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ORIGINAL PAPER

Shu-Mei Jiang · Jun Hu · Wei-Bo Yin · Yu-Hong Chen · Richard R.-C. Wang · Zan-Min Hu

Cloning of resistance gene analogs located on the alien chromosome in an addition line of wheat-*Thinopyrum intermedium*

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Abstract Homology-based gene/gene-analog cloning method has been extensively applied in isolation of RGAs (resistance gene analogs) in various plant species. However, serious interference of sequences on homoeologous chromosomes in polyploidy species usually occurred when cloning RGAs in a specific chromosome. In this research, the techniques of chromosome microdissection combined with homology-based cloning were used to clone RGAs from a specific chromosome of Wheat-Thinopyrum alien addition line TAi-27, which was derived from common wheat and *Thinopyrum intermedium* with a pair of chromosomes from *Th. intermedium*. The alien chromosomes carry genes for resistance to BYDV. The alien chromosome in TAi-27 was isolated by a glass needle and digested with proteinase K. The DNA of the alien chromosome was amplified by two rounds of Sau3A linker adaptor-mediated PCR. RGAs were amplified by PCR with the degenerated primers designed based on conserved domains of published resistance genes (R genes) by using the alien chromosome DNA, genomic DNA and cDNA of *Th. intermedium*, TAi-27 and 3B-2 (a parent of TAi-27) as templates. A total of seven RGAs were obtained and sequenced. Of which, a constitutively expressed single-copy NBS-LRR type RGA ACR3 was amplified from the dissected alien chromosome of TAi-27, TcDR2 and TcDR3 were from cDNA of *Th. intermedium*, AcDR3 was from cDNA of TAi-27, FcDR2 was from cDNA of 3B-2, AR2 was from genomic DNA of TAi-27 and TR2 was from genomic DNA of *Th. intermedium*. Sequence homology analyses showed that the above RGAs were highly homologous with known resistance genes or resistance gene analogs and belonged to NBS-LRR type of R genes. ACR3 was recovered by PCR from genomic DNA and cDNA of *Th. intermedium* and TAi-27, but not from 3B-2. Southern hybridization using the digested genomic DNA of *Th. intermedium*, TAi-27 and 3B-2 as the template and ACR3 as the probe showed that there is only one copy of ACR3 in the genome of *Th. intermedium* and TAi-27, but it is absent in 3B-2. The ACR3 could be used as a specific probe of the R gene on the alien chromosome of TAi-27. Results of Northern hybridization suggested that ACR3 was constitutively expressed in *Th. intermedium* and TAi-27, but not 3B-2, and expressed higher in leaves than in roots. This research demonstrated a new way to clone RGAs located on a specific chromosome. The information reported here should be useful to understand the resistance mechanism of, and to clone resistant genes from, the alien chromosome in TAi-27.

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Shu-Mei Jiang and Jun Hu contributed equally to this work

S.-M. Jiang · J. Hu · W.-B. Yin · Y.-H. Chen · Z.-M. Hu (✉)
Institute of Genetics and Developmental Biology,
Chinese Academy of Sciences, Datun Road,
Beijing 100101, China
E-mail: zmhu@genetics.ac.cn
Tel.: +86-10-64889356
Fax: +86-10-64889783

R. R.-C. Wang
USDA-ARS, FRRL, Utah State University,
Logan, UT 84322-6300, USA

Introduction

To date, 46 resistance genes (R genes) conferring resistance against pathogens, insects, nematodes, and viruses attacking 12 plant species have been cloned (Dilbirligi et al. 2004). Most of the cloned R genes are structurally conserved and can be grouped into four distinct classes on the basis of the presence of one or more of the nucleotide-binding site (NBS), receptor-like transmembrane kinase (RLK), cytoplasmic protein kinase (PK),

and leucine-rich repeat (LRR) domains (Hammond-Kosack and Jones 1997; Meyers et al. 1999; Mondragon-Palomino et al. 2002). Designing degenerated primers based on the conserved domain can help to isolate resistance gene analogs (RGAs), which may be linked with resistance genes or correlated with resistance genes (Kanazin et al. 1996; Leister et al. 1999; Shen et al. 1998; Yu et al. 1996). In recent years, the homology-based cloning method has been extensively applied in isolation of RGAs from total genomic DNAs or cDNA of various plant species, such as rice (Leister et al. 1999; Mago et al. 1999), wheat (Fenillet et al. 1997; Seah et al. 1998; Maleki et al. 2003; Dilbirligi et al. 2004), maize (Collins et al. 1998) and soybean (Kanazin et al. 1996; Yu et al. 1996).

