Sequence analysis of a rice BAC covering the syntenous barley *Rpg1* region

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Abstract: In the course of map-based cloning of the barley stem rust resistance gene *Rpg1*, we identified a rice bacterial artificial chromosome (BAC) containing the *Rpg1* flanking markers. Based on the excellent gene order colinearity between barley and rice in this region, we expected that this rice BAC would contain the barley *Rpg1* homologue. In order to identify the putative rice homologue, we sequenced ca. 35 kb of the rice BAC at random and then an additional 33 kb of contiguous sequence between the two most closely spaced *Rpg1* flanking markers. Sequence analysis revealed a total of 15 putative genes, 5 within the 33-kb contiguous region. A rice *Rpg1* homologue was not identified, although a gene encoding a hypothetical polypeptide with similarity to a membrane protein could not be eliminated as a candidate. Surprisingly, four of the genes identified in the 33-kb contiguous rice sequence showed a high degree of similarity with genes on *Arabidopsis* chromosome 4. The genome regions harboring these genes showed some relatedness, but many rearrangements were also evident. These data suggest that some genes have remained linked even over the long evolutionary separation of *Arabidopsis* and rice, as has also been reported for mammals and invertebrates.

Key words: genes, genome, rice, synteny, sequence.

Résumé : Au cours de travaux visant le clonage du gène *Rpg1* qui confère la résistance à la rouille des tiges chez l'orge, les auteurs ont identifié un chromosome artificiel bactérien (BAC) de riz contenant les marqueurs flanquant *Rpg1*. En raison de l'excellente conservation de l'ordre des gènes dans cette région entre l'orge et le riz, les auteurs s'attendaient à ce que ce BAC contienne l'homologue du gène *Rpg1* de l'orge. Afin d'identifier l'homologue potentiel chez le riz, les auteurs ont séquencé environ 35 kb du clone BAC de riz au hasard et un autre segment 33 kb de séquences contiguës situées entre les deux marqueurs flanquants les plus proches de *Rpg1*. L'analyse de ces séquences a révélé un total de 15 gènes potentiels dont cinq dans la région de 33 kb d'ADN contigu. Un homologue de *Rpg1* chez le riz n'a pu être identifié, bien qu'un gène codant pour un polypeptide hypothétique montrant de la similarité avec une protéine membranaire demeure un candidat possible. Étonnamment, quatre des gènes identifiés dans la région de 33 kb montrent une forte similarité avec des gènes situés sur le chromosome 4 d'*Arabidopsis*. Les régions génomiques contenant ces gènes montraient des similitudes, mais de nombreux réarrangements étaient également évidents. Ces données suggèrent que certains gènes sont demeurés liés sur de très longues périodes d'évolution telles que celle qui sépare l'*Arabidopsis* et le riz, comme cela a été rapporté chez les mammifères et les invertébrés.

Mots clés : gènes, génome, riz, synténie, séquence.

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Introduction

The barley stem rust resistance gene, *Rpg1*, located near the telomere of the short arm of barley chromosome 1 (7H),

has been our major target for map-based cloning. Although barley is an excellent organism for genetic studies, it poses a considerable challenge for map-based cloning because of its large genome size (4.9×10^9 bp, Arumuganathan and Earle

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1991). Discovery of synteny (conservation of gene linkages) among several plant species has suggested the use of the genetic and physical resources of the small genome plants to facilitate map-based cloning in the large genome plants. A general synteny among major grass species has been documented (Devos and Gale 1997; Saghai Maroof et al. 1996) as well as microsynteny between specific regions of the rice and barley genomes, especially in the *Rpg1* region (Han et al. 1998; Kilian et al. 1997, 1995). Comparative sequence analyses of genomes also provides direct information about genome organization and evolution.

