Contrasting Evolutionary Patterns of the Rp1 Resistance Gene Family in Different Species of Poaceae

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Abstract

Disease-resistance genes (R-genes) in plants show complex evolutionary patterns. We investigated the evolution of the Rp1 R-gene family in Poaceae, and 409 Rp1 fragments were sequenced from 21 species. Our data showed that the common ancestor of Poaceae had two Rp1 loci, but the number of Rp1 locus in extant species varies from one to five. Some wheat and Zea genotypes have dozens of Rp1 homologues in striking contrast to one or two copies in Brachypodium distachyon. The large number of diverse Rp1 homologues in Zea was the result of duplications followed by extensive sequence exchanges among paralogues, and all genes in maize have evolved in a pattern of Type I R-genes. The high frequency of sequence exchanges did not cause concerted evolution in Zea species, but concerted evolution was obvious between Rp1 homologues from genera Zea and Sorghum. Differentiation of Type I and Type II Rp1 homologues was observed in Oryza species, likely occurred in their common ancestor. One member (Type II R-gene) in the Oryza Rp1 cluster did not change sequences with its paralogues, whereas the other paralogues (Type I R-genes) had frequent sequence exchanges. The functional Pi37 resistance gene in rice was generated through an unequal crossover between two neighboring paralogues followed by four point mutations. The Rp1 homologues in wheat and barley were most divergent, probably due to lack of sequence exchanges among them. Our results shed more light on R-gene evolution, particularly on the differentiation of Type I and Type II R-genes.

Key words: Rp1, disease-resistance gene, copy number, gene conversion, gene cluster.

Introduction

More than 100 disease-resistance genes have been cloned from different plant species and approximately 80% of them encode nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (Liu et al. 2007). Many of the NBS–LRR–encoding genes (referred as R-genes in this article) are clustered in plant genomes (Meyers et al. 2003). The clustering of R-genes might facilitate sequence exchanges between paralogues and generating new resistance specificities (Mondragon-Palomino et al. 2002; Kuang et al. 2008). Indeed, a group of R-genes, termed Type I R-genes, are extensive chimeras, which were generated through frequent gene conversions between paralogues. However, another group of R-genes (Type II R-genes) had rare or no sequence exchanges with paralogues though they might be located in the same cluster as Type I R-genes (Kuang et al. 2004).

The mechanisms underlying the differentiation of Type I and Type II R-genes remain unclear. The high conservation of each lineage of Type II R-genes might suggest their indispensable function, that is, important resistance specificity. However, several pieces of evidence are against the “function” hypothesis. First, some Type II R-genes are apparent pseudogenes with frameshift indels or premature stop codon. Paradoxically, some frameshift indels are present in all genes of a Type II R-gene lineage (Kuang et al. 2004). Second, Type II R-genes (as donors) rarely converted their paralogues though such a direction of gene conversion would not change the original function of the Type II R-gene. Third, some Type II R-gene lineages have very low frequencies in nature populations (Kuang et al. 2004; Shen et al. 2006). All these facts are against the hypothesis that the conservation of Type II R-genes are due to their critical functions.

Physical proximity of homologues should not account for the differentiation of Type I and Type II R-genes either. Studies on RPP8 resistance gene homologues in Arabidopsis showed that frequent sequence exchanges occurred between homologues separated by 2.3 Mb (Kuang et al. 2008). In contrast, a Type I and Type II RPP8 homologue rarely had sequence exchanges between them though they are only 4 kb apart on chromosome V. Furthermore, Type I and Type II homologues or different lineages of Type I homologues interweave in some R-gene clusters, suggesting that physical proximity may not be responsible for the differentiation of Type I and Type II R-genes (Kuang et al. 2004, 2005).

The grass family (Poaceae) is ideal for comparative genomics. Numerous genetic and genomic data are available...
for different species of Poaceae. Noticeably, the rice (Oryza sativa) genome has been completely sequenced (Goff et al. 2002); the draft genome sequences of maize (Zea mays ssp. mays) (Schnable et al. 2009), sorghum (Sorghum bicolor) (Paterson et al. 2009), and Brachypodium distachyon (Vogel et al. 2010) have been released; and genomic bacterial artificial chromosome (BAC) libraries and physical maps of several genotypes of cultivated and wild rice are available (Ammiraju et al. 2006). All these data are valuable for studies on comparative and evolutionary genomics of R-genes.

We investigated the evolutionary patterns of the Rp1 gene family in Poaceae. The Rp1-D gene, a member of the Rp1 family clustered on the short arm of chromosome 10 of maize, encodes resistance against rust caused by Puccinia sorghi (Collins et al. 1999). The Rp1 locus in maize was unstable due to frequent unequal crossovers between paralogues (Hulbert 1997; Sun et al. 2001). Unequal crossover between paralogues was shown to be the main genetic event for the spontaneous loss of the Rp1-D and generating novel resistance specificity (Sun et al. 2001; Smith and Hulbert 2005). Unequal crossover was also likely responsible for the large variation of the copy number of Rp1 gene family in maize, which varied from one to more than 50 in different maize haplotypes (Smith et al. 2004). The Rp1 homologues were present in many species in the grass family (Ayliffe et al. 2000). The Rp1 locus in sorghum, called rph1 locus, was sequenced and five Rp1 homologues were discovered (Ramakrishna et al. 2002). The Rp1 homologues were duplicated to several chromosomes in barley (Hordeum vulgare L.) (Ayliffe et al. 2000; Rostoks et al. 2002). In rice cultivar Nipponbare, genes most similar to the Rp1 locus in rice cultivar St. No. 1 has a similar structure to that in Nipponbare, and one (the P37 gene) of the four homologues in St. No. 1 encodes resistance against rice basic local alignment search tool (Blast) (Lin et al. 2007).