However, it is difficult to clone a RGA located on a specific chromosome with homology-based method from total genomic DNA or cDNA due to interference of sequences on homoeologous chromosomes in complicated allopolyploid genome, such as wheat. Most wheat genes have multiple orthologs and in some cases paralogs (Dilbirligi et al. 2004). Chromosome microdissection is a direct way to get chromosome and/or chromosome-region-specific DNA (Ludecke et al. 1989; Chen and Armstrong 1995; Zhou et al. 1999; Liu et al. 1999). Combining of chromosome microdissection with homology-based cloning method can eliminate serious interference of homology sequences in genome and help locate and clone gene, especially for disease resistance genes in low or unique copy on a specific chromosome.

Wheat-*Thinopyrum intermedium* alien addition line TAi-27 is one of 14 alien addition lines carrying a pair of chromosomes from *Th. intermedium (syn. Agropyrom intermedium)* in common wheat (He et al. 1988). TAi-27 possesses resistance to barley yellow dwarf virus (BYDV) located on the alien chromosomes (Zhang et al. 1991; Han et al. 1998; Tian et al. 1999). Tian et al. (2000) gave evidence showing that TAi-27 possessed two pairs of St chromosomes; one being disomic addition and the other substituting for a pair of wheat chromosomes. Liu et al. (2001) provided evidence that TAi-27 has one group 2 and one group 7 alien chromosomes. Furthermore, Zhang et al. (2001a, b) showed that a group 2 St chromosome derived from the partial amphiploid "Zhong 4 awnless," the same source for TAi-27, is responsible for conferring BYDV resistance. Therefore, it appears that the BYDV resistance bearing chromosome in TAi-27 is the same group 2 chromosome as in addition line Z1 (Larkin et al. 1995; Han et al. 2003).

Barley yellow dwarf virus causes serious yield losses in all cereals worldwide. Annual yield reductions due to BYDV average 1–3%, although losses are tenfold greater in some seasons (Burnett 1987). Resistance genes against BYDV are not found in common wheat but available in barley, oat and some wild *Triticeae* species. Until now, no resistance genes against BYDV were cloned, but several polypeptide markers and PCR markers linked with BYDV resistance gene have been reported (Holloway and Heath 1992; Paltridge et al. 1998, Wang et al. 2002) and being used routinely in wheat breeding (Ayala et al. 2001; Stoutjesdijk et al. 2001; Xin et al. 2001; Zhang et al. 2000, 2001a, b).

In this research we microdissected the alien chromosome of Wheat-*Th. intermedium* alien addition line TAi-27, got its DNA by LA-PCR (linker adaptor mediated PCR) and cloned the RGAs from microdissected alien chromosome DNA, and as well as genomic DNA and cDNA of TAi-27, *Th. intermedium* and 3B-2 (a maternal parent of TAi-27). Obtained RGAs were compared with published known resistance genes and analogs by Blast. A specific RGA from microdissected alien chromosome DNA were recovered from TAi-27 and *Th. intermedium* and characterized by Southern and Northern hybridization. Results of this research would help formulate a strategy to clone RGAs located on specific chromosomes, understand the mechanism of TAi-27 and *Th. intermedium* resistance against BYDV, and further clone resistant genes from TAi-27 and *Th. intermedium*.

Materials and methods

Plant materials

Germplasm used in this research included wheat-*Thinopyrum* alien addition line TAi-27 (20 pairs of chromosomes of AABBDD + two pairs of St-genome chromosomes from *Th. intermedium*; $2n=44$), along with its two parents, *Th. intermedium* ($E_1E_1E_2E_2StSt$, with the St genome originated from *Pseudoroegneria*, $2n=42$; Liu and Wang 1993) and 3B-2 (*Triticum aestivum*, AABBDD, $2n=42$, the maternal parent). All materials were kindly supplied by Prof. Menyuan He of Northeast Normal University and Prof. Xiangqi Zhang of Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (CAS).

Chromosome microdissection and DNA amplification of alien chromosome of TAi-27

The chromosome microdissection and DNA amplification were performed according to the procedures described by Wan et al. (2001) and Jiang et al. (2004). Briefly, the TAi-27 seeds were immersed in warm water (25°C) for 5–8 h, then germinated on moist filter paper in a petri dish at 25°C in dark. After the seeds sprouted, they were cultured at 4°C for 24 h, then at 25°C in dark until the roots grew up to 0.5–1 cm. The seeds with roots (0.5–1 cm) were treated in ice water (0°C) for 24 h to increase metaphase cells. Then root tips were harvested and fixed in 3:1 ethanol: acetic acid for 5 min, and transferred immediately into 70% ethanol and stored at −20°C. Before being squashed, the root tips were digested with an enzyme mixture of 2% cellulase and 2%

