number per panicle (SPP), seed-setting rate (SSR), 1000-grain weight (TGW) and grain yield per plant (GYP) were evaluated.

Evaluation for bacterial blight resistance

To evaluate the BB resistance of ILs, one Xanthomonas oryza pv. Oryzae (Xoo) strains (PXO112, race 5) was used. The recurrent parent IR24 and 131 ILs were tested for their reactions to the Xoo strain at the experiment farm of Rice Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China in the spring of 2010. IR24 and 131 ILs plants were clonally propagated by growing auxiliary buds from nodal cuttings. Ten clones of each plant were used for each strain inoculation test. IR24 plants were inoculated approximately 50 d after sowing. All plants were inoculated by the leaf clipping method described by Kauffmann et al (1973). The bacterial inoculums were prepared as described previously (Guo et al, 2009). The bacterial cell suspension was applied to the 10 youngest fully expanded leaves of each tiller by clipping 2−3 cm from the tip of the leaf using a pair of scissors dipped in the inoculums. Lesion lengths of the leaves were measured at 14−21 d after inoculation and scoring was done following Machmud (1978).

Evaluation for BPH and WBPH resistance

Evaluation for BPH and WBPH resistance was conducted in the summer of 2010 at Plant Protection Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China. The 131 ILs and the parental line IR24 were tested with two replications in plastic trays of 60 cm × 45 cm × 10 cm. In each replication, 20 germinated seeds from each IL were sown in a row of 20 cm with 3 cm spacing between rows, but only 15 healthy seedlings were retained. Three rows of control varieties were grown at random in each tray. The susceptible control TN1 and the resistant control RH (Rathu Heenati) were used in both experiments. During the 2-leaf stage, 2nd- to 3nd-larva nymphs of plant hopper were released to the trays for infestation at a density of 10 insects per seedling. When the seedlings of the susceptible control TN1 were completely died, seedling mortality was measured for each IL. The reaction against the WBPH and BPH was scored following the guidelines of Standard Evaluation Systems for Rice (IRRI, 1988): 0, No damage; 1, Very slight damage; 3, The first and second leaves of most plants partially yellowing; 5, Pronounced yellowing and stunting or about 10% to 25% of the plants wilting; 7, More than half of the plants wilting or dead and remaining plants severely stunted or dying; 9, All plants dead. The insects used for infestation were collected from the fields at Rice Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China.

RESULTS

Polymorphism between two parents detected by SSR markers

Among the 208 SSR markers distributed throughout 12 chromosomes of rice, 193 (92.8%) could detect polymorphism between IR24 and O. minuta. The ratio was similar to the SSR polymorphism between O. minuta and IR24 in the previous studies (Guo et al, 2009). Finally, 164 polymorphic SSR markers equably distributed throughout 12 rice chromosomes were used to screen the genotypes of ILs.

Development of ILs

To construct the ILs, the F1 plants, derived from a cross between O. minuta and IR24, was backcrossed three times consecutively with IR24 until a BC3 population (186 BC3F1 individuals) was obtained. The genotypes of 186 BC3F1 individuals were surveyed by 150 polymorphic SSR markers in the previous studies (Guo et al, 2009), and all the plants were backcrossed one time consecutively to produce 351 BC3F1 individuals. According to the results of phenotype evaluation and genotypic analysis, 192 BC3F1 individuals were selected to develop primary ILs. During the course of self pollination for three generations, the segregations of partial traits occurred in a few lines. Finally, 131 unique ILs were obtained, and each of the alleles from O. minuta was found to
be in at least one introgression line via genotypic analysis with 164 SSR markers. Based on the genotypes of the 131 ILs, 89 ILs were further selected as the core set of ILs, which substituted segments that covered 85.1% of the whole *O. sativa* genome (Supplementary Fig. 1, see at http://www.sciencedirect.com/science/journal/16726308; www.ricescience.org).