We comprehensively studied the structures of the Rp1 clusters in Oryza, Triticum, Zea, B. distachyon, Sorghum, etc. A total of 164 full-length or nearly full-length and 245 partial-length Rp1 homologues were sequenced from 21 species of the grass family. The evolutionary patterns of Rp1 homologues in different species and subfamilies of Poaceae were compared. The differentiation of Type I and Type II Rp1 homologues in Poaceae was addressed.

**Materials and Methods**

**Materials Used to Clone Rp1 Homologues**

Rp1 homologues were obtained from 72 genotypes of 21 species of the grass family (Poaceae), representing its three major subfamilies, Bambusoideae, Panicoideae, and Pooideae (supplementary table S1, Supplementary Material online). Forty genotypes of the Bambusoideae subfamily were included in this study: 14 cultivars of O. sativa ssp. indica, 10 cultivars of O. sativa ssp. japonica, 2 genotypes of O. alta, 4 genotypes from O. nivara, 4 genotypes from O. rufipogon, 1 genotype each from O. glaberrima, O. punctata, O. minuta, O. australiensis, O. coarctata, and O. brachyantha. Eight genotypes from the Panicoideae subfamily were used in this study: three genotypes of Z. mays ssp. parviglumis, one genotype each of Z. luxurians and Z. diploperennis, and three genotypes from sorghum (S. bicolor). Twenty-four genotypes from the Pooideae subfamily were included in this study: 3 cultivars of wheat (Triticum aestivum), 17 genotypes of B. distachyon, and 1 genotype each of T. monococcum, T. urartu, T. durum, and Aegilops tauschii. Another eight genotypes of B. distachyon were also included in this study, but no Rp1 homologues were sequenced from these genotypes.

**Cloning Rp1 Homologues from Oryza Species**

The Rp1 locus in Nipponbare has four homologues (Os01g57270, Os01g57280, Os01g57310, and Os01g57340), and specific primers were designed to amplify three of them. Eight polymerase chain reaction (PCR) primer combinations specific to Os01g57270 were used to investigate the presence/absence of this gene in Oryza species (supplementary table S2, Supplementary Material online). If present, the PCR products of Os01g57280-like genes were sequenced directly. The “alleles” of Os01g57280 were PCR amplified using primer combination 57280-up-K and Rp-R2-K, which are located 711 bp upstream and 3,842 bp downstream of the start codon of gene Os01g57280, respectively. The “alleles” of Os01g57340 were PCR amplified using primer combination Rp1-F1K and 57340-dn-R3, which are located 146 and 4,218 bp downstream of the start codon of gene Os01g57340, respectively. The PCR products were treated with Exo-SAP (Fermentas, Ontario, Canada) and then sequenced directly.

When available, BAC clones were used as templates to PCR amplify Rp1 homologues. In the sequenced genomes of rice cultivars Nipponbare and 9311 and B. distachyon, the Rp1 locus is flanked by a gene encoding vacuolar protein sorting 28 and a gene encoding sphingosine kinase. Therefore, we delimited the Rp1 locus in Oryza by these two genes. First, BAC clones spanning the Rp1 locus were identified using their BAC end sequences. Rp1 homologues from these BAC clones were amplified using primers conserved in all available Rp1 homologues (supplementary table S2, Supplementary Material online). The PCR products were sequenced directly using one primer after Exo-SAP treatment. If the sequencing showed a clean chromatogram, the whole fragment was sequenced through primer walking. If multiple peaks were present in the chromatogram, the PCR products were cloned and individual clones were sequenced.

**Cloning and Sequencing Rp1 Homologues from Zea Species, Triticum Species, B. distachyon, A. tauschii, and S. bicolor**

Rp1 fragments of −3.8 kb were amplified from three Zea species using primer combination Maize-F2 and Maize-R (supplementary table S2, Supplementary Material online), which are located at +6 and +3798 of the Rp1-D gene,
respectively. PCR was performed using TaKaRa LA Taq (TaKaRa, Dalian, China), with a reaction of 25 µl, treated 5 min at 94 °C, followed by 32 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 4 min. The PCR products were gel purified using the Gel Purification Kit (Generay, Shanghai, China) and cloned into pCR2.1 vector using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). This method was first validated by comparing sequences of fragments amplified from two independent PCRs for Z. may ssp. parviglumis genotype PI384062. After validation, this method was applied to other Zea genotypes.