pectolyase in 75 mM KCl, 7.5 mM EDTA at 37°C for 15–25 min, then rinsed in ddH_2O and stored at 4°C for 15–20 min. After the root tips were squashed in a drop of 1% Carbol Fuchsin solution, they were immediately used for microdissection. The alien chromosome in TAi-27 was identified by its smallest size (Fig. 1) and then microdissected by using the glass needle fixed on the arm of a LeitZ micro-operation instrument on an inverted phase-contrast microscope (OLYMPUS 1M, Japan). The microdissected chromosome was digested with 20 μl of proteinase K solution (19 ng/μl in 1×T4 ligase buffer), then 0.02U Sau3A (Promega) in an Eppendorf tube. Subsequently, the chromosomal DNA was amplified using *Sau*3A Linker adaptor-mediated PCR (LA-PCR). *Sau*3A linker adaptors were prepared with the 23mer DNA sequence 5′-GATCCTGAGCT CGAATTC-GACCC-3′ and the 19mer DNA sequence 5′-GGGT CGAATTCGAATTCGAGCTCAG-3′. The digested chromosomal DNA was linked with *Sau*3A adaptor (2 μl, 5 ng/μl) using T4 DNA 1igase (0.5 μl, 3 U/μl, Promega) in a total volume of 24.5 μl. Two rounds of PCR were performed. The first round of PCR was carried out in the same tube by adding 10 μl of 10X Taq buffer, 6 μl of 25 mM $MgCl_2$, 2 μl of 10 mM dNTPs, 1 μl of 19 mer primer (50 ng/μl), 2 U Taq DNA polymerase (Promega) and double distilled water in a 100 μl total volume. After denaturing at 94° C for 5 min, amplification was performed with 35 cycles of 1 min at 94° C, 1.5 min at 50° C, 3 min at 72° C, followed by a final 15 min extension at 72°C. The second round of PCR was done under the same conditions described above except that only a 2 μl product from the first round of PCR was used as the template.

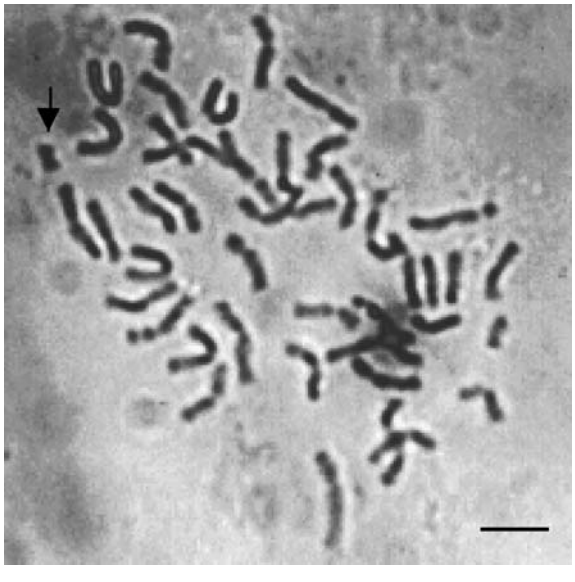

Fig. 1 Metaphase chromosomes of a TAi-27 root tip mitotic cell. The *arrow* indicates the microdissected chromosome. *Bar* represents 10 μm

Genomic DNA extraction, mRNA isolation and cDNA synthesis

The genomic DNA and mRNA of *Th. intermedium*, TAi-27 and 3B-2 were extracted from leaves at three-leaf stage by using the CTAB method and TRIzol kit (Gibco, BRL), respectively. cDNA was synthesized by using Takara RNA PCR kit (AMV) ver 2.1 (TaKaRa, Japan).

Cloning and sequencing of resistance gene analogs

Two degenerated primers were designed based on the conserved P-Loop and hydrophobic domains (GLPLA) of NBS-LRR type resistance genes including *N* of tobacco (Whitham et al. 1994), *L6* of flax (Lawrence et al. 1995), *RPS2* of *Arabidopsis* (Bent et al. 1994; Mindrinos et al. 1994) and *CRE3* of wheat (Lagudah et al. 1997). The sequences of the primers were F1 (P-loop), 5′-GGAATGGGWGGSGTKGGGAARAC-3′; R1 (GLPLA), 5′-ARNGCNARWGGMARNCC-3′; R = A/G, Y = C/T, N = A/C/G/T, W = A/T, S = G/C,K = G/T, H = A/C/T, M = A/C.

PCR was performed in a 20 μl volume, including TaKaRa Premix 10 μl,1 μl F1 (10 μM),1 μl R1 (10 μM), and templates which were the second-round PCR products of the alien additional chromosome of TAi-27, genomic DNA and cDNA of *Th. intermedium*, TAi-27 and 3B-2, respectively. After denaturation at 95°C for 1 min, amplification was performed with 35 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, followed by a final extension at 72°C for 5 min. PCR products were inserted into PUCmT-Vecter (Sagon, China). Recombinant plasmids DNA were isolated by alkaline lysis method. DNA sequencing was carried out by Bio-Asia Company (China).

