Eur J Plant Pathol (2010) 126:333–342
DOI 10.1007/s10658-009-9549-5

Targeted mapping of rice ESTs to the *LmPi1* locus for grey leaf spot resistance in Italian ryegrass

Wataru Takahashi · Yuichi Miura · Tohru Sasaki · Tadashi Takamizo

Received: 14 June 2009 / Accepted: 5 October 2009 / Published online: 21 October 2009

Abstract Epidemics of blast disease, also called grey leaf spot (GLS), in ryegrass species have recently become a serious problem in Japan and the USA. GLS causes serial growth inhibition and the abnormal morphology of withering in ryegrass seedlings. If the lesions expand they can completely destroy the plant at several growth stages. We attempted to construct a partial genetic linkage map around the *LmPi1* locus, which confers resistance to GLS and is located on linkage group (LG) 5 of Italian ryegrass, by using synteny with rice. Because LG5 of Italian ryegrass is syntenic with rice chromosome (Chr) 9, we designed intron-scanning primers with alignment between the rice genome sequence and expressed sequence tag (EST) information on rice Chr 9. These primers were used to genotype an F_1 mapping population, which had been used for detection of the *LmPi1* locus, by detecting single-strand conformation polymorphisms (SSCPs). We successfully constructed a partial genetic linkage map around the *LmPi1* locus, spanning 66.3 cM. A comparative genomic analysis between the partial genetic linkage map and the rice Chr 9 genetic linkage map revealed that the *LmPi1* locus was a candidate for orthology to the *Pi5* locus or *Pi15* locus, both of which are co-located in a resistance gene cluster on rice Chr 9 and are associated with broad-spectrum resistance to rice blast.

Keywords Blast · Comparative genomics · Expressed sequence tag · *Lolium multiflorum* · Single-strand conformation polymorphism

W. Takahashi (✉) · T. Takamizo
Forage Crop Biotechnology Research Team,
National Institute of Livestock and Grassland Science,
768 Senbonmatsu, Nasushiobara,
Tochigi 329-2793, Japan
e-mail: twataru@affrc.go.jp

Y. Miura
Kyushu Experiment Station,
Japan Grassland Agriculture and Forage Seed Association,
1740 Takaba, Koushi,
Kumamoto 861-1114, Japan

T. Sasaki
Forage Crop Research Institute,
Japan Grassland Agriculture and Forage Seed Association,
388-5 Higashiakada, Nasushiobara,
Tochigi 329-2742, Japan

Introduction

Italian ryegrass (*Lolium multiflorum*) originated in the Mediterranean region and is grown mainly for hay and silage. It is one of the most important forage grasses in the temperate zones of Europe and Asia because of its high palatability to and digestibility by livestock.

Ryegrass blast, also called grey leaf spot (GLS), has recently become a very serious problem in Italian ryegrass in Japan (Miura et al. 2005) and in perennial ryegrass (*L. perenne*) in the USA (Han et al. 2006). The causal agent of the disease is the fungal pathogen

Pyricularia oryzae (anamorph of *Magnaporthe oryzae*), which has recently been described as a new species distinct from *P. grisea* (anamorph of *M. grisea*) (Couch and Kohn 2002). Disease symptoms first appear as small brown spots on the leaves and stems; they develop into water-soaked spots that further progress into round or oval lesions with grey centres and dark brown margins. If *P. oryzae* heavily infects a ryegrass stand composed of susceptible genotypes, the stand will be completely destroyed within a matter of days.

It is well known that *P. oryzae* also causes blast disease in rice (*Oryza sativa*), and many researchers have studied rice blast by genetic, pathological, and molecular biological approaches by which the mechanisms of blast resistance in rice are now being clarified. For instance, 85 genes and approximately 350 quantitative trait loci (QTL) for blast resistance have been identified and mapped on the rice genome to date (reviewed by Ballini et al. 2008). These efforts have further progressed into the selection of blast-resistant lines by the use of molecular markers (Yi et al. 2004; Jena and Mackill 2008) and map-based cloning of resistance genes. To the best of our knowledge, 10 resistance genes have so far been cloned: *Pib*, *Pita*, *Pik*^{h}, *Pi9*, *Pi2/Pizt*, *Pid2*, *Pi36*, *Pi37*, and *pi21* (reviewed by Ballini et al. 2008), and *Pi5* (Lee et al. 2009). *pi21* is the only gene for partial resistance to rice blast; the others control complete resistance.