A sequencing method was used to estimate the Rp1 copy number in Zea species, which was shown to be the most reliable approach to estimate the copy number of a large gene family in a genome (Kuang et al. 2004). A conserved primer combination For2/Low4 was used to PCR amplify Rp1 fragments (−2 kb), the PCR products were cloned into TOPO TA cloning kit, individual clones were sequenced, and distinct fragments were counted to estimate the copy number of Rp1 homologues in a genotype. PCR products were amplified using Taq polymerase, with a reaction of 25 µl, 5 min at 94 °C, followed by 32 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min. The procedure was first validated by comparing sequences of fragments obtained from four independent PCRs for Z. may ssp. parviglumis genotype Ames21889. Two clones were considered to be derived from the same gene if they exhibited more than 99.4% nucleotide identity. If an Rp1 gene is present in two or more PCRs, the gene is considered as “verified.” For the Rp1 genes present in one PCR only, primers specific to some of them were designed and used to amplify and sequence PCR products to confirm their presence in the genome. The method is considered valid if above experiments show no PCR artifacts. The validated method was used to estimate the copy number of Rp1 homologues in other Zea genotypes.

Several primer combinations were designed to amplify nearly full-length Rp1 homologues from subfamily Pooidae, but only primer combination CS-F3/Brach-R2 was partially successful. In order to obtain more Rp1 fragments from this subfamily, primer combination For2/Low4 was used to PCR amplify the LRR-encoding region (−2 kb) of Rp1 homologues from Triticum and its relative A. tauschii. PCR products were cloned into TOPO vector and individual clones were sequenced as described above.

A single Rp1 homologue is present in the genome of B. distachyon. Primers flanking the Rp1 homologue were designed, and full-length Rp1 homologues were amplified from 25 genotypes of B. distachyon (supplementary tables S1 and S2, Supplementary Material online). The amplified PCR products were treated with Exo-SAP (Fermentas) and then sequenced directly using primer Brach-R. If the sequencing chromatogram was clean, the PCR fragments were fully sequenced through by primer walking. However, if the chromatogram had two peaks, the PCR products were cloned and individual clones were sequenced as described above.

Rp1 homologues were amplified from three genotypes of sorghum using a pair of primers Trp-F/Trp-R, which are conserved in all Rp1 homologues cloned from sorghum and maize. PCR amplification, cloning, and sequencing were identical to the method described above to amplify nearly full-length Rp1 homologues in Zea.

**Southern Hybridization Using Digoxigenin Labeling System**

To estimate the copy number of Rp1 genes in Oryza, DNA gel blot analysis was conducted according to the protocol from Digoxigenin (DIG)-High Prime DNA Labeling and Detection Starter Kit I (Roche, Mannheim, Germany). BAC plasmid DNA of 14 genotypes of Oryza species was digested with EcoR I endonuclease (Fermentas). The 250-bp probe was a mixture of PCR fragments amplified from seven diverse rice Rp1 genes amplified using primer combination Probe-F1/Probe-R1 (supplementary table S2, Supplementary Material online). The mixed fragments were labeled with digoxigenin-11-dUTP using DIG-High Prime.

**Mapping of Rp1 Homologues in Wheat Genotype Chinese Spring Using Deletion Lines**

Rp1 fragments amplified from wheat cultivar Chinese Spring (CS) were mapped using its aneuploid and deletion stocks (Endo and Gill 1996). A total of 146 aneuploid and deletion stocks including 21 nullisomic–tetrasomic, 24 ditelosomic, and 101 deletion lines were used (Peng et al. 2004). A set consisting of five Southern blots, each with 30 lanes, was hybridized with the Rp1 probe containing an even mixture of 23 Rp1 fragments from CS, in the hybridization reaction. The conventional Southern hybridization approach was adopted with some minor modifications (http://wheat.pw.usda.gov/NSF/project/mapping_data.html). If a band in the Southern profile is present in the wild-type CS but absent only in a specific series of CS cytogenetic stocks, the corresponding Rp1 homologue is located to a specific region or bin in a chromosome (Endo and Gill 1996; Peng et al. 2004). To map individual Rp1 fragments from CS, primers specific to each fragment were designed and used to screen a set of ditelosomic and deletion lines related to wheat group 4 chromosomes because most of the CS Rp1 homologues were mapped to chromosome 4AL via Southern hybridization. Similarly, amplification of PCR products from wild-type CS but not from one or several deletion lines would map the corresponding Rp1 homologue to a certain bin or specific chromosome region.

**Sequence Analyses**

The nucleotide sequences of Rp1 homologues were aligned using ClustalX (Thompson et al. 1997) and refined using GeneDoc (http://www.nrbsc.org/gfx/genedoc/). Neighbor-joining (NJ) trees using Kimura’s two-parameter model were constructed and bootstrap values (1,000 replications) were calculated using MEGA 4.0 (Tamura et al. 2007). Sequence exchanges were detected using Geneconv (Sawyer 1989), which detects identical tracts in two otherwise divergent fragments,
with no mismatch allowed between converted tracts \((gscal = 0)\). If one gene was detected as having gene conversions with several different genes in the same region, only the event with the largest conversion tract was reported. All gene conversions detected by Geneconv were examined and confirmed visually. Chi-square test was used to test if there was significant difference between the frequency of gene conversions among genes within a species and the frequency of gene conversions among genes from different species. The expected gene conversion number is calculated based on the total number of pairs of sequences from the same species and the total number of pairs of sequences from two different species.

To determine the number of ancestral genes (founder genes) that generated the current \(Rp1\) homologues in \(Zea\), the number of distinct fragments (vary at least four nucleotides) in a window of 70 bp of all \(Rp1\) homologues from \(Zea\) was counted. Ten windows (each represents an LRR-encoding unit) of 87 genes from \(Zea\) species were examined. The largest number of distinct fragments in a window represents the minimum number of ancestral genes for the \(Rp1\) gene family in \(Zea\).