Characterization of the RGA specific to alien chromosome of TAi-27

Recovery of the RGA specific to alien chromosome of TAi-27

According to the sequence of ACR3, a RGA cloned from microdissected alien chromosome DNA of TAi-27, a pair of primers was designed to further characterize whether it is present in *Th. intermedium*, TAi-27 and 3B-2. The sequences of the primers were F2, 5′-GG GCGTAGGCAAGACCACAC-3′; and R2, 5′-AGCG AGAGGAAGGCCCCAAGC-3′. PCR was performed in a 20 μl volume, including TaKaRa Premix 10 μl,1 μl F2 (5 μM),1 μl R2 (5 μM), and the templates which were the second-round PCR products of the alien chromosome of TAi-27 and genomic DNA and cDNA of *Th. intermedium*, TAi-27 and 3B-2, respectively. The PCR procedures were same as those described above, except the annealing temperature was 65°C. The

obtained PCR products were sequenced by Bio-Asia Company (China).

Southern hybridization

Genomic DNA of *Th. intermedium*, TAi-27 and 3B-2 digested by restrictive enzymes—*Hae*III, *xsp*II and *Sau*3AI and the second-round PCR products of the alien chromosome of TAi-27 were separated by 0.8% agarose gel and transferred onto nylon membrane (Hybond$^+$, Amersham). Southern hybridization was performed using recovered ACR3 from TAi-27 labeled by $\alpha-^{32}$ P-dCTP (Random Primer DNA Labeling Ver.2, TaKaRa, Japan) as the probe. After hybridization at 65°C for overnight, the nylon membrane was washed with wash buffer I (2×SSC, 0.1%SDS) for two times, each for 15 min at room temperature, then washed with wash buffer II (0.1×SSC, 0.1%SDS) for two times, each for 15 min at 65°C. The detection was done according to the procedure described by Sambrook et al. (1989).

Northern hybridization

Total RNA from leaves, stems and roots of *Th. intermedium*, 3B-2 and TAi-27 at three-leaf stage were denatured, then separated by electrophoresis in formaldehyde gel (1.5%) and transferred onto nylon membrane (Hybond$^+$, Amersham). Northern hybridization was done with the probe ACR3 labeled by $\alpha-^{32}$ P-dCTP. The hybridization and detection procedures were same as those for Southern hybridization described above.

Data analysis

The DNA sequence and their deduced amino acid sequence of RGAs obtained in this research were compared with those in GenBank database using the Blast search program (http://www.ncbi.nlm.nih.gov/). Polygenetic analysis was performed with program DNAman (DNAman for windows, version 4.0, Lynnon Biosoft).

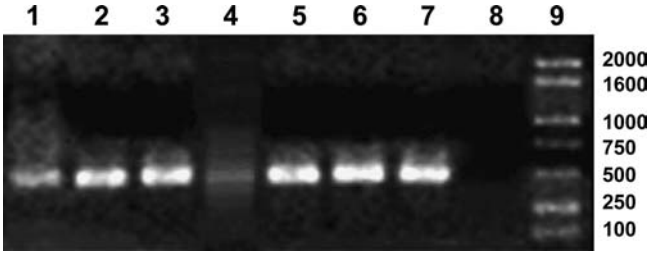

Fig. 2 Electrophoresis of PCR products by using primers F1 and R1 (5′-GGAATGGGWGGSGTKGGGAARAC-3′ and 5′-ARNGCNARWGGMARNCC-3′; R = A/G, Y = C/T, N = A/C/G/T,W = A/T,S = G/C,K = G/T,H = A/C/T,M = A/C). *Lanes 1 to 7*: The amplified products with the DNA of the alien chromosome of TAi-27, the genomic DNA of TAi-27, *Th. intermedium* and 3B-2, and the cDNA of TAi-27, *Th. intermedium* and 3B-2 as the templates, respectively. *Lane 8*: The negative control (with ddH2O as the template). *Lane 9:* The molecular weight marker DL2000

Results

Amplification and identification of resistance gene analogs

Using primers F1 and R1, a strong single band of about 500 bp was amplified from the second-round PCR products of the alien chromosome of TAi-27, genomic DNA of *Th. intermedium*, and TAi-27, and cDNA of *Th. intermedium*, TAi-27 and 3B-2, and a weak band from genomic DNA of 3B-2, respectively (Fig. 2). The bands from those different templates were recovered and inserted into PUCmT-Vector. Five clones were randomly picked from recombinant clones of each band obtained above and the inserted fragments were sequenced. The sequence data were analyzed by Blast search program. Seven different RGAs were obtained (Table 1), and other sequences were not RGAs. Of seven RGAs, one (ACR3) came from the alien chromosome of TAi-27, two (TcDR2,TcDR3) from cDNA of *Th. intermedium*, one (AcDR3) from cDNA of TAi-27, one (FcDR2) from cDNA of 3B-2, one (AR2) from genomic DNA of TAi-27 and one (TR2) from genomic DNA of *Th. interme-*

