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ORIGINAL PAPER

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Microdissection and molecular manipulation of single chromosomes in woody fruit trees with small chromosomes using pomelo (*Citrus grandis*) as a model. II. Cloning of resistance gene analogs from single chromosomes

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Abstract Amplification of resistance gene analogs (RGAs) is both a useful method for acquiring DNA markers closely linked to disease resistance (R) genes and a potential approach for the rapid cloning of R genes in plants. However, the screening of target sequences from among the numerous amplified RGAs can be very laborious. The amplification of RGAs from specific chromosomes could greatly reduce the number of RGAs to be screened and, consequently, speed up the identification of target RGAs. We have developed two methods for amplifying RGAs from single chromosomes. Method 1 uses products of *Sau*3A linker adaptor-mediated PCR (LAM-PCR) from a single chromosome as the templates for RGA amplification, while Method 2 directly uses a single chromosomal DNA molecule as the template. Using a pair of degenerate primers designed on the basis of the conserved nucleotide-binding-site motifs in many R genes, RGAs were successfully amplified from single chromosomes of pomelo using both these methods. Sequencing and cluster analysis of RGA clones obtained from single chromosomes revealed the number, type and organization of R-gene clusters on the chromosomes. We suggest that Method 1 is suitable for analyzing chromosomes that are unidentifiable under a microscope, while Method 2 is more appropriate when chromosomes can be clearly identified.

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Introduction

Many plant disease resistance (R) genes share several common domains in their encoded proteins (Baker et al. 1997; Hammond-Kosack and Jones 1997). One of the domains is the nucleotide-binding site (NBS), the motifs of which are well conserved in many R genes, such as *RPS2* of *Arabidopsis* (Bent et al. 1994; Mindrinos et al. 1994), *N* of tobacco (Whitham et al. 1994) and *L6* of flax (Lawrence et al. 1995). In various plant species, degenerate primers have been designed—based on the NBS domain—to amplify resistance gene-like sequences or resistance gene analogs (RGAs). This approach has been applied to soybean (Kanazin et al. 1996; Yu et al. 1996; Graham et al. 2002), potato (Leister et al. 1996), *Arabidopsis* (Aarts et al. 1998; Speulman et al. 1998; Meyers et al. 1999, 2003), wheat (Seah et al. 1998; Maleki et al. 2003), maize (Collins et al. 1998), lettuce (Shen et al. 1998), rice (Wang et al. 1998; Mago et al. 1999; Zheng et al. 2001), tomato (Grube et al. 2000; Pan et al. 2000), flax (Dodds et al. 2001), grapevine (Donald et al. 2002) and citrus (Deng et al. 2000).

Amplification of RGAs is a useful protocol for genetic research and plant breeding. First, RGAs can serve as molecular markers. Studies have shown that R-gene families are usually distributed as many clusters throughout the genome (Meyers et al. 1998). Each cluster usually consists of many members. Therefore, it may be easier to find markers closely linked to given R genes from amplified RGAs (Donald et al. 2002). Second, for a

similar reason, RGAs can also be R-gene candidates. If an RGA cosegregates with an R gene, it will quite possibly be the R gene and thus can be selected as a candidate for further analysis (such as a genetic complementation test). Therefore, amplification of RGAs is a potential approach for the cloning of new R genes (Dodds et al. 2001). Third, RGA analysis is a useful approach for elucidating the organization and evolution of R-gene families or clusters (Dodds et al. 2001; Graham et al. 2002; Meyers et al. 2003), which has been a very active research field in plant science in recent years.

R-gene families are large multigene families. For example, RGAs constitute about 2% of the genome in *Arabidopsis* (Michelmore and Meyers 1998). To date, RGA amplification is usually performed based on the whole genome, resulting in a very large number of different RGAs. This makes the identification of any one specific RGA (for example, those closely linked to a given R gene or candidates of a target R gene) very laborious.