**Average number and length of introgressed segments**

Genotypes of 131 ILs were determined by using 164 polymorphic SSR markers distributed evenly across all 12 rice chromosomes, with an average distance of 10.6 cM between neighbor markers. A total of 1,309 substituted segments were detected in the set of ILs, and 1,081 (82.6%) introgressed segments were homozygous and 228 (17.4%) were heterozygous. The number of introgressed segments in each individual ranged from 2 to 19, with an average of 9.99 (Fig. 1). The number of introgressed segments in 68.53% ILs was less than eight. The length of introgressed segments ranged 1.05 cM to 70.4 cM, with an average of 14.78 cM. The length of 79.64% introgressed segments was less than 20 cM (Fig. 2).

**Ratio of introgression in each individual and each locus**

The *O. minuta* chromosomal segments in each introgression line harbored chromosomal fragments of *O. sativa* ranging from 1.15% to 27.6%, with an overall average of 8.57% (Fig. 3). In 86 ILs (65.65%), each contained less than 10.0%. Each *O. minuta* allele was present in 131 ILs with ranges of 2–33, and an average of 10.9 (Fig. 4).

The results did not coincide with other studies by Chetelat and Meglic (2000) and Tian et al. (2006a) that concluded the distribution of the introgressed segments...
along the chromosomes was not random and the majority of introgressed segments were often at the terminal position, but it was similar with the results obtained by Tan et al (2007). This may have resulted from selecting different target traits during the course of developing the introgression lines.

**Phenotypic evaluation of introgression lines**

As shown in Table 1, there were a wide range of phenotypic variation in nine traits including day to heading, plant height, and seven yield-related traits in the introgression line populations. Among these nine investigated traits, the variation range of grain yield per plant was the largest in ILs, while days to heading were the smallest.

**BB resistance of ILs**

After inoculation with *Xoo* strains, IR24 was susceptible to PXO112 (lesion lengths of more than 15.0 cm), while amongst 131 ILs, different reaction to PXO112 was observed (Fig. 5). Of 131 ILs, 41 introgression lines showed high resistance to *Xoo* (lesion lengths of less than 3.0 cm).

**BPH resistance and WBPH resistance of ILs**

All of the 131 ILs and IR24 were tested for the resistance to WBPH and BPH. A number of ILs was shown to have high resistance to WBPH or BPH.

**DISCUSSION**

**Development of ILs**

More and more evidence suggests that ILs are useful genetic materials for the identification of new
genes (Eshed and Zamir, 1995; Chetelat and Meglic, 2000; Kubo et al, 2002), for distinguishing pleiotropy versus linkage as well as pseudo-overdominance versus true-dominance (Monfore and Tanksley, 2000a), and for the map-based cloning of QTLs (Alpert and Tanksley, 1996). However, three key factors, the donor genome, the range of phenotypic variation in the ILs population and the coverage ratio of the donor genome, should be considered during the course of developing ILs. In fact, all of the rice ILs reported in previous studies had AA genome species as a donor, and a larger part of the ILs only had a lower coverage of donor genomes. For example, Tian et al (2006a) developed a set of 159 ILs carrying only 67.5% of the genome of *O. rufipogon* (AA), and a few chromosomal regions were not covered by the ILs. Similar results were also reported by Kubo et al (2002) in a chromosome substitution series derived from a japonica (AA) and indica (AA) cross. A lower coverage of the donor genome might result from the hybrid sterility, gametophyte genes, heading date genes, or less efficient use of MAS during backcrossing.

Introgressions from distantly related genomes other than AA genomes are usually difficult to obtain because of low crossability and abnormality of chromosome pairing and recombination. *O. minuta* is non-AA genome wild rice. The interspecific hybrid and backcross progenies from the cross of *O. sativa* and *O. minuta* were obtained by embryo rescue and subsequent backcrosses (Guo et al, 2009). In the present study, we developed a set of 131 ILs, and each of the alleles from *O. minuta* (BBCC) were found to be in at least one introgression line via genotypic analysis with 164 SSR markers. Based on the genotypes of the 131 ILs, 89 ILs were further selected as the core set of ILs, which substituted segments that had covered 85.1% of the whole *O. sativa* genome (Supplementary Fig. 1, see at http://www.sciencedirect.com/science/journal/16726308; www.ricescience.org). We believe that a four-time backcross was reasonable to obtain a higher representation of the donor genome in ILs, and phenotypic selections in advanced backcross and selfing generation were necessary to ensure a wide range combined consecutive selfing after consecutive backcrossing and trait-performance at multiple environments was used during the development of *O. minuta*-*O. sativa* ILs.