Table 1 Seven resistance gene analogs (RGAs)

RGA	Source	Length (bp)	Acc number	Alignment	Homology (%)
TcDR2	cDNA of *Th .intermedium*	506	AY242388	RGA [*Hordeum vulgare*]	91
				RGA[*Avena vaviloviana*]	97
TcDR3	cDNA of *Th. intermedium*	518	AY249524	R 4 protein [*Glycine max*]	78
TR2	Genomic DNA of *Th. intermedium*	509	AY249525	RGA [*Triticum aestivum*]	82
				KR1 [*Glycine max*]	77
AcDR3	cDNA of wheat-*Thinopyrum* alien addition line TAi-27	509	AY238935	RGA [Glycine max]	82
				RGA [*Triticum aestivum*]	82
AR2	Genomic DNA of wheat-*Thinopyrum* alien addition line TAi-27	518	AY249526	LM6 [*Glycine max*]	75
				RGA [*Triticum aestivum*]	82
ACR3	DNA of alien chromosome in wheat-*Thinopyrum* alien addition line TAi-27	509	AY249527	RGA [*Glycine max*]	82
				RGA [*Triticum aestivum*]	82
FcDR2	cDNA of 3B-2	518	AY249528	R 4 protein [*Glycine max*]	78

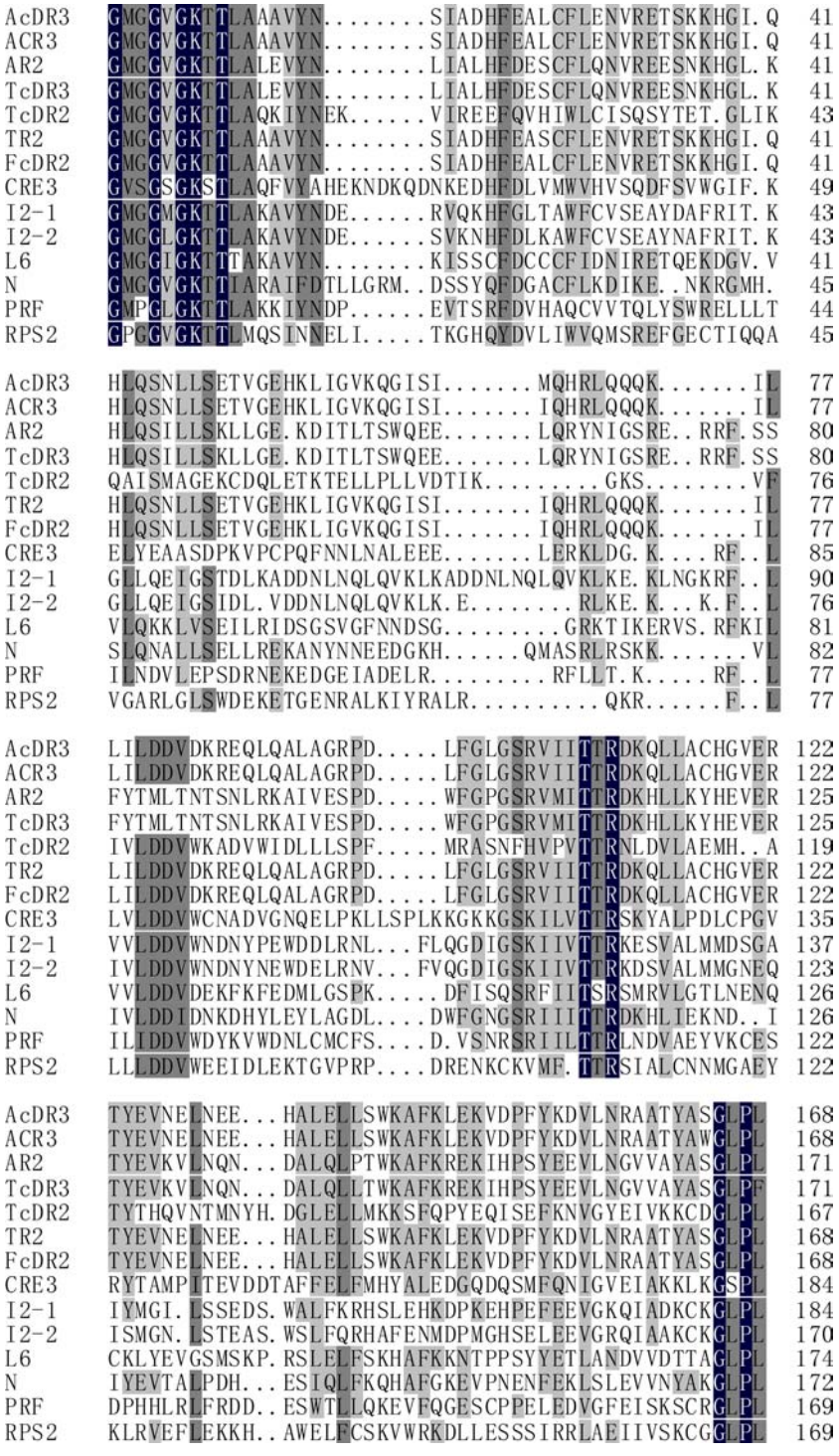

Fig. 3 Deduced amino acid homology comparisons among RGAs cloned in this research and some known genes (CRE3, I2-1, I2-2, L6, N, PRF, RPS2)

dium. All of the seven RGAs were registered in GenBank (Table 1).

Homology comparison and classification of RGAs

The seven RGAs were compared with seven known R genes in the GenBank database for DNA sequences and deduced amino acid sequences by the BLASTN and BLASTX. The sequences were aligned with other plant RGAs in GenBank with high homology (Table 1). The cloned seven RGAs