Transgenic Rice Plants Harboring Genomic DNA from *Zizania latifolia* Confer Bacterial Blight Resistance

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**Abstract:** Based on the sequence of a resistance gene analog FZ14 derived from *Zizania latifolia* (Griseb.), a pair of specific PCR primers FZ14P<sub>1</sub>/FZ14P<sub>2</sub> was designed to isolate candidate disease resistance gene. The pooled-PCR approach was adopted using the primer pair to screen a genomic transformation-competent artificial chromosome (TAC) library derived from *Z. latifolia*. A positive TAC clone (ZR1) was obtained and confirmed by sequence analysis. The results indicated that ZR1 consisted of conserved motifs similar to P-loop (kinase 1a), kinase 2, kinase 3a and GLPL (Gly-Leu-Pro-Leu), suggesting that it could be a portion of NBS-LRR type of resistance gene. Using *Agrobacterium* -mediated transformation of Nipponbare mature embryo, a total of 48 independent transgenic T<sub>0</sub> plants were obtained. Among them, 36 plants were highly resistant to the virulent bacterial blight strain PXO71. The results indicate that ZR1 contains at least one functional bacterial blight resistance gene.

**Key words:** *Zizania latifolia*; transformation-competent artificial chromosome library; resistance-gene analog; *Oryza sativa*; bacterial blight resistance; gene transfer

*Zizania latifolia* (Griseb.) belongs to Poaceae family like rice (*Oryza sativa* L.). This wild rice is a perennial food crop native to China and possesses many elite traits such as strong stem, high ability of tillering, rapid filling of grain, high quality of grain, high biomass, as well as resistance to blast, bacterial blight and sheath blight. All these characteristics make it a potential valuable source to improve the germplasm for modern rice breeding.

Genomic library construction, characterization and screening are the fundamental steps for isolation of genes for many species with genomes largely unknown. Yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), P<sub>1</sub>-derived artificial chromosome (PAC), binary-BAC system and transformation-competent artificial chromosome (TAC) have been frequently and successfully used for isolation of plant and animal genes. Among them, TAC possesses the characteristics of PAC and binary-BAC system and can be directly used for transformation (Liu et al, 1999). TAC is able to harbor a large foreign DNA fragment and can propagate stably both in *Escherichia coli* and *Agrobacterium tumefaciens*. The main advantage of TAC compared to other systems is its direct transformation without tedious steps of error prone sub-cloning. Kong et al (2006) obtained 91 584 TAC clones covering five times of the genome of *Z. latifolia* with an average insert of 45 kb, providing a step forward for isolation of genes from the species. Using the pooled PCR technology, a Type I thionin gene and a couple of CAB genes have been isolated from a TAC library of Chinese spring wheat (*Triticum Aestivum*) (Liu et al, 2000). Furthermore, a positive TAC clone was also obtained from a diploid wheat (*Dasypyrum villosum*) using a pair of degenerate primers (Qin et al, 2000).

Plant resistance genes (R gene) and their proteins are usually conserved in several structures and domains including leucine-rich repeats (LRR), serine-threonine kinase (STK), nucleotide-binding site (NBS), leucine zippers (LZs) and toll/interleukin-receptor like (TIR). These structures provide a new solution for isolation of genes using the approach of resistance gene analog (RGA). The basic principle is to design degenerate primers based on the conserved domains of plant resistance genes, and then using the specific primers to amplify the homozygous fragments from the targeted...
species. The isolated fragments can be used as RFLP probes for gene mapping and screening of genomic libraries for isolation of potential positive clones. In fact, a number of plant resistance genes, such as the leaf rust disease resistance gene \textit{Lr10} and potato virus \textit{X} disease resistance gene \textit{Rx2}, have been isolated from wheat and potato using this strategy (Fenillet et al, 1997; Bendahmane et al, 2000). All these genes belong to the NBS-LRR class of genes, the largest R-gene class that confers resistance to bacteria, fungi, virus and insect pests (Staskawicz et al, 1995; Hulbert et al, 2001). No function other than disease resistance has yet been assigned to this large class of genes (Hulbert et al, 2001). Besides the functional genes mentioned above, a number of RGAs have been also identified and mapped in various crop species (Chen et al, 1998; Yuksel et al, 2005; Chen et al, 2006).