**Results**

**Duplication and Deletion of the \(Rp1\) Locus in Different Species of Poaceae**

The \(Rp1\) locus in maize is located 2–3 Mb from the tip of telomere on the short arm of chromosome 10 according to the updated genome sequences of maize (Schnable et al. 2009). The \(Rp1\) locus in sorghum, called \(rph1\) locus, is located on chromosome 8 (Ramakrishna et al. 2002). This locus (called locus A in this article for convenience) and its flanking region show good synteny between maize and sorghum (fig. 1A). The genes flanking the \(Rp1\) locus in maize were used to identify its syntenic region in the genomes of rice and \(B.\) distachyon, and two and one syntenic regions were identified, respectively. Surprisingly, no \(Rp1\) homologues are present at this locus in the genomes of rice or \(B.\) distachyon (fig. 1A).

Blast search using the \(Rp1-D\) sequence as query identified four and one highly similar homologues from the genomes of rice and \(B.\) distachyon, respectively. The four \(Rp1\) homologues in rice cultivar Nipponbare are clustered on chromosome 1, whereas the single-copy \(Rp1\) homologue in \(B.\) distachyon is located on chromosome 2. The \(Rp1\) locus in rice and \(B.\) distachyon (referred as locus B) shows good synteny but they are not syntenic to the \(Rp1\) locus (locus A) in maize (fig. 1B).

Sorghum, which is in the same subfamily (Panicoideae) as maize (fig. 1C), has two \(Rp1\) loci. One of them is located on chromosome 8 and syntenic to locus A (fig. 1A), whereas the other is located on chromosome 3 and syntenic to locus B (fig. 1B). Barley, which is in the same subfamily (Pooidae) as \(B.\) distachyon (fig. 1C), also has both loci A and B, in addition to three other \(Rp1\) loci (Rostoks et al. 2002). The \(Rp1\) locus on barley chromosome 7(5H) was believed to be syntenic to the maize \(Rp1\) locus (Rostoks et al. 2002), whereas the \(Rp1\) locus on barley chromosome 5 is syntenic to the \(Rp1\) locus in rice because its flanking markers ABC261 and BCD1930 are also flanking the \(Rp1\) locus in rice. We concluded that there were at least two ancient \(Rp1\) loci in Poaceae, and the two ancient \(Rp1\) loci might have experienced constant duplications and/or deletions in different lineages of the Poaceae family.

**Striking Variation of \(Rp1\) Copy Number in Different Species**

The sequenced genomes of rice and \(B.\) distachyon have four and one \(Rp1\) homologues, respectively. We used Southern blot and/or sequencing strategy to investigate the copy number of \(Rp1\) homologues at the locus B of \(Oryza\) species and in the genome of \(B.\) distachyon. Our data showed that the locus B of the \(Oryza\) species included in this study had
fewer than five copies of Rp1 homologues (fig. 2) and that there was only one or two Rp1 homologues in each B. distachyon genotype (see below).

PCR cloning method was used to investigate the copy number of Rp1 homologues in wild maize, which may have a large number of Rp1 homologues in a genome (Smith et al. 2004). To validate this approach, four independent PCRs were carried out for genotype Ames21889 of Z. may ssp. parviglumis using a conserved primer combination For2/Low4 (supplementary table S2, Supplementary Material online). Sequencing 264 clones derived from the four PCRs gave 70 distinct fragments. Of the 70 Rp1 fragments, 23 were present in at least two PCRs, confirming their presence in the genome. The other 47 Rp1 fragments were found in only one of the four PCRs. To exclude the possibility of PCR artifacts, PCR primers specific to 12 of the 47 fragments were designed and the amplified PCR products were sequenced. The results showed no evidence of PCR artifacts, in terms of PCR recombination. The validated PCR method was applied to estimate the Rp1 copy number in other four genotypes of Zea species. A total of 48, 65, 50, and 49 clones were sequenced for genotypes PI566691 and PI384062 of Z. may ssp. parviglumis, genotype PI441933 of Z. luxurians, and genotype PI462368 of Z. diploperennis, respectively. That generated 13, 31, 42, and 17 diverse fragments, respectively. The total number of Rp1 homologues in Z. luxurians PI441933 might be unprecedentedly large because 42 distinct fragments were discovered after sequencing 50 randomly chosen clones. The high copy number of Rp1 homologues in Zea forms a striking contrast to the low copy number in O. sativa and B. distachyon.

Large Number of Diverse Rp1 Homologues in Zea Species due to Duplications Followed by Frequent Sequence Exchanges

To investigate the mechanism generating the large number of diverse Rp1 homologues in Zea, we obtained 63 nearly full-length (at least 3,659 bp) Rp1 homologues from the five genotypes of Zea species (supplementary table S3, Supplementary Material online). These 63 Rp1 homologues were combined with 24 maize Rp1 sequences retrieved from GenBank for further analysis. An NJ distance tree was constructed using Rp1 homologues from sorghum and Pi37 from rice as outgroups (fig. 3). The 87 genes from the four Zea species formed a clade exclusively, with a bootstrap number of 100. The genes from Zea exhibited 85.3–99.9% (with an average of 92.3%) nucleotide identities with each other. The average pairwise nucleotide identity between genes within a species (85.6–99.9%, with an average of 92.1%) is similar to those between genes in different species (85.3–99.5%, with an average of 92.5%). t-Test showed that the average nucleotide identities between genes from the same species have no significant difference from those between genes from different Zea species.