contained the conservative regions in known NBS-LRR resistance protein, such as conservative P-loop, *Kinase2a*, *Kinase3a* and hydrophobic domain (GLPLA) (Fig. 3). Therefore, the seven RGAs belonged to NBS-LRR type of R genes.

The cloned seven RGAs were highly homologous to each other and can be divided into three groups. The first group included AcDR3, ACR3, TR2 and FcDR2, which differ from each other by 1–2 amino acids. The

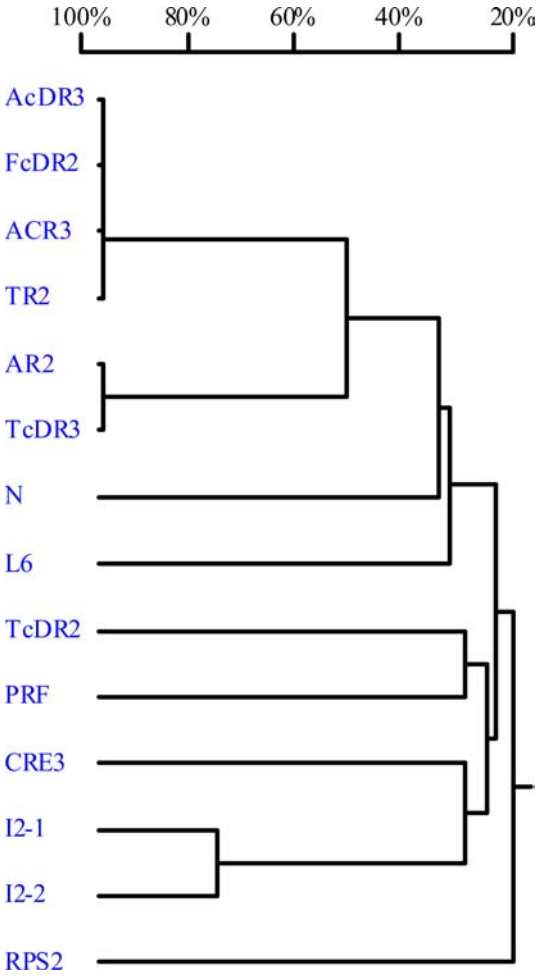

Fig. 4 Homology analysis based on alignment of the deduced amino acid sequences of seven RGAs with seven known resistance genes (CRE3, I2-1, I2-2, L6, N, PRF, RPS2)

second group included AR2 and TcDR3, also differ by 1–2 amino acid. The third group consists of only TcDR2. Similarity between the first group and the second group was 54%, but only 27% between the third group and the other two groups (Fig. 4).

Recovery of ACR3 from alien chromosome of TAi-27, *Th intermedium* and 3B-2

Primers F2 and R2, designed according to the sequence of ACR3, were used to amplify ACR3 from microdissected chromosome DNA, genomic DNA and cDNA of TAi-27, *Th. intermedium* and 3B-2. A PCR product of about 500 bp was amplified from alien chromosome DNA of TAi-27, genomic DNA and cDNA of TAi-27 and *Th. intermedium*, but not from genomic DNA and cDNA of 3B-2 (Fig. 5). The bands of PCR products were recovered and sequenced. All sequences were same as that of ACR3.