In this paper, we present the isolation and functional validation of a resistance gene analog from \textit{Z. latifolia}. So far, no functional RGAs have been reported from this species. Our study would facilitate the application of resistance genes from \textit{Z. latifolia} in rice breeding programs.

**MATERIALS AND METHODS**

**Materials and reagents**

The genomic TAC library from \textit{Z. latifolia} was constructed and maintained in eighty-one 96-well plates (Kong et al, 2006). The \textit{A. tumefaciens} strain EHA105 was kindly provided by Prof. Tu from Zhejiang University, Hangzhou, China. The \textit{E. coli} strain DH10B and the bacterial blight strains PXO99 and PXO71 were provided by Dr. Vercruz from the International Rice Research Institute. Another bacterial blight strain JS97-2 was maintained in our laboratory. Both pGEM-T vector and \textit{E. coli} strain JM109 were purchased from Promega, whereas DNA purification kit was purchased from QiAgen (Germany). Restriction enzymes \textit{HindIII} and \textit{EcoRI} were purchased from TaKaRa (Japan).

**Primer design**

Eight RGAs were isolated from \textit{Z. latifolia} using degenerate primers designed according to the conserved motifs of the tobacco \textit{N} gene and \textit{Arabidopsis RPS2} gene (Chen et al, 2006). Each of these eight sequences was subjected to BLAST (http://www.signal.salk.edu). The analog, FZ14, was chosen for primer design using the DNAMAN5 software. A pair of primers (forward primer FZ14P1: 5'-AAGGCACCACTGTTTG AA-3', reverse primer FZ14P2: 5'-ATATCCTGCAT GTTCCCAGCA-3') was synthesized at Invitrogen (Shanghai) with an expected PCR product of 386 bp.

**Screening for positive TAC clone**

The pooled-PCR approach (Liu et al, 2000) was adopted for screening the targeted clones from the genomic TAC library using the primers FZ14P1/P2. The PCR was carried out in a total volume of 10 µL containing 1 µL of 10×PCR buffer, 5 ng of pooled plasmid DNA, 1 µL of dNTPs (2 mmol/L), 0.2 µmol/L of each primer, and 1 U of Taq DNA polymerase (Sangon Biotech, Shanghai) using the following profile: initial denaturation at 94°C for 2 min; 35 cycles consisting of 45 s at 94°C, 45 s at 56.5°C and 1 min at 72°C; a final extension of 10 min at 72°C.

**Validation of positive clone**

The targeted single colonies were used as template for PCR using the primers FZ14P1/P2 with the method mentioned above, but in the total volume of 50 µL. The PCR products were recovered and purified following the manufacture’s instruction (QiAgen). The purified products were ligated in pGEM-T Easy vector and transformed into competent JM109 cells following the method provided by the manufacture (Promega). The recombinant clones were sequenced at Invitrogen and digested by \textit{EcoRI} to release the insert. The sequence was analyzed and compared with the sequence of FZ14 using BioEdit 7.0.

**Transformation of rice**

The positive TAC clone was used directly for transformation. Rice calli were induced from the embryos of mature seeds of the wild type Nipponbare and transformed using the \textit{Agrobacterium}-mediated transformation method (Zhu et al, 2001).

**Evaluation of bacterial blight resistance**

Transgenic rice plants (\(T_0\)) were grown in a
greenhouse at the China National Rice Research Institute, Hangzhou, China and evaluated for bacterial blight resistance at the maximum tillering stage using three races (PXO71, PXO99 and JS97-2) following the method by Kauffman et al (1973). The lesion length (LL) and the full length of the inoculated leaf (FL) were measured 20 days after inoculation and the ratio of LL/FL (percentage of diseased lesion area) was calculated. The ratio of LL/FL<10% was considered as resistant (R), 10%–20% as moderately resistant (MR), 20%–30% as moderately susceptible (MS), and >30% as susceptible (S).