Chromosome microdissection is a physically based approach used for isolating specific chromosomes or chromosomal regions. The merit of this technology is to concentrate genetic analysis on a part—instead of the whole—of a genome. It was first developed for isolating polytene chromosome bands from *Drosophila* salivary gland cells (Scalenghe et al. 1981) and was later extended to the manipulation of mammalian chromosomes (Röhme et al. 1984). The combination of chromosome microdissection and PCR has become a very useful molecular cytogenetic technique for genetic and genomic studies and has been widely utilized in human, animal and a few plant species. One of the important applications of this technology is the construction of chromosome-specific or chromosomal region-specific DNA libraries (Jung et al. 1992; Albani et al. 1993; Schondelmaier et al. 1993; Vega et al. 1994; Chen and Armstrong 1995; Liu et al. 1997; Stein et al. 1998), which in turn can be used for screening chromosome-specific DNA probes for restriction fragment length polymorphism (RFLP) analysis (Chen and Armstrong, 1995) and gene tagging (Jung et al. 1992; Schondelmaier et al. 1993).

Huang et al. (2004, accompanying paper) demonstrated that the combined technology of chromosome microdissection and linker adaptor-mediated PCR (LAM-PCR) is suitable for constructing chromosome-specific DNA libraries in fruit trees possessing small chromosomes. In the investigation reported here, we developed a technical system that combines chromosome microdissection and homologous sequence amplification in order to acquire RGAs from single chromosomes. Since the source of the DNA templates is narrowed down from the whole genome to a single chromosome, the workload of identifying specific RGAs can be greatly reduced. As this investigation was an extension of the previous one (Huang et al. 2004), we continued using pomelo as a model. Our results have value as an example that is especially relevant for plants with small chromosomes and lacking a solid base of genetic studies. As far as we know, this is the first report on RGA cloning from single chromosomes in plants.

Materials and methods

Plant material

A pomelo variety, *Guanximiyou* (*Citrus grandis* cv. Guanxi), was used, as noted in Huang et al. (2004).

Chromosome preparation and microdissection

Young pomelo embryos at approximately 100 days after fertilization were used for chromosome preparation and microdissection. See Huang et al. (2004) for details.

Amplification of DNA fragments of single chromosomes

The method of *Sau*3A linker adaptor-mediated PCR (LAM-PCR) was used to acquire DNA fragments from single chromosomes. This procedure is described in detail in Huang et al. (2004), with the exception that only one round of LAM-PCR was carried out in the present investigation.

Amplification of RGAs from single chromosomes

A pair of degenerate oligonucleotide primers designed by Zheng et al. (2001) on the basis of two motifs of the NBS amino acid sequences conserved in several plant R genes were adapted. The forward primer P1 was designed based on the conserved P-loop amino acid sequence (GMGGVGKTT); the reverse primer P2 was designed based on another conserved sequence of amino acids (GLPLAL) residing in a transmembrane region in the RPS2 protein. The sequences of the two primers were: (P1) 5′-GGNATGGGNG-GNNTNGGNAA(AG)ACNAC-3′ and (P2) 5′-NAC(CT)TTNAG-NGCNAGNGNAGNCC-3′, where letter N stands for (AGCT). These primers were previously proven to be very efficient for amplifying RGAs from total genomic DNA in pomelo (Huang and Lu 2002).

Two methods were used for amplifying RGAs from single chromosomes.

- Method 1. RGAs were amplified using the LAM-PCR products of single chromosomes as templates.
- Method 2. Naked single chromosomal DNA molecules (Huang et al. 2004) were directly used as templates for RGA amplification, but two rounds of PCR were performed, with the products of the first round as templates for the second round.

In both methods, PCR was performed in a total volume of 25 μl using the following conditions: an initial denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 2 min. Each reaction contained 50 m*M* Tris-HCl pH 8.3, 2 m*M* $MgCl_2$, 800 μM dNTPs, 66 ng P1 primer, 66 ng P2 primer, 1 U *Taq* polymerase and 2 µl template DNAs (for the first-round PCR of Method 2, the template was an intact single chromosomal DNA).

