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Full Length Research Paper

Isolation of candidate disease resistance genes from enrichment library of *Oryza minuta* based on conserved domains

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A strategy was described for the isolation of disease resistance genes from *Oryza minuta* by integrating the techniques of transformation-competent genomic library, RecA-mediated magnetic bead enrichment library and candidate disease resistance gene cloning. The principal advantages of this method were: simple, rapid and suitable. In this research, a transformation-competent genomic library with a volume of 2.68 × 10^5 clones was constructed for *O. minuta*; an enrichment library of disease resistance genes was further constructed with a volume of 4992 clones, from which 26 positive clones were screened by colony *in situ* hybridization. The end-clone sequencing of 13 representative positive clones showed that 6 clones were well matched with cloned disease resistance genes or located near the existing disease resistance genes. Full sequencing of a clone revealed a gene similar to a putative brassinosteroid LRR receptor kinase in japonica rice; the protein structure analysis suggested that it may be a disease resistance gene or functionally involved in a signal transduction pathway. These results indicate these clones may include new *R* genes and the strategy is feasible to clone new *R* genes from wild rice species.

Key words: *Oryza minuta*, disease resistance gene, transformation-competent genomic library, magnetic bead enrichment library, colony *in situ* hybridization, sequence analysis.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important food crops in the world, providing staple food for nearly one-half of the global population. To meet the increasing demand of the world food supply, we will have to produce up to 40% more rice by 2030 with less water, land, labor and chemicals (Khush, 2005). Besides improving the yield per plant, reducing losses caused by biotic and abiotic stress is also considered as an important factor. It was reported that fungal diseases can cause up to 10% losses regionally, and the rice blast disease can cause one million hectares lost annually in China alone (Savary et al., 2000; Khush et al., 2009). Great efforts have been invested in improving disease resistance. Recently, about 70 disease resistance (*R*) genes from plants have been cloned and characterized (Liu et al., 2007). These cloned *R* genes are very useful novel resources for improving resistance by means of genetic engineering. However, most of the cloned *R* genes confer high level of race-specific resistance in a gene-for-gene manner, and the resistance is effective against one or a few related races or strains of the pathogens. There is an urgent need to broaden the rice gene pool by introgressing genes from diverse sources.

Wild *oryza* species represent a rich, largely untapped reservoir of resistance to biotic and abiotic stresses, most notably to disease resistance (Brar, 2005). Up till now, several *R* genes cloned from wild rice species have been widely used (Song et al., 1995; Gu et al., 2005; Qu et al., 2006; Zhang et al., 2010), including the broad spectrum

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R gene *Xa21* with resistance to 29 isolates of *Xanthomonas oryzae pv. oryzae* (*Xoo*) (Wang et al., 1996) and the *pi9* with resistance to 43 *Magnaporthe grisea* (*M. grisea*) isolates (Qu et al., 2006). However, low cross-ability and limited recombination between chromosomes of cultivated and wild species limited the use of such genes in traditional breeding (Brar and Khush, 1986, 2002). The present plant gene cloning technology also has limits because the genomic research of wild species is still in its infancy. Recently, BAC libraries of wild *oryza* species have been developed (He et al., 2003; Wing et al., 2005; Ammiraju et al., 2006; Li et al., 2008), which facilities the map-based cloning of useful genes from wild species. But previous study suggested the stability of BAC clones in *Agrobacterium* and the transformation efficiency had decreased when the DNA insert size was increased (Wang et al., 1995; Song et al., 2003), and the functional complementation by candidate clone transformation was difficult in these studies. Thus, there is a great need to develop novel strategies for cloning *R* genes from wild rice species.

Oryza minuta, an allotetraploid species native to Asia (2n = 48), with the genome of BBCC, is a potential source of resistance to two devastating rice diseases: rice blast disease caused by the fungus *M. grisea* and bacterial blight caused by pathogen *Xoo* (Ou, 1985). RecA is a component of the DNA repair system in *Escherichia coli* that promotes genetic recombination by forming triple complexes between a single-stranded DNA probe and homologous double stranded DNA molecules (Honigberg et al., 1986; Rigas et al., 1986; Teintze et al., 1995; Hasegawa et al., 2006). The principle of using RecA to facilitate cDNA clones' screening from libraries has been previously described (Hakvoort et al., 1996; Taidi-Laskowski et al., 1988). To discover the rice disease resistant genes from *O. minuta*, we reported here an approach of cloning wild rice *R* genes by integrating the techniques of transformation-competent genomic library, RecA mediated magnetic bead enrichment library and candidate *R* gene cloning.