**PCR assay of transgenic rice plants**

Total genomic DNA of transgenic rice plants was extracted using a mini-prep method (Lu and Zheng, 1992). PCR was performed as mentioned above using the primers FZ14P1/P2.

**RESULTS**

**Sequence analysis of FZ14**

DNA sequences of eight RGAs reported previously (Chen et al, 2006) were subjected to BLAST. The results indicated that one of the RGAs, FZ14 (Accession no. DQ239432), was highly homologous to a rice NBS-LRR clone Os07g29820 with 91% identity (Supplement Fig. 1, see at http://www.ricescience.org; http://www.sciencedirect.com). Because the NBS-LRR class of resistance genes has been well known for the characteristics of conserved domains in their proteins (Merers et al, 1999; Dangl and Jones, 2001), we therefore searched for any conserved motifs both in the NBS and LRR regions. Interestingly, the P-loop (kinase la), kinase 2, kinase 3a and Gly-Leu-Pro-Leu (GLPL) were identified in the sequence of FZ14 (Fig. 1).

**Screening of positive TAC clone**

Firstly, we screened 162 primary clone pools using the specific primers FZ14P1/P2. One of the pools, T40, was identified with the expected band of 386 bp (Fig. 2-A). Because each of the primary clone pool consisted of clones from 48 wells, we then screened each well (the secondary clone pool) using the same primers and identified wells 7-3 and 7-4 as the positive ones with the expected band of 386 kb (Fig. 2-B). We therefore amplified the original 12 colonies forming each well in the stock plate. The results showed that both colonies 5 and 6 gave the expected band (Fig. 2-C). Colonies 5 and 6 were the same when digested by HindIII (unpresented data). Thus, we tentatively name colonies 5 and 6 as ZR1 (Zizania resistance clone 1) because of its structure similarity to plant resistance genes.

**Validation of ZR1**

The positive clone ZR1 was used as the template for PCR with the primers FZ14P1/P2. The expected product was purified and ligated into pGEM-T easy vector to form a new plasmid pGEM-ZR1 which then was transformed into the competent JM109. After

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**Fig. 1.** Deduced amino acid sequence of FZ14 from Z. latifolia by BioEdit 7.0. The shaded are conserved motifs in the NBS region.

**Fig. 2.** Screening for positive clones ZR1 from Z. latifolia genomic TAC library.

M, 100 bp DNA ladder; A: Lanes 1 to 10, Primary clone pool, among which Lane 5 is the positive primary clone pool T40; B: Lanes 1 and 2, Positive secondary clone pool; C: Lanes 1 to 8, Single clone, among which lanes 5 and 6 are positive clone ZR1.
overnight culture at 37°C, five white colonies were selected for further culture. The recombinant plasmid was extracted and sequenced in Invirtogen (Shanghai). The plasmid DNA was also digested with EcoRI for validation of the insert. The results showed that the sequence of the insert was completely the same as the RGA clone FZ14 (Supplement Fig. 2, see at http://www.ricescience.org; http://www.sciencedirect.com). Restriction enzyme digestion showed an insert band with the expected size of 386 bp and an extra band with the size of empty vector (Fig. 3). Thus, we concluded that the positive clone ZR1 would probably contain a NBS-LRR type of resistance gene from Z. latifolia.

Performance of transgenic rice to bacterial blight and PCR validation

A total of 48 independent transgenic rice plants (T0) was obtained through Agrobacterium-mediated transformation. The transgenic plants were inoculated with three races of bacterial blight, respectively. The results indicated that all transgenic plants and the wild type Nipponbare were susceptible to both races PXO99 and JS97-2. However, 36 out of 48 transgenic plants (75%) were resistant to PXO71 whereas the wild type was susceptible (Table 1; Fig. 4-A). All these 36 plants were subjected to PCR analysis using the specific primers FZ14P1/P2, and the results showed that all the resistant plants exhibited the expected band of 386 bp while the wild type did not give any amplification (Fig. 4-B). These results clearly indicated that ZR1 harbored at least one NBS-LRR class of resistance gene conferring bacterial blight resistance to PXO71.