Cloning and sequence analysis of amplified RGAs

PCR products of single chromosomes were separated by agarose gel electrophoresis, and DNA fragments with the expected sizes were recovered, ligated to the pGEM-T Easy vector (Promega, Madison, Wis.) and then transferred into DH5α-competent cells. Classification of clones was performed by restriction analysis using two enzymes (*Hae*III and *Taq*I) that recognize 4-bp sites. Clones

showing the same restriction patterns were presumed to be identical and therefore sorted into the same class. Some clones were randomly selected and sent to the Genomics and Bioinformatics Center (Chinese Academy of Sciences, Beijing, China) for sequencing. The sequences were examined by means of an alignment search in the GenBank using BLAST in order to identify the RGAs among them. Open reading frames of the cloned RGAs were examined using the software DNASTAR. Multiple-sequence alignments at the amino acid level among the cloned RGAs and known R genes were performed, and a phylogenetic tree was established using the CLUSTAL W program.

Results

Six chromosomes were isolated randomly from the same metaphase and transferred into microcentrifuge tubes numbered from 1 to 6. Tubes 1 and 2 were used to amplify RGAs with Method 1, while tubes 3–6 were used to amplify RGAs with Method 2. With the exception of tubes 1 and 4, all other tubes produced PCR products, each with a single bright band of approximately 500 bp (Fig. 1). As the size of all fragments amplified was expected to be approximately 500 bp from primers P1 and P2, the results indicated that both methods were effective for obtaining RGAs.

To confirm the results, we recovered candidate RGA bands from tubes 2 and 6, which represented Method 1 and Method 2, respectively, and cloned them for further analysis. More recombinant clones were obtained from tube 6 than from tube 2. From each tube, 50 or more clones were randomly selected for restriction analysis. The selected clones from tube 2 and those from tube 6 were grouped into 8 and 36 classes, respectively, according to their restriction patterns.

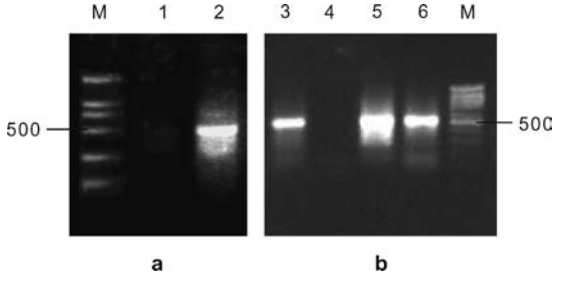

Fig. 1 Resistance gene analogs (RGAs) obtained from single chromosomes by Method 1 (**a**) and Method 2 (**b**). The *lane number* corresponds to the tube number. *M* Standard molecular-weight marker (in basepairs)

Ten clones representing different classes were sequenced, five from tube 2 (s34, s49, s50, s51, s7-4) and five from tube 6 (s75, s76, s8-3, s8-4, s8-6). The conserved motifs used for priming the PCR were absent in s50, s75, s76 and s8-4. This might be due to an incomplete sequence from the sequencing step. In spite of this, all sequences showed strong overall similarities to known R genes or published RGAs that had been recently cloned from other plant species (data not shown). The similarities of these sequences to known R genes were particularly high at the three NBS motifs: P-pool, kinase-2 and kinase-3a (Fig. 2). The sequence similarities and the presence of the two highly conserved motifs, kinase-2 and kinase-3a, in addition to the primer motif P-pool were indicative that all of the sequences should be RGAs of the NBS-LRR class. However, while all the other sequences could be potentially translated into polypeptides, s75 and s8-4 were found to possess a stop codon, suggesting that they might be pseudogenes.