A rice bacterial artificial chromosome (BAC) containing the markers flanking the barley stem rust resistance gene *Rpg1* was previously characterized, demonstrating excellent microsynteny between rice and barley in this region (Kilian et al. 1997). In order to identify a putative rice *Rpg1* homologue, we sequenced and analyzed this rice BAC clone. A rice *Rpg1* homologue was not identified, although a membrane protein-like gene, designated PG2, could not be eliminated as a candidate. Surprisingly, four of the five genes identified in the 33-kb contiguous rice segment (including the two closest *Rpg1* flanking markers) showed a high degree of similarity with genes on *Arabidopsis* chromosome 4.

Materials and methods

BAC DNA preparation and subcloning

The isolation and subcloning of the rice BAC clone 10-N-24 including the markers flanking the *Rpg1* gene was previously reported (Kilian et al. 1997). Plasmid libraries were generated with the restriction enzymes *Bam*HI, *Eag*I, *Mlu*I, *Sal*I, *Bam*HI + *Eco*RI, and a contig with selected large insert clones was constructed.

Sequencing

Sequencing was performed using the Dye Terminator cycle sequencing kit (Perkin-Elmer) and an Applied Biosystems 373 DNA sequencer at the Bioanalytical Center, Washington State University, Pullman, Wash. Sequencing of rice cDNAs or ESTs was done at the Rice Genome Research Program (RGP), Institute of the Society for Techno-innovation of Agriculture, Forestry, and Fisheries (STAFF), Tsukuba, Japan.

Genetic mapping

High resolution genetic mapping in barley was performed with 3000 gametes. Fifteen hundred gametes are doubled haploid (DH) lines derived from the crosses: Steptoe × Morex, Harrington × Morex, Steptoe × Q21861, Dicktoo × Morex, and Q21861 × SM89010. The other 1500 gametes are represented by F₂ plants from a Steptoe × Q21861 cross. DH lines with recombination between the *Rpg1* flanking markers ABG704 and ABG077, and F₂ plants with recombination between the flanking markers B118 (cosegregates with ABG704 in a 150-gamete population) and ABG077 were identified and used for high resolution mapping.

Phenotyping

All recombinants were scored for resistance or susceptibility to the stem rust pathogen *Puccinia graminis* f.sp. *tritici*, pathotype MCC, as previously described (Kilian et al. 1994). For recombinant F₂ plants, 15 F₃ seedlings from each F₂ plant were phenotyped as above, and the data were used to determine the *Rpg1* genotype in the F₂.

Data analyses

Sequencing data were assembled by GCG program supported by the VADMS (Visualization, Analysis and Design in the Molecular Sciences) center at Washington State University. Assembled DNA sequences were searched first for open reading frames (ORFs) by ORF FINDER, a program on the Home page of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov:80/>). The identified ORFs (amino acid sequence) were used to search data bases using the new BLAST program (Altschul et al. 1997) implemented also on the NCBI home page. The similarity score and *E*-value (probability of a chance similarity) for the unknown sequence are reported in the text. Repetitive sequences were identified by visual analysis and REPEAT-MASKER (<http://ftp.genome.washington.edu/RM/RepeatMasker.html>).

Results

Initially, we sequenced the rice BAC 10-N-24 subclones at random in order to identify a putative *Rpg1* candidate gene. A total of 125 subclones were sequenced and assembled into 43 contigs, yielding a 34 728 - bp sequence. Data base searches identified 9 contigs containing 10 ORFs, which showed similarity to existing data base sequences (Table 1). The clones B106 and B156 are likely to be parts of the same gene since they show similarity to the same hypothetical protein gene in *Arabidopsis*. One interesting clone, B138, which showed strong homology to the cell division inhibitor gene *MinD* in *Chlorella vulgaris*, contained a perfect P-loop motif (GKGGVGKT). With the conservation of the P-loop in the class of NBS-LRR R genes, this clone was considered to be a potential candidate for *Rpg1*. However, genetic mapping placed B138 1.3 cM proximal to *Rpg1* (based on 150 gametes). Other putative genes also failed to cosegregate with *Rpg1*.