GLS resistance in ryegrasses is also being studied, although this research lags conspicuously behind that of rice blast resistance. In perennial ryegrass, the partial resistance phenotype is controlled by a major gene with a high level of broad-sense heritability (Han et al., 2006). In Italian ryegrass, Trevathan (1982) found that some genotypes from Europe showed resistance to GLS. Variable GLS resistance has also been observed among cultivars (Reith et al. 2003). Most recently, we have developed an accurate inoculation method, the 'filter-paper method', for the assay of GLS in Italian ryegrass, and we have selected GLS-resistant genotypes from among Japanese and USA commercial cultivars and lines provided by the National Plant Germplasm System of the US Department of Agriculture (Takahashi et al. 2009). Attempts at molecular breeding of GLS-resistant ryegrass cultivars are under progress, and a few reports have been published thus far. We used bulk segregant analysis to develop the molecular marker P56, which is tightly linked to the *LmPi1* locus for GLS resistance, located on linkage group (LG) 5 of Italian ryegrass (Miura et al. 2005). Similarly, Curley et al. (2005) have identified four QTL for GLS resistance from a genetic mapping population derived from parental clones of Italian × perennial ryegrass hybrids.

In these contexts, the availability of the whole genome sequences of the model plants *Arabidopsis thaliana* (The Arabidopsis Genome Initiative 2000) and rice (International Rice Genome Sequencing Project 2005) and the recent enrichment of datasets of expressed sequence tags (ESTs) in various plant species facilitate comparative genomics between model and non-model plants by which the colinearity of molecular markers and genes in syntenic loci can be revealed. This approach has led to the high-resolution mapping of agronomically useful traits in non-model crops. For instance, saturation mapping of barley (*Hordeum vulgae*) ESTs to the targeted genomic region of the barley leaf rust resistance gene has been performed by comparative genomics with rice (Perovic et al. 2004; Mammadov et al. 2005). The most challenging topic in comparative genomics was recently tackled in sugarcane (*Saccharum* spp.) by Le Cunff et al. (2008). Sugarcane is highly polyploid (~12×) and aneuploid, and it contains approximately 10 Gb/2C of DNA; it is likely that this is why sugarcane has been recalcitrant to the map-based cloning of its agronomically important genes. To isolate the brown rust resistance gene (*Bru1*) of sugarcane, Le Cunff et al. (2008) proposed diploid/polyploid syntenic shuttle-mapping with two diploid model species such as sorghum (*Sorghum bicolor*) and rice, with the expectation that this would overcome the constraints associated with high ploidy. They succeeded in high-resolution mapping, including markers at 0.25 and 0.14 cM on both sides of *Bru1* (Le Cunff et al. 2008). Comparative genomics approaches in ryegrasses to the genetic loci relevant to resistance to diseases such as GLS (Curley et al. 2005; Sim et al. 2007; Jo et al. 2008) and to flowering (Armstead et al. 2005) and fertility (Armstead et al. 2008) have been used, and syntenies with rice or with other plant species have been revealed.

We attempted here to enrich the molecular markers in the genetic region of the *LmPi1* locus, located on LG5 of Italian ryegrass, by utilising synteny with rice. We mapped EST markers derived from rice chromosome (Chr) 9 (Wu et al. 2002), which is syntenic to

ryegrass LG5 (Jones et al. 2002; Sim et al. 2005), onto the target region containing the *LmPi1* locus. Interestingly, comparative genomics between the resultant partial genetic linkage map and the genetic linkage map of rice Chr 9 suggested that the *LmPi1* locus is a putative counterpart of the recently cloned *Pi5* locus (Lee et al. 2009) or the finely mapped *Pi15* locus (Lin et al. 2007), which are co-located in a resistance gene cluster on rice Chr 9 (Pan et al. 2003) and are associated with broad-spectrum resistance to rice blast.

Materials and methods

Plant materials

A two-way pseudo-testcross F_1 population of Italian ryegrass (*L. multiflorum*) was generated from a single cross between two heterozygous individuals: a GLS-resistant individual of cv. 'Sachiaoba' as the female parent and a GLS-susceptible individual of cv. 'Minamiaoba' as the male parent. The F_1 population, composed of 156 individuals, had been previously used for the development of DNA markers linked to the *LmPi1* locus (Miura et al. 2005). DNAs extracted from the first 88 individuals were used in this study.