The multiple-sequence alignment at the amino acid level (Table 1) and subsequent phylogenetic analysis (Fig. 3) showed that these ten RGAs could be classified into four separate clusters. The five RGAs from tube 2 (s34, s49, s50, s51 and s7-4) were highly similar among themselves (percent identities >96%) and thus formed a 'compact' group, while the five RGAs from tube 6 exhibited quite diverse and generally low similarities among themselves (percent identities ranging from 16.7% to 85.7%) and were classified into three distinct groups—s75 and s76, s8-3 and s8-4, and s8-6. RGA similarities across the two chromosomes (or tubes) were generally low (percent identities <32%). According to the phylogenetic tree, s8-6 showed the highest similarity (56.6%

Table 1 Percentage amino acid identities and divergences of ten classes of pomelo RGAs when compared to each other and to six known R genes

Name	Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
s8-6	1															
s49	2	31.0														
s50	3	24.3	96.6													
s51	4	31.0	96.4	97.3												
s7-4	5	30.4	97.6	96.6	96.4											
s75	6	23.1	31.0	25.7	31.0	31.0										
s76	7	16.7	26.8	25.7	26.2	26.2	85.7									
s8-3	8	28.7	27.4	18.9	26.8	28.0	21.6	20.2								
s8-4	9	35.8	29.1	22.3	28.5	28.5	21.8	23.6	59.4							
s34	10	30.5	97.6	98.6	98.2	97.6	32.3	26.9	27.5	29.1						
RPS2	11	25.1	35.1	23.6	35.7	35.7	35.7	30.4	24.0	27.3	36.5					
L6	12	23.7	26.8	22.3	26.8	26.8	14.5	13.7	24.6	26.1	26.9	22.8				
N	13	26.6	28.0	14.9	28.6	26.8	19.1	16.1	28.1	23.0	26.9	22.2	35.1			
Prf	14	36.8	26.8	18.9	26.8	26.2	13.5	22.0	28.1	29.7	26.9	22.2	18.1	26.9		
RPP13	15	56.6	27.4	17.6	27.4	26.8	19.7	19.0	28.7	30.9	26.9	25.7	17.3	27.2	23.7	
I2C-1	16	30.6	31.0	23.0	31.0	30.4	23.7	20.8	44.4	44.8	30.5	29.2	24.7	31.0	33.3	33.5