MATERIALS AND METHODS

The leaves of *O. minuta* introduced from the International Rice Research Institute were collected and immediately frozen in liquid nitrogen for genomic DNA extraction.

Construction of *O. minuta* transformation-competent genomic library

According to a mega-based method of Liu and Whittier (1994), high molecular weight DNA (>1 Mb) was isolated from *O. minuta*. The DNA was digested with *Hind*III and size-fractionated in the 10 to 23 kb size range as described (Liu et al.,1995; Peterson et al., 2000). The digested and size-selected DNA fragments were ligated into *Hind*III-digested pCAMBIA1301 vector and then used for transformation of *E. coli* DH10B by electroporation. Transformants carrying inserts were selected on LB agar plates containing 50 mg/L kanamycin (Kan), and the inserts were analyzed by means of restriction digestion with *BamH*I. To investigate structural stability of the plasmid in *E. coli*, 2 plasmids were randomly selected and analyzed with *Hind*III, *BamH*I and *EcoR*V in generations 0 and 100. Finally, the library was stored in TE buffer at -80°C with the concentration of 1 µg/µl.

The generation of probes

The primers were designed according to the conserved sequence of nucleotide binding site (NBS) and serine-threonine kinase (STK) conserved domains (Table 1). For the amplification of the probe templates, 50 pmol of each primer set and 50 ng of *O. minuta* genomic DNA were added to 25 µl of PCR reaction mixture with 10 × reaction buffer (Mg^{2+}, Takara), 0.2 mM of each dNTP and 1.25 units of *Taq* DNA polymerase. 30 cycles of PCR, consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1.5 min, and extension at 72°C for 10 min, were performed. The products were purified and sub-cloned into pGEM T-easy vector followed by sequencing. Then, the suitable clones were selected and quantified as the template for the synthesis of the biotin-labeled probe. The products of STK domain amplification were also extracted and used as probe template without sub-cloning and sequencing. The probe for enrichment library construction and hybridization was labeled by PCR with 21-biotined-dUTP and according to the NEBlot Phototope Kit, respectively (NewEngland Biolabs).

Construction and screening of enrichment library

The wild rice genomic enrichment library of *R* genes was constructed by the magnetic bead enrichment method with the biotin probe mediated by RecA. The biotin-labeled probe, RecA protein-coated probe and the magnetic beads were used. The enrichment process was performed according to Zhumabayeva et al. (1999). A control model plasmid library and a control biotin-labeled DNA probe were also prepared. In order to enhance the efficiency of the enrichment, we undertook the second enrichment which was performed as the first one. The enriched liquid genomic library was transformed into *E. coli* DH10B competent cells and was then selected on LB-agar containing Kan, X-Gal with IPTG and incubated at 37°C for 20 h. The confirmation of positive clones was performed by using colony *in situ* hybridization (NEBlot Phototope Kit and Phototope-Star Detection Kit, NewEngland Biolabs).

DNA and protein sequence analysis

DNA sequence location and similarity analysis were performed using BLAST (http://www.ncbi.nlm.nih.gov/). The gene prediction program used was FGENESH (http:// www.softberry.com.cn), and the domains were predicted by the SMART program (http://smart.embl-heidelberg.de/) and CDD program (http://www.ncbi.nlm.nih.gov/sites/entrez?db=cdd).

RESULTS

The generation of *O. minuta* transformation-competent genomic library

By ligating genomic DNA fragments (10 to 23 kb) of *O. minuta* into the *Hind*III site of the pCAMBIA 1301 vector

Table 1. Oligonucleotide primers used for the amplification of the probe templates.