Design of intron-scanning primers and polymerase chain reaction (PCR)

We previously reported that the molecular marker P56, which is tightly linked to the *LmPi1* locus, is located on LG5 (Miura et al. 2005, 2007). Moreover, synteny between ryegrass LG5 and rice Chr 9 has been shown by other research groups (Jones et al. 2002; Sim et al. 2005). We therefore selected EST clones of rice Chr 9 at a genetic distance of about every 5 cM or less, for as far as possible, from public EST map data (Wu et al. 2002) in the online database of the Rice Genome Research Programme (RGP; http://rgp.dna.affrc.go.jp/E/). Subsequently, genomic clones [P1-derived artificial chromosome (PAC) clones] that contained the nucleotide sequence information for the selected EST clones were retrieved by using WhoGA, a rice genome annotation viewer from the RGP (http://rgp.dna.affrc.go.jp/whoga/index.html.en). A coding sequence (CDS) of the EST clone was concomitantly obtained with the nucleotide sequence features of the retrieved PAC clone. The exon/intron structure of the target gene was predicted by generating CDS-to-PAC clone sequence alignments with Spidey, an online tool for mRNA-to-genomic alignment (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). Primer pairs in predicted exon regions were designed to amplify across predicted intron regions by the primer analysis software OLIGO v. 6.7 (Molecular Biology Insights, Inc., Cascade, CO, USA). PCR was performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) with a 10-µl reaction mixture containing 0.05 µl HotStarTaq (5 units μl^{-1}; Qiagen, Hilden, Germany), 1 µl 10× PCR buffer, 0.4 µl 25 mM $MgCl_2$, 0.8 µl dNTPs (2.5 mM each), 0.2 µl each primer (20 pmol μl^{-1}), 20 ng genomic DNA, and 5.35 µl sterile distilled water. After the first treatment of the reaction mixture at 95°C for 15 min, the following PCR programmes were performed: two cycles of 94°C for 1 min and 72°C for 2.5 min; two cycles of 94°C for 1 min and 68°C for 2.5 min; two cycles of 94°C for 1 min, 65°C for 30 s, and 72°C for 2 min; and 30 cycles of 94°C 1 min, 55°C for 30 s, and 72°C for 2 min.

Single-strand conformation polymorphism (SSCP) analysis

The SSCP analysis was carried out with precast polyacrylamide gel (GeneGel Excel 12.5/24; GE Healthcare UK Ltd., Buckinghamshire, England), a peltier temperature-regulated electrophoresis unit (GenePhor; GE Healthcare), and an electrophoresis power supply (EPS3501XL; GE Healthcare) in accordance with the manufacturers' instructions. Denaturing solution was made in a ca. 25-ml total volume containing 23.75 ml 99% formamide, 1.25 ml 1% xylene cyanol, and 10 mg bromophenol blue. To denature the PCR products, equal amounts of PCR products and denaturing solution were mixed to make 6 µl of mixture. The mixture was treated at 95°C for 5 min for denaturing and was then cooled rapidly on ice. The denatured sample was loaded onto a polyacrylamide gel, which was temperature-regulated at 5 or 15°C, and was electrophoresed for 100 min at 600 V, 25 mA, and 15 W. Isolated PCR products were visualised by silver staining with a sliver staining kit (GE Healthcare) in a Hoefer automated gel stainer (GE Healthcare).

Construction of partial genetic linkage map

Polymorphic markers were scored in each individual of the F_1 population. The following segregation types were adopted: locus heterozygous in either female or male parent representing two alleles (lm × ll or nn × np), and locus heterozygous in both parents representing three or four alleles (ef × eg or ab × cd), where the parental genotypes were coded according to JoinMap 4 (Kyazma B.V., Wageningen, Netherlands). The segregation types that were heterozygous in both parents were used as bridge markers. For map construction, the segregation data were inputed and calculated with the CP algorithm in JoinMap 4, and genetic distances calculated by Haldane's mapping function. All other calculation conditions of JoinMap 4 were default settings. The genetic linkage map was drawn with MapChart 2.2 software (Voorrips 2002).

Results

Amplification of target gene by PCR and SSCP analysis

EST clones selected from the rice EST map are shown in Table 1. A total of 42 EST clones were selected, and primer pairs designed from these EST clones; the average expected product length in rice was 465 bp. Twenty-seven primer pairs (64.3%) successfully amplified clear PCR products from the female and/or male parent. Twenty-five primer pairs (59.5%) successfully amplified fragments that were polymorphic between parents in SSCP analysis.

Most SSCP analyses of the mapping population showed multiple bands (data not shown). However, most banding patterns from the SSCP analyses could be categorised into four segregation types (Table 1): eight primer pairs indicated a segregating banding pattern representing two alleles from the female parent (lm × ll); five primer pairs indicated a segregating banding pattern representing two alleles from the male parent (nn × np); six primer pairs indicated a segregating banding pattern representing three alleles derived from heterozygosity in both parents (ef × eg); and one primer pair indicated a segregating banding pattern representing four alleles derived from heterozygosity in both parents (ab × cd). Four primer pairs showed a non-segregating banding pattern, and the remaining primer pair showed an unexpected segregating banding pattern in the mapping population. No primer pair that showed heterozygosity in both parents representing two alleles (hk × hk) was observed. The 20 markers categorised into the four segregation types were used for the genetic map construction described below.