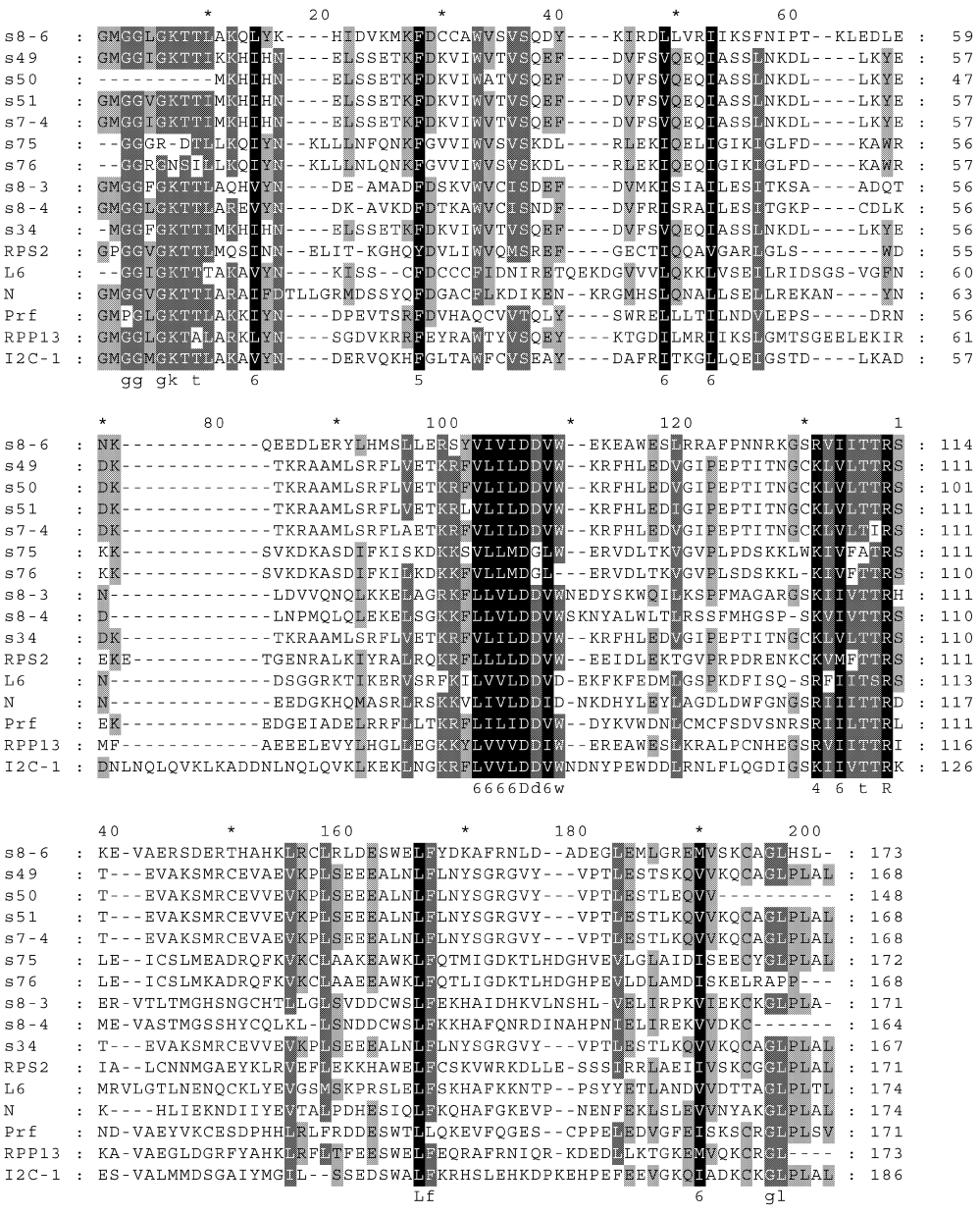

Fig. 2 Alignment of deduced amino acid sequences among ten pomelo RGAs (*s34*, *s49*, *s50*, *s51*, *s7-4*, *s75*, *s76*, *s8-3*, *s8-4*, *s8-6*) and the most similar R genes—tomato *N* (Whitham et al. 1994), *I2C* (Ori et al. 1997) and *Prf* (Salmeron et al. 1996), flax *L6* (Lawrence et al. 1995), *Arabidopsis RPS2* (Bent et al. 1994) and *RPP13* (Bittner-Eddy et al. 2000)

Fig. 3 Phylogenetic tree based on the alignment of deduced amino acid sequences of ten pomelo RGAs (see Fig. 2 legend) and the NBS domains of six R genes (*N*, *I2C*, *Prf*, *L6*, *RPS2*, *RPP13*)

identity) to a known R gene, *RPP13* in *Arabidopsis*, followed by the group of s8-3 and s8-4, which was similar to a tomato gene, *I2C-1*, with an average percent identity of about 44.6%. Both group s75 and s76, group s34, s49, s50, s51 and s7-4 showed some similarity to an *Arabidopsis* gene, *RPS2*.

Discussion

We report here the development of two methods for amplifying RGAs from single chromosomes. The two methods are useful for different situations. Method 2 uses an intact single chromosomal DNA molecule directly as the template for RGA amplification. Hence, it is relatively simple and, in principle, all RGAs on the isolated

chromosome can be amplified with degenerate primers designed from highly conserved sequences of R genes. However, Method 2 has a shortcoming in that the isolated chromosome can only be used as template once. For this reason, Method 2 is not suitable for a chromosome that cannot be identified under a microscope, if confirmation of the chromosome's identity is hoped for. Nevertheless, for a chromosome that is identifiable under a microscope, it would be preferable to use Method 2 for its simplicity. In addition, since the target chromosome is identifiable under a microscope, more than one copy of the target chromosome can be isolated for RGA amplification, if the microdissection of the target chromosome is not difficult. This would further guarantee the amplification of all RGAs on the target chromosome, although it has been shown in this study that RGAs can be amplified well based on a single chromosome.