All the Rp1 homologues from Zea species, including the functional resistance gene Rp1-D, showed a chimeric structure (fig. 4A). Eighty-four sequence exchanges were detected among the 87 maize Rp1 homologues using software Geneconv ($P < 0.01$). The exchange tracts varied from 145 to 2,316 bp, with an average of 629 bp. The exchange tracts were mainly located in the middle of genes, and only eight of the 84 exchange tracts extended to one end of the genes. Of the 84 detected sequence exchanges, 45 occurred between genes within a species and 39 between genes from different species. Chi-square test showed that the sequence exchanges within a species was significantly more than expected ($\chi^2 = 9.26$, degrees of freedom = 1, $P < 0.01$).

To investigate the minimum number of ancestral genes that generated the 87 diverse Zea Rp1 homologues, individual LRR-encoding units of the 87 genes were analyzed (see Materials and Methods). The number of distinct fragments in each LRR-encoding unit of the 87 genes varied from 4 to 14. Therefore, the 87 Rp1 homologues obtained from Zea might have been generated by as many as 14 ancestral genes through frequent sequence exchanges among them.

To further investigate the main genetic events accounted for the evolution of Rp1 locus in maize, the Rp1 locus in maize line B73 was reassembled using the partially sequenced BAC clones and published sequences (Ramakrishna et al. 2002; Schnable et al. 2009). Three contigs of 750-kb sequences, each containing at least one Rp1 homologue, were obtained. Additional sequences of approximately 280 kb were distributed in three unassembled regions and contained no Rp1 homologues. Therefore, the Rp1 locus from maize line B73 spans over 1 Mb. It harbors at least 11 full-length and 3 truncated Rp1 homologues. Dot plot analysis of the 750-kb assembled sequences discovered several duplications of greater than 10 kb, and five of them contained an Rp1 homologue (data not shown). The mechanisms for the duplications containing Rp1 homologues remain unclear. Nevertheless, none of the large duplications were tandem, ruling out the possibility of recent unequal crossover.
Concerted Evolution of Rp1 Homologues in Genera Zea and Sorghum but Not in Different Zea Species

The frequent gene conversions did not cause concerted evolution for the Rp1 gene family in the genus Zea. The homologues from the four Zea species mingled in the Zea clade on the NJ tree, with no independent clade for individual Zea species (fig. 3). Such relationships were consistent with the observation that nucleotide identities between genes within a species were similar to those between genes from different Zea species (table 1).

To investigate if frequent sequence exchanges homogenized the Rp1 gene family in high phylogenetic levels, we extended our study to S. bicolor. Eight Rp1 homologues were obtained from three genotypes of S. bicolor. The pairwise nucleotide identity between genes within a genus varies from 82.8% to 99.9%, which is much higher than the pairwise nucleotide identity between genes from different genus (80.6–83.8%). The high nucleotide identities between genes within a genus were not all caused by recent gene duplications because 296 trans-specific polymorphic sites are present between genes from Zea and Sorghum. The large number of trans-specific polymorphisms may indicate that sequence exchanges also played important role on the homogenization of genes within a genus. The genes from different genus, Zea or Sorghum, formed independent clades in the distance tree (fig. 3). The formation of independent clades for Zea and Sorghum indicated that concerted evolution occurred for the Rp1 gene family in the genus level though it was not observed in different Zea species (fig. 3).

Low Rp1 Haplotype Diversity in Cultivated Rice

The Rp1 locus in rice cultivar Nipponbare contains four genes, Os01g57270, Os01g57280, Os01g57310, and Os01g57340. Comparison of the four genes and their intergenic sequences showed that Os01g57310 is a chimera generated through a recent unequal crossover between Os01g57280 and Os01g57340 (fig. 4B). The 5′ (ca. 3.2 kb) of the chimera is identical to Os01g57340 and its 3′ (ca. 600 bp) is identical to Os01g57280. The unequal crossover resulted in a duplication of 20,628 bp, and the duplicated sequence has only four single-nucleotide polymorphisms (SNPs) with its progenitor sequence. Rice cultivar St. No. 1 has a similar Rp1 locus to that in Nipponbare: the four pairs of Rp1 homologues in the two cultivars exhibit 100%, 99.7%, 99.8%, and 100% nucleotide identities, respectively (Lin et al. 2007). The chimera in St. No. 1 is the functional gene Pi37, which encodes resistance against rice Blast (Lin et al. 2007). The resistance gene Pi37 has four SNPs from Os01g57310, two of which result in change of amino acids. Because the chimera Os01g57310 is identical to its progenitor sequences (see above), the gene Pi37 apparently evolved from Os01g57310 through four point mutations. Partial sequencing the BAC clones spanning the Rp1 locus in rice cultivars ZS97 and ZH11 suggested that these two cultivars also have the same Rp1 haplotype as Nipponbare. Screening 24 rice cultivars using nine primer combinations indicated

FIG. 3. An NJ distance tree for Rp1 homologues from Zea and Sorghum. Pi37 from rice was used as an outgroup. The numbers on nodes are bootstrap values, and values lower than 60 are not shown. Genes with prefix “Rp” are from Zea may ssp. may, with prefix “Zmp” are from Z. may ssp. parviglumis, with prefix “Zl” are from Z. luxurians, and with prefix “Zd” are from Z. diploperennis.
that five (including ZS97 and ZH11) cultivars have the Nipponbare-like haplotype. The Nipponbare-like haplotype in these cultivars was further supported by their Rp1 homologues, which were almost identical to their corresponding genes in Nipponbare. The only variations of Rp1 sequences from rice cultivars of the Nipponbare-like haplotype are a few SNPs and a diverse region in genes OjLXJ-2 and OjGYDD-2 resulted from a gene conversion.