**Favorable alleles in *O. minuta***

*O. minuta*, a tetraploid wild relative of cultivated rice, is rich in gene sources, such as resistance to blast blight, BB, WBPH, and BPH. BB resistance, blast resistance and BPH resistance had been transferred into cultivated rice from *O. minuta* (Amante-Bordeos et al, 1992; Rahman et al, 2009). In the present study, we developed a set of 131 ILs. Based on the evaluation of the phenotypes of these ILs, a wide range of alterations in morphological and yield-related traits were also found. These results indicated the value of wild germplasm in rice improvement. Similar results were obtained in wide crosses between *O. sativa* and *O. officinalis* (Jena and Khush, 1990), *O. sativa* and *O. australiensis* (Multani et al, 1994), and *O. sativa* and *O. latifolia* (Multani et al, 2003). More importantly, BB resistance, BPH resistance and WBPH resistance were also identified in the ILs. After inoculation, three lines, line 41, 11, 7 ILs showed high resistance to BB, WBPH, and BPH, respectively. Five lines, line 41, 71, 114, 171 and 172 were resistant to WBPH and BPH.

**Potential of the application of ILs**

Due to its simple genetic background, ILs could be used to construct a new platform for genetic and functional genomic analysis. Li et al (2005) explained the theory and practice and illustrated their potential for genetic analysis behind ILs construction. Notably, the process of construction of ILs was also the process of construction of QTL-NILs, which effectively assesses the location and effect of QTLs (Tanksley and Nelson, 1996). In addition, QTL fine mapping may be rapidly finished by using the secondary F₂ population derived from the cross between introgression line and the recipient. Tian et al (2006b) reported that one QTL (*gpa7*) for number of grains per panicle was fine mapped in a 35-kb region on chromosome 7 using a secondary F₂ population derived from a cross between Guichao 2 and its one introgression line (SIL040), showed significantly less grains per panicle than Guichao 2. He et al (2006) fine
mapped qGY2-1 for grain yield per plant from wild rice by developing a set of NILs of the target QTL using an introgression line (BIL19). These results confirmed that ILs were ideal genetic materials in fine mapping QTLs. In our study, some significant differences, including morphological, physiological and yield-related traits, were found in ILs compared to those of the recipient. Using these specific phenotypic ILs, the genes/QTLs controlling resistance to BB, BPH, WBPH, yield and yield components, will be mapped in latter study. Further isolation and assessment of these genes/QTLs would provide ideal chances to understand the molecular mechanism of rice domestication and to make use of the favor genetic resource in a modern rice breeding program. Some specific introgression lines with other important traits including resistance to blast blight, drought-tolerance, grain quality improvement and so on, were identified in this ILs population (unpublished data). By means of the new analysis platform, combining with development of introgression line, QTL mapping and DNA array technology, possible candidate genes in the QTL regions can be rapidly identified.

CONCLUSIONS

We developed a set of 131 ILs derived from a backcross between an accession of O. minuta as the donor, and an elite indica cultivar IR24 as the recurrent parent, constructed by MAS. Each of the alleles from O. minuta was found to be in at least one introgression line via genotypic analysis with 164 SSR markers. Based on the evaluation of the phenotype of these ILs, 41, 11, 7 ILs showed high resistance to BB, BPH, WBPH, yield and yield components, will be mapped in latter study. Further isolation and assessment of these genes/QTLs would provide ideal chances to understand the molecular mechanism of rice domestication and to make use of the favor genetic resource in a modern rice breeding program. Some specific introgression lines with other important traits including resistance to blast blight, drought-tolerance, grain quality improvement and so on, were identified in this ILs population (unpublished data). By means of the new analysis platform, combining with development of introgression line, QTL mapping and DNA array technology, possible candidate genes in the QTL regions can be rapidly identified.

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