**DISCUSSION**

Rice breeding especially hybrid rice breeding has reached a very high level in China, but the increase of population and the level of living standard require a stable and increasing output in rice production since rice is the staple food in the country. Evaluation and characterization of genes from related foreign species and make them available in rice breeding programs are considered as one of the strategies to achieve the goal. Z. latifolia is such a closely related species to O. sativa and possesses a number of elite traits such as resistance to rice blast, bacterial blight and sheath blight, making it an ideal source for rice improvement.

Identification and isolation of plant resistance genes through map-based and transposon tagging approaches have been considered popular ways to characterize and utilize the genes. However, both

<table>
<thead>
<tr>
<th>Line</th>
<th>Lesion length (cm)</th>
<th>Diseased lesion area (%)</th>
<th>Phenotype</th>
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<tr>
<td>Nipponbare (CK)</td>
<td>11.0±0.4</td>
<td>48.2±0.2</td>
<td>S</td>
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<tr>
<td>ZR1-1</td>
<td>0.3±0.1</td>
<td>1.1±0.8</td>
<td>R</td>
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<tr>
<td>ZR1-3</td>
<td>1.0±0.4</td>
<td>4.3±1.4</td>
<td>R</td>
</tr>
<tr>
<td>ZR1-5</td>
<td>2.6±0.4</td>
<td>9.6±2.1</td>
<td>R</td>
</tr>
<tr>
<td>ZR1-6</td>
<td>1.7±0.8</td>
<td>7.0±4.2</td>
<td>R</td>
</tr>
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<td>ZR1-8</td>
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<td>18.6±1.7</td>
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<tr>
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<td>ZR1-45</td>
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Data are Mean±SD. R, Resistant; MR, Moderately resistant; S, Susceptible.
methods require numerous steps and were effective only for species including model plants with a small and simple genome. Fortunately, the conserved structures only for species including model plants with a small genome. Fortunately, the conserved structures identified in many plant resistance genes provide a new solution for isolation of similar genes from much more complicated genomes (Xu et al, 2004). Isolation of candidate genes and functional genes from several species has proved that the RGA approach is not only possible but a simple and economic way for gene isolation (Fenillet et al, 1997; Bendahmane et al, 2000; Ramalingam et al, 2003; Chen et al, 2006).

So far, more than 50 plant resistance genes have cloned from monocots and dicots (Han et al, 2005). Among them, the largest group is NBS-LRR class of resistance genes. In rice, the blast resistance genes is isolation (Fenillet et al, 1997; Bendahmane et al, 2000; Qu et al, 2006; Zhou et al, 2006; Lin et al, 2007). In present study, the positive clone ZR1 contained four conserved motifs within the NBS region (P-loop, kinase 2, kinase 3a and GLPL). In addition, it was highly similar to the NBS-LRR gene Os07g29820 identified in the Nipponbare genome with the sequence identity level of 91%. The result indicates that ZR1 from Z. latifolia could probably harbor a NBS-LRR class of gene and support the close relation between Z. latifolia and O. sativa.

Bacterial blight resistance genes mainly come from the wild relatives of rice (Jin et al, 2007; Zheng et al, 2009) although a few genes from other species confer bacterial blight resistance have also been noticed (Yu et al, 2006). To our knowledge, this is the first report on a functional clone from Z. latifolia conferring bacterial blight resistance in rice, and would facilitate the isolation and application of resistance genes from Z. latifolia in rice breeding programs. Fully characterization of ZR1 and the performance of transgenic progenies to a wide range of X. oryzae pv. oryzae are currently underway.

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