A systematic sequencing of the rice BAC region between the *Rpg1* flanking markers B122 to B24 was then undertaken to assure that the putative homologous gene was not missed. A detailed physical map was established, and sequencing was carried out using specific primers and subcloning of large insert plasmid clones. A total of 160 fragments were sequenced and assembled into a 33 178 - bp contig, termed Bam33 contig. Detailed analyses of the Bam33 contig sequence identified five putative genes designated PG1–5 (Fig. 1). The PG1–4 translation products showed similarity with a hypothetical protein in *Arabidopsis*, a myb-related protein in *Arabidopsis*, and the product of *Arabidopsis* cDNA T20924, respectively (Table 1). The myb-related protein gene PG3 included the myb-like domain WTDPEQGQV with only one mismatch from the consensus sequence W[ST]XXE[DE]XX[LIV]. The PG4 sequence included the rice EST S1558. The PG5 sequence ORF encoded 317 amino acids that showed homology to a fertilin alpha subunit in *Oryctolagus cuniculus*. The probe isolated from the rice PG5 region hybridized strongly to a Southern blot containing a wide range of grass species, including barley, but data base searches did not identify an *Arabidopsis* homologue. *Arabidopsis* genes with high similarity to the rice genes PG1 and PG2 are on the same *Arabidopsis* BAC, ATFA6. These two genes are ca. 16 kb apart and are translated in opposite directions, but with the same orientation in both species. Furthermore, the *Arabidopsis* genes with simi-

larity to the rice genes PG3 and PG4 are on another *Arabidopsis* BAC, IG002N01, and are separated by a very short physical distance from each other (1.5 kb in *Arabidopsis* and 0.5 kb in rice, Fig. 1). These two genes are also transcribed in opposite directions as well as in reverse orientations in the two species. These results suggest possible conservation of gene order and spacing between these gene pairs in rice and *Arabidopsis*.

Short di- and trinucleotide repeats were found dispersed throughout the 33-kb sequence, especially in the intergenic regions. Two sequence repeats are noteworthy. A 281-bp sequence (from 9939 to 10 220 bp) was tandemly repeated in the ca. 14-kb sequence between PG1 and PG5 with only 10 base changes and 1 addition or deletion. It is possible that this repeat represents the long terminal repeats of an ancient transposon. A short inverted repeat, (GAA)₄ and (TTC)₄ separated by 233 bp, was found in the 477-bp sequence between PG3 and PG4. The significance of this repeat is not known, but it is noteworthy that the same repeat was also found in the related *Arabidopsis* region (Fig. 2).

High resolution genetic mapping (3000 gametes) showed that all of the identified rice genes recombined away from *Rpg1* except PG2, which could not be mapped because of very poor hybridization with barley genomic DNA and lack of polymorphism. Thus, the PG2 gene remains as a potential candidate for *Rpg1* because it could not be eliminated.

Discussion

Genome organization in rice

Based on detailed physical mapping of the rice BAC 10-N-24, the sequenced 68 kb covers a region of about 90 kb. The 15 putative genes identified in this region represent a minimum of one gene per 6 kb. Based on a genome size of 4.5×10^8 bp and an estimated 24 000 genes, an average of one gene per 20 kb has been calculated (Inoue et al. 1994). Since the analyzed region is near the telomere, it would be expected to have a higher than average gene/kb ratio, assuming that plant centromeric regions are primarily comprised of heterochromatin and therefore have reduced recombination and fewer functional genes (Moore et al. 1993). Remarkably, PG3 and PG4 are separated by only 0.5 kb and transcribed in different directions. Assuming that PG3 and PG4 are both functional genes, they either share a common promoter region, which directs transcription in an opposite orientation or have a promoter region in a portion of the other gene as suggested for *Arabidopsis* (Bevan et al. 1998).

Gene distribution was uneven in the region analyzed. In the sequenced Bam33 contig, five putative genes were identified with four of them located in a 15-kb region, but none in the adjacent 14-kb region. The uneven gene distribution phenomenon may also hold true for the syntenic barley region. This is indicated by a very high recombination and low physical/genetic distance ratio proximal to *Rpg1* and a very low recombination and high physical/genetic distance ratio distal to *Rpg1* (unpublished data). Genes per se have been suggested to be the preferential sites for recombination (Civardi et al. 1994).