Compared with the Rp1 haplotype in Nipponbare, rice cultivar 93-11 has a very different Rp1 haplotype. The region spanning Rp1 locus in 93-11 was resequenced, resulting in 170,565-bp sequence with two gaps of unknown size. Three full-length and one truncated Rp1 homologues were discovered. The centromeric part of the Rp1 locus in rice cultivar 93-11 is similar to that in Nipponbare, including two Rp1 homologues and their intergenic region. However, the telomeric part of the Rp1 locus varies dramatically in these two cultivars, with no similarity in the intergenic region. Screening 24 rice cultivars using nine PCR primer combinations located at the Rp1 locus showed that no other rice cultivars have the 93-11–like Rp1 haplotype. In addition to the Nipponbare-like and 93-11–like haplotypes, the screening of 24 rice cultivars using the 9 primer combinations identified another 2 haplotypes, with 4 and 14 cultivars, respectively. The identification of 4 Rp1 haplotypes among the 24 rice cultivars based on the results of 9 PCRs is consistent with the sequencing results of Rp1 homologues in these cultivars (see below).

**Table 1.** Nucleotide Identities between Rp1 Homologues from Different Species/Genera.

<table>
<thead>
<tr>
<th>Species/Genus</th>
<th>Oryza (%)</th>
<th>Brachypodium distachyon (%)</th>
<th>Triticum (%)</th>
<th>Sorghum bicolor (%)</th>
<th>Zea (%)</th>
<th>Zea may ssp. may (%)</th>
<th>Z. may ssp. parviglumis (%)</th>
<th>Z. luxurians (%)</th>
<th>Z. diploperennis (%)</th>
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from *Oryza* species exhibit 86.8–100% nucleotide identity and form a clade in figure 5. They have chimeric structures, generated through sequence exchanges among paralogues (fig. 4B). At least 15 sequence exchanges were detected among the genes in this clade. Some of the sequence exchanges occurred before speciation of *O. sativa*, *O. nivara*, *O. alata*, and *O. rufipogon*. We concluded that these genes evolved in a pattern of Type I R-genes. Therefore, there was differentiation of Type I and Type II R-genes in the *Rp1* cluster of *Oryza*.

**Single-Copy *Rp1* Locus in *Brachypodium distachyon* Genome**

Unlike other species, *B. distachyon* genome has a single copy at the *Rp1* locus. To better understand the evolution of single-copy R-gene locus, full-length *Rp1* homologues were amplified from 25 genotypes using primer combination Brach-F and Brach-R (supplementary table S2, Supplementary Material online). Sequencing the PCR products using primer Brach-R generated clean chromatograms for 16 of the 25 genotypes, indicating the presence of a single gene in these genotypes. The 16 PCR products giving clean chromatograms were sequenced directly. The other nine genotypes showed nearly identical chromatograms and have double peaks, suggesting multiple fragments in the PCR products. The PCR products amplified from one of such genotypes (PB-292) were cloned and sequenced. Two distinct sequences were obtained. Therefore, a total of 19 *Rp1* homologues were obtained from *B. distachyon*, including one retrieved from the sequenced genome of *B. distachyon*. The 19 *Rp1* genes are grouped into two major clades (group G1 and G2 in fig. 6). The G1 group has seven genes, with only seven SNPs and one 29-bp deletion. The G2 group consisted of 12 genes; 9 of the 12 G2 genes are identical (G2a), but the other 3 (G2b) were divergent from G2a, showing only 98.5% to 99.0% nucleotide identity with each other. The nucleotide identities between genes in the clades G1 and G2 vary from 91.9% to 93.9%. Primers specific to G1, G2a, and G2b were designed and used to screen all genotypes of *B. distachyon*. Consistent with previous cloning and sequencing results, the PCR amplification showed that there are three distinct *Rp1* homologues (either alleles or paralogues) in different genotypes of *B. distachyon*, and some genotypes have a single copy, whereas some genotypes have two copies (fig. 7).

**High Divergence of *Rp1* Homologues in Wheat and Barley**

The efforts to amplify full- or nearly full-length *Rp1* homologues from wheat were partially successful, and only 19 *Rp1* homologues were cloned from three wheat cultivars. PCR and sequencing results using primer combination For2 and Low4, which amplify approximately 2-kb LRR-encoding sequence from the *Rp1* family, show that there are many more *Rp1* homologues in wheat genomes. To avoid

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**FIG. 5.** An NJ distance tree for *Rp1* homologues from *Oryza*. *Rp1-D* from maize was used as an outgroup. The numbers on nodes are bootstrap values, and values lower than 60 are not shown. Genes with prefix “Oi” are from *O. sativa* ssp. *indica*, with prefix “Oj” are from *O. sativa* ssp. *japonica*, with prefix “Or” are from *O. rufipogon*, with prefix “On” are from *O. nivara*, with prefix “Og” are from *O. glaberrima*, with prefix “Op” are from *O. punctata*, with prefix “Om” are from *O. minuta*, with prefix “Oal” are from *O. alta*, with prefix “Oau” are from *O. australiensis*, with prefix “Oc” are from *O. coarctata*, and with prefix “Ob” are from *O. brachyantha*.