Motif	Forward primer (5′-3′)	Motif	Reverse primer (5′-3′)
GGVGKTT	AGCGCCAGCGGCAGCCC AGGGCGAGCGGGAGGCC AGCGCCAGTGGCAGCCC AGGGCGAGTGGGAGGCC AAGGCCAACGGCAAGCC AAGGCTAATGGTAAGCC	GLPLAL	GGGGGGGTGGGGAAGACGAC GGCGGCGTCGGCAAGACCAC GGGGGGGTGGGGAAAACGAC GGCGGCGTCGGCAAAACCAC
FG(K/V/I/S)VY(K/R)G	TTC GGC AAG GTG TAC AAG GG TTC GGC AAG GTG TAC AAA GG TTC GGC AAG GTC TAC AAG GG TTC GGC ATC GTG TAC AAG GG	D(V/I)YS(F/Y)G(V/I/M)	AT GCC GAA GCT GTA GAT GTC AT GCC GAA GCT GTA GAT ATC AT GCC GAA GCT GTA CAC GTC AT GCC GAA GCT GTA GAC GTC

The primers were designed according to NBS and STK conserved domains. They were the conserved motif L-loop with the consensus sequence GGVGKTT and GLPL with sequence of GLPLAL in NBS domain (Bai et al., 2002), and the conserved motif catalytic sub-domain I with the sequence FG (K/V/I/S) VY (K/R) G and IX with the sequence of D (V/I) YS (F/Y) G (V/I/M) in STK domain (Hanks et al., 1988).

and subsequently transforming into *E. coli* DH10B, we have constructed an *O. minuta* DNA transformation-competent genomic library with approximately 4.5× coverage, which is represented by 2.68 × 10^5clones with average insert size of 13 kb (Figure 1A). Restriction analysis of randomly selected plasmids indicated that both clones remained completely intact in *E. coli* (Figure 1B).

The generation of probes

After sequence analysis of the TA clones, 12 fragments including 3 with the NBS conserved domain (Figure 2A), 8 with the STK conserved domain (Figure 2B) and 1 with the leucine rich repeat (LRR) conserved domain (Figure 2C) were selected and mixed as the template for the amplification of the biotin probe. The products of STK domain amplification were also prepared directly as the templates for the generation of the biotin probe.

The construction and screening of the enrichment library

In our experiment, the control library enrichment efficiency reached between 3000 and 5000 times. Finally, the capacity of the enrichment library with a volume of 1536 clones and 3456 clones were constructed for the NBS and STK type probes respectively; all 4992 clones were stored in 384 blots. After screening by colony *in situ* hybridization, we got 5 and 21 positive clones for NBS and STK, separately (Figure 3). Restriction digestion analysis of these 26 clones showed that they can be categorized into 13 classes (Figure 1C).

DNA and protein sequence analysis

By comparing the end sequence of 13 representative clone of each class, we found that 8 clones could be located on the japonica rice genome. Though XET3A8 and XET5B11, XES3-1G16 and XES3-3H18, XES3-3B6 and XES3-4H3 have different restriction digestion patterns, they are located at the same position, respectively probably because they are the same fragments with different insert directions. Among the 8 located clones, 6 showed high similarity with known *R* genes or located near the existing *R* genes, and the corresponding proteins contained conserved domains, such as STK and LRR. Two clones could be mapped in the japonica rice genome with no *R* gene fragments nearby (Table 2). The other 5 clones could not be located on the japonica rice because of multiple matches.

The whole-genome sequenced clone, XET5B11, contains an 11.9 kb fragment located on the corresponding japonica rice gene Os07g0166700, which encodes a putative brassinosteroid LRR receptor kinase. Open reading frame (ORF) analysis revealed that it contains a 697 amino acid (aa) protein with the similarity of 94% to the probe of NBF2R2. Analysis of the predicted 697 aa revealed several known regions and domains. There is a LRR domain between aa 113 to 298, a transmembrane region located at aa 366 to 388, and aa 452 to 688 containing a putative cytoplasmic protein kinase domain. The regions of the kinase domain are similar to the consensus sequence of sub domains of serine/threonine protein kinase (Hanks et al., 1988). The LRR domain is not a classic LRR; the predicted protein contains no matches to the LxxLxxLxxLx (N/C/T) x(x) LxxLPxx motif identified in dicot *R* gene products (Jones and Jones, 1996). The N-terminal region contains 20%