Table 1. Summary of putative genes identified from the rice BAC 10-N-24.

Clone (contig)	Sequence length (bp)	Open reading frame*	Homologue	Organism	Score (E-value)	GenBank acc. no.
B98	1538	-2 (2-1165)	<i>Sci1</i> protein	<i>Oryza sativa</i>	208 (4e-53)	AF149807
B106	440	-1 (48-410)	Hypothetical protein	<i>Arabidopsis thaliana</i>	123 (4e-28)	AF149808
B124	486	+1 (121-485)	Circumsporozoite protein precursor	<i>Plasmodium knowlesi</i>	53 (7e-07)	AF149809
B138	674	-2 (1-537)	Cell division inhibitor <i>MinD</i>	<i>Chlorella vulgaris</i>	131 (4e-30)	AF149810
B156	389	+2 (2-154)	Hypothetical protein	<i>Arabidopsis thaliana</i>	82 (5e-16)	AF149811
B175	1250	-3 (815-1111)	Predicted protein	<i>Caenorhabditis elegans</i>	59 (7e-09)	AF149812
Bam7	4062	+1 (3397-4014)	Retrotransposon del1-46	<i>Lilium henryi</i>	200 (4e-51)	AF149813
		+3 (621-1406)	DeltaSAC-synaptojanin1	<i>Rattus norvegicus</i>	55 (7e-07)	
BE3-14	619	+1 (1-128)	Gene Bp10 protein	<i>Brassica napus</i>	130 (2e-30)	AF149814
Mlu33	1293	+2 (2-193)	<i>Rcc3</i> (root-specific protein gene)	<i>Oryza sativa</i>	95 (6e-20)	AF149815
Bam33	33178	PG1 + (417-3070)	Hypothetical protein	<i>Arabidopsis thaliana</i>	159 (3e-38)	AF149806
		PG5 - (17479-18432)	Fertilin alpha subunit	<i>Oryctolagus cuniculus</i>	66 (3e-10)	
		PG2 - (19343-21742)	Membrane protein homologue	<i>Arabidopsis thaliana</i>	79 (2e-14)	
		PG3 - (27096-27900)	Myb-related protein	<i>Arabidopsis thaliana</i>	61 (2e-09)	
		PG4 + (28377-32572)	<i>Arabidopsis</i> cDNA T20924 product	<i>Arabidopsis thaliana</i>	53 (2e-06)	

Note: Data base searches were completed February 16, 1999. Blast scores were based on amino acid homology.

*, forward ORF; -, reverse ORF. The number in parentheses indicates the starting-ending position of the ORF. For PG1 to PG5, the joined ORFs were used for data base searches.

Fig. 1. Comparative physical maps of rice and *Arabidopsis* relative to a barley genetic map. The putative genes identified by sequencing of the contiguous 33-kb rice BAC 10-N-24 fragment were compared with similar *Arabidopsis* genes. All putative rice genes, except PG2, were located by high resolution (3000 gametes) mapping in barley. The bars with diagonal stripes indicate the putative gene ORFs. The horizontal arrows indicate the direction of transcription. The gene size in rice was calculated based on similarity with the *Arabidopsis* ORFs. There may be additional ORFs that are part of the genes, but which do not have sufficient similarity to the *Arabidopsis* ORFs to be identified. The solid circles on the rice BAC indicate the location of probes used for mapping in barley. The vertical lines with double arrows indicate the ORFs with amino acid sequence similarity between rice and *Arabidopsis*. There are three additional putative genes between the hypothetical protein and membrane protein homologue in *Arabidopsis* BAC ATFCA6. *Arabidopsis* BAC ATFCA6 and IG002N01 have GenBank accession nos. Z97341 and AF007269, respectively, and are from *Arabidopsis* chromosome 4.

Is there a rice *Rpg1* homologue?