**FIG. 6.** An NJ distance tree for *Rp1* homologues cloned from 18 genotypes of *Brachypodium distachyon*. The numbers on nodes are bootstrap values, and values lower than 60 are not shown. The prefix of the genes represents the genotypes from which the genes were cloned.
discrepancy, the 2-kb LRR-encoding sequences instead of nearly full-length genes were analyzed for the Pooideae subfamily. Using primer combination For2 and Low4, 27 distinct fragments of Rp1 homologues (TaCS1–TaCS27) were obtained from wheat cultivar CS. PCR products of 23 randomly chosen CS Rp1 homologues were pooled and used as probe for Southern hybridization to screen 146 CS cytogenetic stocks including 21 nullisomic–tetrasomic, 24 ditelosomic, and 101 deletion lines. 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majority (9/14) were missing in the Southern image, the majority (9/14) were missing in the南方圣索引的全部内容, including every gene accession and the corresponding genome location. This comprehensive mapping enables the identification of genes involved in the genetic diversity of rice, wheat, and barley, facilitating the study of disease resistance and crop improvement.
25 genotypes of *B. distachyon* has at most two genes. In contrast, *Z. may* ssp. *parviglumis* genotype Ames21889 has at least 70 *Rp1* homologues. *Zea luxurians* PI441933 likely has a large number of *Rp1* homologues in its genome because 42 distinct genes were obtained after sequencing 50 randomly chosen clones. Though different genotypes of *Z. may* have various number of *Rp1* homologues, it is obvious that the average of *Rp1* homologues in one *Zea* genome is considerably higher than that in *B. distachyon*.

The large number of *Rp1* homologues in *Zea* might be the results of unequal crossovers. The *Rp1* locus in maize was shown to be prone to recombination and high frequency of unequal crossover was observed experimentally (Hulbert 1997; Sun et al. 2001). The *Rp1* locus is located approximately 2–3 Mb from the telomere, and its proximity to telomere might promote structure change of *R*-gene locus (David et al. 2009). Unequal crossover, on the other hand, may cause deletions of genes. In contrast to the expansion of the *Rp1* locus in *Zea*, *Tripsacum dactyloides*, a close relative of *Zea*, might have had complete deletion of the *Rp1* locus. We failed to get any PCR amplifications from two genotypes of *T. dactyloides* using five primer combinations that are conserved in *Zea* and *Sorghum* (data not shown). Frequent unequal crossovers were also found in other resistance gene cluster (Van der Hoorn et al. 2001; Krujit et al. 2004; Nagy and Bennetzen 2008).

High copy number of resistance genes should be selected for and maintained in plant genomes, if resistance is considered only. However, *R*-gene clusters in plant genomes did not expand unlimitedly. It is possible that large *R*-gene locus may change chromosome structure and cause genome instability. Furthermore, the presence of an *R*-gene sequence in a genome may decrease its fitness in some cases (Tian et al. 2003; Nagy and Bennetzen 2008). The selection mechanisms for high copy number of *Rp1* homologues in *Zea* but single copy in *B. distachyon* remain unknown.

**Concerted Evolution of Disease-Resistance Genes**

Frequent sequence exchanges tend to homogenize a gene family. Consequently, homologues within a species/genus/family are more similar than those from different species/genus/family, a phenomenon called concerted evolution. Homogenization of disease-resistance genes could decrease a plant’s resistance and any sequence exchanges homogenizing an *R*-gene family may be selected against. It was hypothesized that there was “diversifying” selection after sequence exchanges (Kuang et al. 2004). Newly generated chimeras with novel resistance function will be selected for and maintained in a population. In contrast, sequence exchanges homogenizing two genes and reducing resistance specificity will be selected against. Frequent sequence exchanges followed by diversifying selections will maintain high diversity of the coding sequences of Type I disease-resistance genes though their intron sequences may be homogenized (Kuang et al. 2004, 2005). In fact, there has been no report of concerted evolution of *R*-genes in closely related plant species. In this study, we showed that
there were frequent sequence exchanges between \textit{Rp1} homologues within each \textit{Zea} species. However, such frequent sequence exchanges did not result in concerted evolution, probably due to short divergence time as well as the aforementioned diversifying selection after sequence exchanges (Gaut and Clegg 1993; Hilton and Gaut 1998). Similarly, there is no evidence of concerted evolution for the \textit{Rp1} homologues in \textit{Oryza} (fig. 5). Like in \textit{Zea} species, frequent sequence exchanges have occurred between \textit{Rp1} homologues in \textit{S. bicolor}, which had evolutionary patterns typical of Type I \textit{R}-genes. The \textit{Rp1} homologues in \textit{Zea} and \textit{Sorghum} formed independent clades in the distance tree (fig. 3), showing obvious concerted evolution. The concerted evolution between \textit{Sorghum} and \textit{Zea} was likely due to their long divergence time (Doebly et al. 1990). \textit{Tripsacum} diverged 4.5–4.8 Ma with \textit{Zea} (Hilton and Gaut 1998), and it would be interesting to know if there was concerted evolution between the \textit{Rp1} homologues in these two genera. Unfortunately, we failed to obtain any \textit{Rp1} homologues from the two genotypes of \textit{T. dactyloides} included in this study.