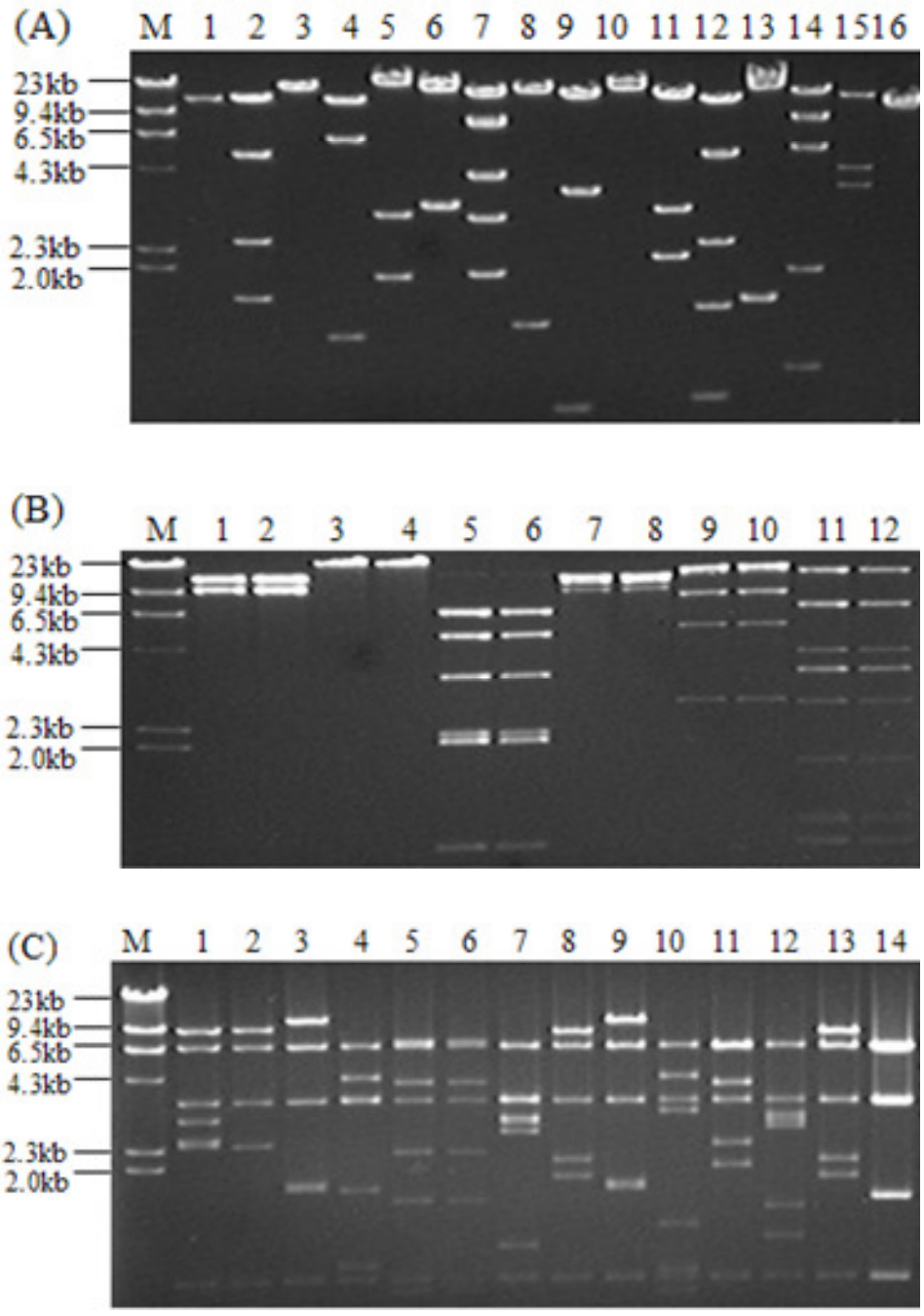

Figure 1. Restriction analysis clones selected from the library. (A) Restriction analysis of 15 clones randomly selected from the genomic library. Plasmid DNA was completely digested with *BamH*I and separated on 1% agarose gel. M, Lambda DNA/*Hind*III marker; lane 1, pCAMBIA1301 digestion by *BamH*I; lanes 2 to 16, plasmid DNA digestion by *BamH*I. (B) Comparison of digestion patterns of 2 randomly selected clones from the library in generations 0 and 100. M, Lambda DNA/ *Hind*III marker; lanes 1, 3, 5, 7 and 9, generation 0; lanes 2, 4, 6, 8, 10 and 12, generation 100; lanes 1, 2, 7 and 8, digestion by *Hind*III; lanes 3, 4, 9 and 10, digestion by *BamH*I; lanes 5, 6, 11 and 12, digestion by *EcoR*V. (C) Analysis of partial positive clones from the enrichment library. Plasmid DNA was digested completely with *EcoR*V and then electrophoresis in 1% agaros gel. M, Lambda DNA/ *Hind*III marker; lanes 1 to 13, positive plasmid DNA digestion by *EcoR*V; lane 14, pCAMBIA1301 digestion by *EcoR*V.

leucine which corresponds approximately to the LRR of Os07g0166700 protein; we refer to this region as a