In many plant species, especially those with small chromosomes such as pomelo and other fruit trees, most of the chromosomes are not identifiable under a microscope. For these chromosomes, it is not practical to isolate several copies of the same chromosome for RGA amplification. An appropriate strategy would be to isolate single chromosomes randomly for RGA amplification and identify the chromosomes afterwards using molecular biological methods (e.g. hybridization with known DNA probes). Method 1 can be used for this purpose because the products of LAM-PCR from single chromosomes cannot only serve as templates for RGA amplification, but they also can be used for chromosome identification as well as for other research purposes such as homologous amplification of other genes and the construction of single chromosomal DNA libraries (Huang et al. 2004). These are the advantages of Method 1.

However, Method 1 also has an intrinsic weakness. The procedure of LAM-PCR is complicated, involving the preparation of linker adaptors and primers, the digestion of single chromosomes, the ligation of chromosomal DNA fragments with linker adaptors and so on. In addition, the experimental conditions for LAM-PCR, especially the digestion time, should be strictly controlled. The sizes of the chromosomal DNA fragments obtained by LAM-PCR are crucial for RGA amplification. Since the predicted sizes of RGAs amplified with primers P1 and P2 are approximately 500 bp, only those LAM-PCR products with a size greater than 500 bp can potentially serve as templates for RGA amplification. Therefore, single chromosomal DNAs should be partially rather than completely digested with *Sau*3A. In the present study, in which single chromosomes were digested at 37°C for 4 h, the sizes of the amplified DNA fragments were approximately 300 bp to 2,000 bp and fell predominantly in the range 500—1,500 bp (Huang et al. 2004). This size range proved to be suitable for RGA amplification. In spite of this, however, some RGAs might still be missed because the DNA fragments obtained by LAM-PCR cannot cover the whole chromosome. Moreover, RGAs containing *Sau*3A sites in the region between the two sites complementary to the primers P1 and P2 cannot be amplified.

In short, both Method 1 and Method 2 have advantages and disadvantages, and each is suitable to particular situations. Hence, the two methods should be utilized flexibly according to the situation and the research goal. It is necessary to point out that both methods may sometimes fail to produce PCR products as we have seen in tube 1 and tube 4 in this study. Possible reasons for this failure are that there are no RGAs on the chromosomes to be amplified or that there are RGAs on the chromosomes but the primers used are not appropriate.

Although in this study we sequenced only a few RGAs (ten classes of clones) for technical exploration, the results still provided us with some interesting insights into the numbers and organization of R genes on the two chromosomes studied. Genomic sequencing of *Arabidopsis* has revealed that NBS-encoding sequences tend to be clustered in the genome, and numerous R genes have been mapped to the clusters of NBS-encoding sequences on *Arabidopsis* chromosomes IV and V (Michelmore 2000). This clustering appears to occur also in pomelo. In this study, all five RGAs from the chromosome in tube 2 were highly similar to each other (percent identity >96%), suggesting possible gene duplication during evolution. In contrast, all of the RGAs from the chromosome in tube 6 were much more diverse in their amino acid sequences, but they were similar to several known R genes in *Arabidopsis* and tomato. It is possible that there are more R-gene clusters on the chromosome in tube 6. This remains to be determined.

Generating DNA probes is one of the crucial starting points for genome research because defining chromosome regions is a prerequisite for genetic and physical mapping. While chromosome-specific DNA probes can be obtained from chromosome-specific DNA libraries (Huang et al. 2004), this approach is expensive, time-consuming and laborious. The screening of single- and low-copy probes from chromosome-specific libraries is generally difficult. In addition, the contamination of exogenous DNAs can interfere with the quality of chromosome-specific DNA libraries when minute amounts of initial DNA are used. RGAs have been widely used as DNA probes. Therefore, the methods developed in this research can also be used for generating chromosome-specific DNA probes. By comparison, the methods are much simpler yet efficient. The possibility of exogenous DNA contamination is relatively low when specific primers are used.

Although the methods developed in the present study were designed for acquiring RGAs from specific chromosomes, they are readily applicable to isolating homologs of other known genes. With more information becoming available in genome databases, the methods described in this report will be a powerful tool for chromosome and genome research and will ultimately allow the study of the distribution and organization of various genes on chromosomes.

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