Experiments described here were designed to identify a putative rice *Rpg1* homologue. High resolution genetic mapping in barley eliminated all of the identified rice genes as putative candidates for *Rpg1*, except the membrane protein-like gene, PG2. Unfortunately, we have not been able to map this gene in barley because of poor hybridization and lack of polymorphism. The PG2 gene does not show sequence similarity to cloned R genes and thus would have to represent a novel disease resistance gene if it were an *Rpg1* homologue.

It is perhaps more probable that *Rpg1* is the exception to the high degree of synteny between rice and barley in this region. An insertion event in barley or a deletion event in rice is a possibility. An intraspecies insertion and (or) deletion event has been described in *Arabidopsis* for the *Rpm1* gene conferring resistance to the bacterium *Pseudomonas syringae* (Grant et al. 1995). Ectopic recombination of plant disease resistance genes has also been postulated due to the frequent nonsyntenic map locations for R-like genes mapped in different grass species (Leister et al. 1998). If the *Rpg1* gene is a member of the NBS-LRR class of disease resis-

tance genes, it is clearly not present in the rice BAC, which includes the *Rpg1* flanking markers. An NBS-LRR R-like gene has been reported to cosegregate with *Rpg1* (Leister et al. 1998). However, this probe mapped ca. 0.5 cM proximal to *Rpg1* in our segregating population (3000 gametes, unpublished data). Therefore, the question of whether rice has an *Rpg1* homologue and, if so, where is it located still remains to be determined.

Comparison between rice and *Arabidopsis*

Synteny between barley and rice in the barley stem rust resistance gene *Rpg1* region, and some other regions, is well established (Han et al. 1998; Kilian et al. 1997). In our analysis of the rice *Rpg1* syntenous region, we were surprised to note that short regions appeared to be syntenous between rice and *Arabidopsis*. It is noteworthy that in addition to the sequence and position conservation of these genes, the genome regions in which the genes are embedded also appears to be related. This is particularly true for the PG3 and PG4 genes, which are separated by a short distance in both rice and *Arabidopsis*, but are transcribed in inverted orientation

Fig. 2. Schematic representation of an inverted repeat conserved in rice and *Arabidopsis*. The figure is aligned on the rice genes PG3 and PG4 (not to scale).

(Fig. 1). A careful examination of the sequence around the myb-related protein gene (PG3) in *Arabidopsis* revealed a short inverted repeat identical to that found in rice (Fig. 2). In *Arabidopsis*, the (GAA)₄ repeat is between the myb-related protein gene (similar to PG3) and T20924 gene product (similar to PG4), and (TTC)₄ is 17 bp beyond the start codon, whereas in rice, both elements are between PG3 and PG4 (Fig. 2). These data indicate that the rice and *Arabidopsis* genomic regions containing PG3 and PG4 are related, but also have undergone considerable rearrangement(s). Distant evolutionary relationships have also been reported for mammals and invertebrates (Trachtulec et al. 1997).

The putative genes PG1 and PG2 are also separated by approximately the same distance in rice and *Arabidopsis*. In this case, however, the intervening sequences appear to be very different. In rice, the putative gene PG5 was found between PG1 and PG2, but an *Arabidopsis* homologue for this gene was not identified in data base searches. Since substantial sequence data for this region has been deposited, the PG5 homologue either does not exist or is found elsewhere in the *Arabidopsis* genome. In *Arabidopsis*, three putative genes are located between the PG1 and PG2 homologues. Homologues for these three putative *Arabidopsis* genes were not found in the sequenced rice BAC.

These data indicate an intriguing relationship between the order of some putative homologous genes on the rice and *Arabidopsis* chromosomes 6 and 4, respectively. However, they also indicate that extensive rearrangements have taken place resulting in the movement of some genes to unidentified positions and the reorganization of those that have remained linked. Are these observations attributable simply to chance or are they evolutionary relics maintained by yet undefined mechanisms of genome organization and evolution? Perhaps the answers will be found as the sequencing of the *Arabidopsis* and rice genomes proceeds.

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