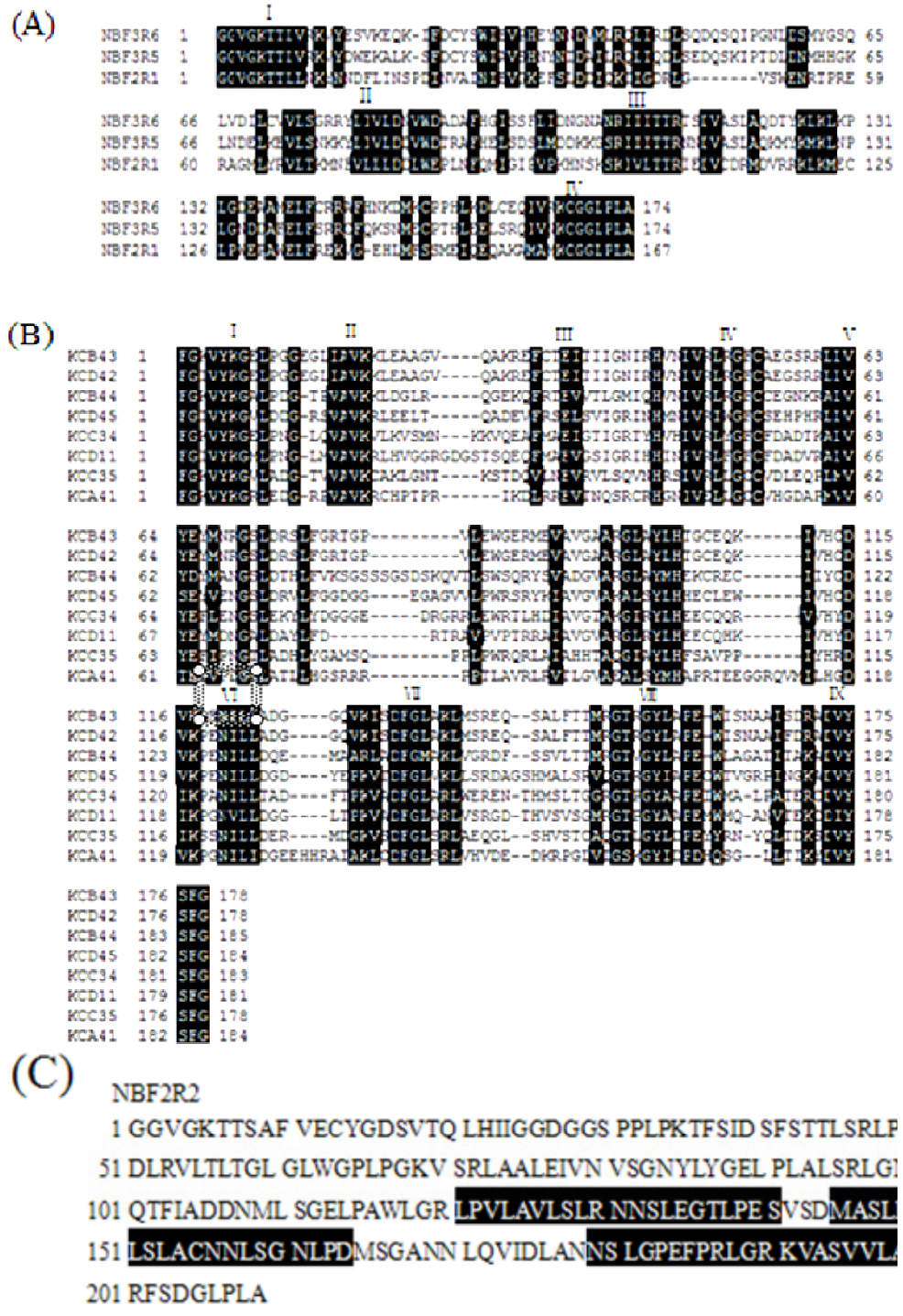

Figure 2. The deduced amino acid of the probes with NBS and STK conserved domains. (A) The conserved motif P-loop (I), kinase-2 (II), kinase-3a (III) and GLPL (IV) in NBS conserved domain (Bai et al., 2002); (B) The conserved motif (I-IX) in STK conserved domain (Hanks et al., 1988). (C) The probe with LRR conserved domain. The shadow represents the conserved motifs.

leucine-rich domain on the basis of its structure (Figure 4). These structural characteristics suggest that this gene may be an *R* gene or involved in a signal transduction pathway (Baker et al., 1997).

DISCUSSION

We have described a RecA-mediated genomic DNA library screening method for identifying novel resistance

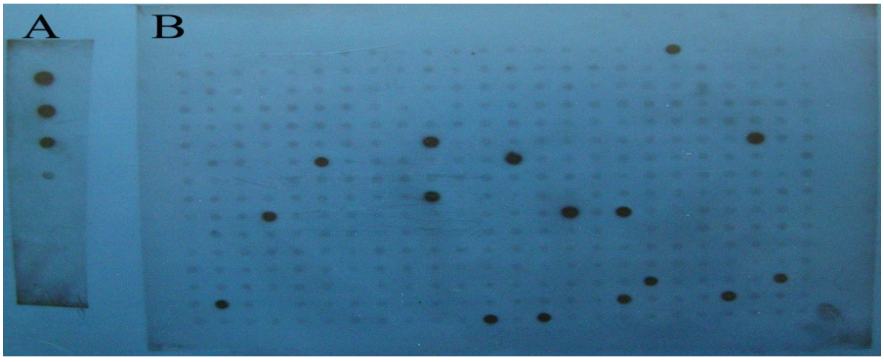

Figure 3. Colony *in situ* hybridization of the enrichment library with STK type biotin-labeled probe. A, The probe template positive control; B, 384-pore plate *in situ* hybridization of the enrichment library.