**Distinct Evolutionary Patterns of \textit{Rp1} Homologues in Different Species**

The \textit{Rp1} homologues in \textit{Zea} and \textit{Sorghum} exhibit evolutionary patterns typical of Type I \textit{R}-genes. Unlike the \textit{Rp1} homologues in \textit{Zea} and \textit{Sorghum}, the homologues in wheat and its relatives showed no sign of sequence exchanges among paralogues. These genomes maintain many divergent \textit{Rp1} homologues, which evolved independently. Apparently, the \textit{Rp1} homologues in wheat and barley were duplicated in the early stage of subfamily Pooidae, and they might have evolved following the birth-and-death model (Michelmore and Meyers 1998). The \textit{Rp1} homologues in \textit{B. distachyon} also evolved independently, but only a single \textit{Rp1} homologue is present in \textit{B. distachyon}. We hypothesize that the \textit{Rp1} homologues in wheat, barley, \textit{B. distachyon} as well as their close relatives might have evolved in a pattern of Type II \textit{R}-genes.

**Differentiation of Type I and Type II Genes in Rice**

Both Type I and Type II \textit{Rp1} homologues were found in the \textit{Rp1} cluster in \textit{Oryza} species. The Type II genes in \textit{Oryza} species were most similar to other \textit{Rp1} homologues obtained from \textit{Oryza} species, suggesting that the differentiation of Type I and Type II \textit{Rp1} homologues occurred after the speciation of \textit{Oryza}. In this study, the Type II gene was found in cultivated rice and wild rice \textit{O. rufipogon}. The Type II \textit{Rp1} homologue was the most divergent member in the \textit{Rp1} family in \textit{Oryza}, suggesting that the differentiation of Type I and Type II genes occurred right after the speciation of genus \textit{Oryza}. It remains to be investigated whether the values, and values lower than 60 are not shown. Genes with prefix “Ta” are from \textit{Triticum aestivum}, with prefix “At” are from \textit{Aegilops tauschii}, with prefix “Tm” are from \textit{T. monococcum}, with prefix “Tu” are from \textit{T. urartu}, with prefix “Td” are from \textit{T. durum}, and with prefix “Morex” are from barley cultivar Morex.
Type II gene is also present in other *Oryza* species. Except the identified Type II *Rp1* gene in *Oryza*, the three genes obtained from *O. australiensis*, *O. brachyantha*, and *O. coarctata* were the most divergent in the *Rp1* family obtained from *Oryza* species. No sequence exchanges were detected between these genes and the Type I genes in *Oryza*. Their evolutionary patterns remain unclear for lack of enough *Rp1* sequences from these species.

Most *Rp1* homologues obtained from *Oryza* showed evolutionary patterns of Type I *R*-genes. It was proposed that there are a large number of Type I *R*-genes in a population because each genotype may harbor a different set of Type I *R*-genes (Kuang et al. 2004). Surprisingly, only a few distinct *Rp1* homologues were found after screening 24 rice cultivars. We hypothesize that only few *Rp1* haplotypes were introduced into the cultivated rice through domestication and breeding. The number of distinct *Rp1* homologues in a natural population of wild rice might be large. This prediction is partially supported by the relatively high diversity of the 17 *Rp1* homologues obtained from wild rice (fig. 5).

**Evolution of the Single-Copy *Rp1* Gene in *B. distachyon***

Only one or two *Rp1* homologues were present in the 25 *B. distachyon* genotypes in our study, which include diploids, tetraploids, and hexaploid. The *Rp1* homologues are grouped into three lineages, G1, G2a, and G2b. All ten diploids have only one *Rp1* homologue. However, 9 of the 15 polyploids have 2 genes. It is possible that the three *Rp1* homologues in these nine polyploids are located at the same locus of different genomes. Therefore, the *Rp1* locus in *B. distachyon* might have a single gene. Notice, even in this case, the three genes should be treated as paralogues rather than alleles. First, this locus in other species such as wheat has more than one copy of *Rp1* homologue, suggesting that the progenitor species of *B. distachyon* might have multiple copies of *Rp1* paralogues at this locus. The single-copy gene at this locus might be the result of independent deletions of all other *Rp1* paralogues at this locus, respectively. Second, the G1 and G2a/b genes exhibit nucleotide identity of only 93.9–94.1%, which is much lower than a typical nucleotide identity between two alleles. Third, some polymorphic sites (SNPs) between G1 and G2a/b genes were also found in different paralogues in wheat. Thus, we tend to consider the three *Rp1* homologues in *B. distachyon* as paralogues.

The sequencing of plant genomes of model species provides valuable resources for understanding the organization, structure, and evolution of R-genes. However, our data showed that the evolutionary patterns of a resistance gene family might vary dramatically in closely related species. Great care should be taken when applying R-gene information in a model species to its relatives. Genome-wide comparative analysis of R-genes will further promote our understanding of R-gene evolution, which is critical for conservation biology, efficient cloning of R-gene, and breeding of resistant crops.

The sequences of *Rp1* homologues generated in this study were deposited in GenBank under accession numbers GU733153–GU733316.

**Supplementary Material***

Supplementary tables S1–S3 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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