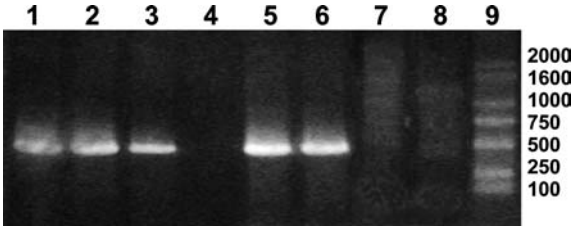

Fig. 5 Electrophoresis of PCR products by using primers F2 and R2 (5′-GGGCGTAGGCAAGACCACAC-3′ and 5′-AGCGAGAGGAAGGCCCCAAGC-3′). *Lanes 1 to 4:* The amplified products with the DNA of the alien chromosome of TAi-27, the genomic DNA of TAi-27, *Th. intermedium* and 3B-2 as the templates, respectively. *Lanes 5 to 7*: The amplified products with the cDNA of of TAi-27, *Th. intermedium* and 3B-2 as the templates, respectively. *Lane 8*: The negative control (with ddH2O as the template). *Lane 9*: Molecular weight marker DL2000

Characterization of ACR3 by southern hybridization

Southern hybridization was carried out for identifying the origin and copy numbers of ACR3 by using the digested genomic DNA of TAi-27, *Th. intermedium* and 3B-2 as templates and ACR3 labeled with $\alpha-^{32}$ P-dCTP as the probe. There was one hybridization band each from genomic DNA of *Th. intermedium* and TAi-27 but none in 3B-2 (Fig. 6), indicating that ACR3 is present as a single copy gene in *Th. intermedium* and TAi-27 but is absent in 3B-2.

Expression of ACR3

Northern hybridization analysis was performed to investigate the expression of ACR3 by using total RNA from leaves of *Th. intermedium* and 3B-2, and total RNA from leaves, stems and roots of TAi-27 as the templates and ACR3 labeled by $\alpha-^{32}$ P-dCTP as the probe. The expression was detected in *Th. intermedium*

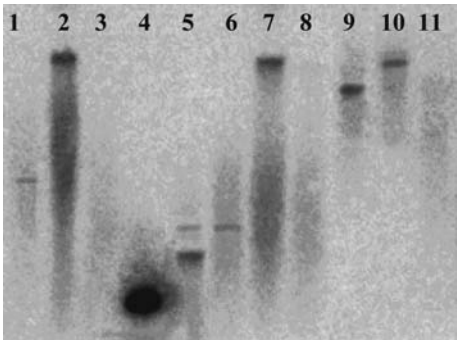

Fig. 6 Southern hybridization by using ACR3 as the probe labeled with α-32P-dCTP. *Lanes 1 to 3*: The genomic DNA of *Th. intermedium*, TAi-27 and 3B-2, respectively, digested with *Hae*III; *lanes 6 to 8*: same as *lanes 1 to 3*, but digested with *xsp*II; *lanes 9 to 11*: same as *lanes 1 to 3*, but digested with *Sau*3A. *lane 4*: positive control. *lane 5:* The second-round LA-PCR product of the alien chromosome DNA of TAi-27

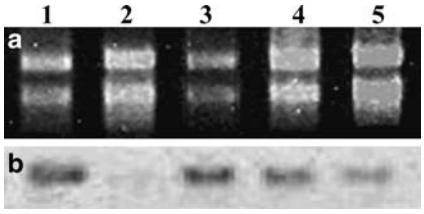

Fig. 7 Northern hybridization by using ACR3 as the probe. **a** Electrophoresis of total RNA. (*lane 1*) total RNA of *Th. intermedium* leaves; (*lane 2*) total RNA of 3B-2 leaves; (*lanes 3, 4, and 5*) total RNA of leaves, stems and roots of TAi-27. **b** Northern hybridization of RNAs in (**a**) using ACR3 as the probe labeled with α-32P-dCTP

and TAi-27 and not in 3B-2 (Fig. 7). Because RNA was extracted from plants not infected with BYDV, the results suggested that ACR3 would be constitutively expressed in TAi-27 and *Th. intermedium*. The Northern hybridization further proved that ACR3 was from *Th. intermedium*, and that ACR3 was expressed in leaves, stem and roots of TAi-27, but stronger in leaves than in roots.