1 MAMVPGSTQLQASQTWSLLKIQQMLGY
28 PAVLGHWHNYTDFCYGGDYKTTSAFVE
55 CYGDSVTQLHIIGGGSPAPSPPLPKTFSID
85 SFFTTLSRLPDLRVLTLTGLGLWGPLPG
LRR
113 KVSRLAALEIVNVSGNY
130 LYGELPLALSRLGNLQTFIADDNM
154 LSGELPAWLGRLPVLAVLSLRNNS
178 LEGTLPESVSDMASLRSLSLASNN
202 LSGNLPDMSGAKNLQVIDLANNS
225 LGPEFPRLGRKVASVV
241 LAGNRFSDGLPPELASFYL
260 LERLDVSRNRFVGPFMPAL
279 LSLPSIEYLSVAGNRFTGML
299 SGNMSCGDNLKFKTDSESSKTVLFSANCL
328 ATGDDTQHPSPFCKNQAIAVSIVPDQARK
357 KPNGAKSGLVAGVVAAAIAAAVLAGVAI
385 FLAVRKASMRRAQARPPRRLVEHASSAYP
414 SKLFADARYISQTVKLGALGIPAYRSFSLVE
445 LEAATND
Kinase
452 FEVSNMMGQDSHGQMYRGRLSNGTPV
478 TIRSLKVKRSQTSQSFNRHIEMISKLRHRH
508 LVSALGHCFEYNLDDSTVTQLYLVFEYV
536 QNGNLRGRISQGTEGRKLTWVQRISTAIG
565 VAKGIQFLHGGIIPGLFANNLKIHQHSSGP
595 EPCKIQATLIIVPNGDKIDIFDFGVILLEVV
626 SGRPITSIYEVEIMKEQLQSALTAEPAKRS
656 FVDPSGEQGICLRCLAKEAVQRPSVEDVL
685 WNLQFAAQVQDD

Figure 4. Deduced amino acid sequence of the XET5B11 ORF encoded protein. The LRR domain at aa 113 to 298 is not a classic LRR, the N-terminal region contains 20% leucine, we referred to this region as a leucine-rich domain. A transmembrane region is double underlined; aa 452 to 688 contains a putative cytoplasmic protein kinase domain, the conserved regions are underlined, they are intalics and similar to the sub domain of the serine/threonine protein kinase.

gene from *O. minuta*. This method greatly reduces tedious filter hybridization screening, while also avoiding the problems associated with PCR-based cloning. Using this method, we successfully identified 26 candidate clones from the *O. minuta* DNA transformation-competent genomic library. Sequence analysis showed that 6 candidate genes had similar structures as *R* gene conserved domains, suggesting that the method of conserved domains mediated by RecA protein homologous recombination is advisable for isolating candidate *R* gene from *O. minuta*. This strategy also suits the gene cloning of other species with no or rare genetic background, since we just require the genetic information of its related species. Finally, the positive clones were introduced into Agrobacterium *tumefaciens* strain EHA105, and the callus of susceptible japonica variety Taipei309 was used for transformation according to published methods (Hiei et al., 1994).

The predicted encoding product of XET5B11 has a structure of LRR receptor-like protein kinase, the transmembrane region and protein kinase domain, indicating it may be including a new *R* gene. The function screening was also performed continuously. The self progenies T_2 lines were challenged by inoculation 9 *Xoo* strains from China (contributed by Nanjing Agricultural University). The homozygous transformation plants were inoculated in the field during summer; the disease was scored at 2 weeks after inoculation for young seedlings and 2 to 3 weeks after inoculation for adult plants. The result reveals that the 121 transformats of XET5B11 had no resistance to the strains (unpublished). The inoculation with *M. grisea* in nature at Jishou in Hunan Province was also performed and no resistance transformat was verified. It was reported that the LRR domain of disease resistance proteins of plants directly interact with the product of avirulence gene or its indirect product, and the race-specificity is determined by the LRR domain. Good examples are the *Xa21* and *Xa26,* which are the only two *R* genes confirmed so far to encode LRR receptor-like protein (Song et al., 1995, Sun et al., 2004). The

Table 2. The location of 8 positive clones and note of the genes on NCBI.

The clone	The gene	NCBI description	The domain
XET3A8	Os07g0166700	Protein kinase-like domain containing protein	STK and LRR
XET5B11	Os07g0166700	Protein kinase-like domain containing protein	STK and LRR
XET7E11	Os11g0692100	Bacterial blight resistance protein	LRR
XES3-1G16	Os05g0318600	Similar to Protein kinase (Pto kinase)	STK
XES3-3H18	Os05g0318600	Similar to Protein kinase (Pto kinase)	STK
XES3-1E4	Os06g0574600	Resistance protein candidate (Fragment)	STK
XES3-3B6	Os06g0705100	Similar to Thylakoid lumenal 13.3 kDa protein	-
XES3-4H3	Os06g0705100	Resistance protein candidate (Fragment)	-

The positive clone was located on or near the gene described and the conserved domain of the gene was also shown.

difference between them is the number of the LRR domains. Since the structure of the XET5B11 is not the typical LRR domain, the resistance spectra would be different from *Xa21* and *Xa26*, or would even disappear. This result partially suggests that the resistance is determined by structure. Since the rice diseases and its races or strains are very diverse, the function screening with more *Xoo* and *M. grisea* races or strains will continue.

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