Discussion

Usually there are many different genes distributed on different chromosomes of plant genome. In NBS-LRR type resistance genes, there are conserved NBS sequences. However NBS sequences are very abundant in plant genome; such as in *Arabidopsis* genome, there are approximately 1% NBS sequences in *Arabidopsis* genome and tend to distribute in clusters (Meyers et al. 1999). It is difficult to get an R gene located on a specific chromosome from genomic DNA by PCR using degenerated primers designed based on the conserved domains of R genes. Furthermore there are one or many resistance genes or gene families often existing on one chromosome; for instance, the short arm of chromosome 1D of wheat contains at least two genes that confer resistance to *Puccinia recondita* and several resistance genes effective against rust pathogens (Spielmeyer et al. 2000). In soybean, a genomic region rich in resistance genes is found on molecular linkage group F (MLG-F) (Peñuela et al. 2002). Therefore, it would be easy to clone resistant genes located on a specific chromosome by PCR using microdissected specific chromosome DNA as the template and degenerated primers designed based on the conserved domains of R genes. Wan et al. (2000) tried to clone RGAs from microdissected alien chromosome DNA of TAi-27 by PCR, but failed. Huang et al. (2004) got RGAs from some randomly picked chromosomes of *Citrus grandis*, but did not proved that the obtained RGAs really came from and specific to the picked chromosome. In this paper, ACR3, an NBS-LRR type RGA, was cloned from microdissected alien chromosome DNA of TAi-27. By Southern hybridization and Northern hybridization, further proved that it originated from *Th. intermedium*. This is the first report that an NBS-LRR type RGA was cloned from a specific chromosome of plant genome.

In this research, ACR3 was amplified from microdissected alien chromosome DNA of TAi-27, but not from genomic DNA and cDNA of *Th. intermedium* and TAi-27 by using the degenerated primers. It further proved that there is serious interference of homology sequences when amplification was performed. Whereas, ACR3 was recovered from *Th. intermedium* and TAi-27 by using the specifically designed primers based on the sequences amplified from microdissected alien addition chromosome DNA. Southern hybridization result proved that there is one copy of ACR3 in *Th. intermedium* and TAi-27. It suggested that ACR3 should be specific to alien chromosome of TAi-27 and could be used as a marker of the alien chromosome of TAi-27, and as a probe to clone the correspondent full length R gene.

There might be other RGAs of NBS-LRR type on the alien chromosome of TAi-27 in addition to ACR3. Although chromosome microdissection is a quick way to obtain chromosome-specific or chromosome-region-specific DNA, a lot of DNA sequences of microdissected chromosome were lost due to the use of the restriction enzyme *Sau*3A for digesting the microdissected chromosome DNA. *Sau*3A is cytosine-methylation-sensitive so that it results in preferential selection of low/unique copy sequences that spread over unmethylated regions in the genome (Cheung et al. 1992). To solve this problem, different DNA restriction enzymes can be used to digest microdissected chromosome DNA, correspondent linker adapters are used to link with digested microdissected chromosome DNA, and subsequently the microdissected chromosome DNAs would be amplified with high coverage by different correspondent primers.

Researches on molecular markers and specific probes of alien addition lines had been carried out since wheat-*Th.* alien addition lines were created (Tian et al. 2000; Zhang et al. 2000; Jiang et al. 2004). Jiang et al. (2004) cloned nine ESTs from a subtractive library of TAi-27 infected by *Schizaphis graminum* carrying GAV strain of BYDV. Of which, seven were located on the alien addition chromosome, but none possessed NBS-LRR conserved domains of RGAs. In this research we got four RGAs from *Th. intermedium*, of which ACR3 was located on an alien chromosome of TAi-27, probably from the St genome of *Th. intermedium* (Liu and Wang 1993). Jiang et al. (2004) proved that the tolerance to BYDV of *Th. intermedium* is stronger than that of TAi-27. Understandably, there are other BYDV resistance genes on other chromosomes in *Th. intermedium*. Although we do not know the relationship between the four RGAs from *Th. intermedium* (this paper) and seven ESTs (Expressed sequence tags, Jiang et al. 2004) from alien addition chromosome of TAi-27 obtained by our group and the tolerance to BYDV, these genes will eventually lead to understanding of the mechanism of BYDV tolerance in *Th. intermedium* and TAi-27.

Resistance genes against BYDV are not found in common wheat but available in barley, oat and some wild *Triticeae* species. The similarity between the sequences we got in this research and known R gene analogs or genes ranged 75%∼97%. Interestingly, the similarities between TcDR2 from cDNA of *Th. intermedium* and RGAs from *Hordeum vulgare* and *Avena vaviloviana* were up to 91% and 97% (Table 1, this paper), respectively. More importantly, four of seven obtained RGAs are highly homologous with RGAs from *Glycine max*, which has not been reported to be infected by BYDV. It is worth to further clarify the phenomena described above.

In conclusion, an efficient way to clone RGAs from a specific chromosome of plant was established in this research and a RGA specific to the alien chromosome of TAi-27 was cloned and characterized. Six additional RGAs with high similarity with known RGAs were obtained from *Th. intermedium* and TAi-27, which provided useful information for further investigating the resistance to BYDV in *Th. intermedium* and TAi-27.

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