Comparative genomics between rice Chr 9 and LG5 of Italian ryegrass

The data from the SSCP analysis of the 20 markers and the marker P56 tightly linked to the *LmPi1* locus (Miura et al. 2005) were analysed by JoinMap 4. The analysis yielded a linkage group with a logarithm of odds threshold of 7.0 with 17 markers, including the marker P56, and we succeeded in constructing a genetic linkage map covering 66.3 cM (Fig. 1). Comparative analysis against rice Chr 9 indicated that markers around the centromere region (0.8 cM) of rice Chr 9 were located and concentrated in the central region of the ryegrass genetic linkage map (Fig. 1). Although some colinearity of markers between the long arm region of rice Chr 9 and the ryegrass genetic linkage map was observed, clear rearrangement was also observed in markers such as R10290 and C0506, which are located in the distal region of the long arm of rice Chr 9 (Fig. 1).

The *Pi5* and *Pi15* loci for rice blast resistance coexist in a resistance gene cluster on rice Chr 9 and are tightly linked to marker C1454 (Jeon et al. 2003; Lin et al. 2007; Lee et al. 2009). We therefore show the location of C1454 on the rice Chr 9 genetic linkage map (Fig. 1). The genetic distance between C1454 and marker E61706 is 0 cM, as is shown on the rice EST map (Wu et al. 2002) in the online database of the RGP (http://rgp.dna.affrc.go.jp/E/). Accordingly, E61706 is genetically associated with the *Pi5* locus and the *Pi15* locus. Interestingly, in Italian ryegrass, E61706 was tightly linked to marker P56 (ca. 0.1 cM), indicating that E61706 is also associated with the *LmPi1* locus of Italian ryegrass (Fig. 1).

Discussion

Whole-genome sequencing of the model plants *A. thaliana* (The Arabidopsis Genome Initiative 2000) and rice (International Rice Genome Sequencing

Table 1 Summary of EST clones selected from rice chromosome 9, and the results of the SSCP analysis

EST clone	Genetic distance (cM)[a]	Primer sequences		Product length (bp)[b]	Electrophoresis temperature (°C)	Segregation types[c]
		Forward primer	Reverse primer			
C62013	0	AGAGCATCCACGGGACGAAC	GGTGGCAGTGAGGCGAAGGTA	402	15	ef × eg
C61788	0	CTTGAGGGAGGAACCTAACAG	GCCATGTGCGACCAACA	504	15	lm × ll
S13769	0.5	TGCTCCGTTGATGAAGTTAG	CGATACTTCTGATAACCCATA	416	15	lm × ll
S12681	0.8	GGATCATGTATTCTAACCGTG	TCATAAGCATGCCATTGAGT	324	15	ef × eg
E20434	0.8	TCAAGATGGCAAGATGAGGTT	GCAAGATGGGTCGCAGAA	432	5	lm × ll
E31558	0.8	CGCCGCCCTGCTCGATCTT	TCCTGGTTCGCCTCCGGGTAC	302	–	NC
S20740	0.8	GCCAAATTTGAAGTTCGAAGT	TGTGGAGTGCCGATAAT	841	–	NC
C11804	2.1	CAACGGGCTCCCCAAC	TTTCCGCAGGAGGTCCAAAGT	311	15	NS
E50550	2.1	GTCCATGAGCACGGTAGGTAC	ATCCCCTTCCGTAGGTA	316	15	nn × np
C11325	2.4	TGAAGATGCCCATGGATACCG	GAACCCCATGCCCATGCC	404	5	U
C61171	3.2	CGGGTTTGTCACGATGAG	GAAACTGCAGCCTGAACTTCT	432	–	NC
R1164	4.4	GGCATATGAGGGTAGTTCTT	GTGCAGCTTTCTTCATGTAGA	472	15	lm × ll
R10783	6.8	CATGCGCTTCTGGAGCTAAC	CAAAGACGTGCCGTTTATGTT	564	–	NC
R2722	20.7	AGTTCTTGAGGATGCCGTTGA	CCGGACATAAGGCATGACCAT	423	15	NS
E61706	30.6	GAGTGGCCAATCTTGTAAGAG	TAATCGATAGCCGTAATCAC	439	15	ef × eg
E61041	33	CGATGATCTCGAAGACTCTAA	CGGTGGTGATTCAACAGA	785	–	NC
S6106	34.9	GCTTGGCAGCCGTATTTGA	GCTTGAGCTTAGGCAATAGGG	503	–	NC
S13828	36	TGGAGGCTGGCAACATC	TGTGCGATCATCAGAACTACA	670	15	lm × ll
S21273	36	GAGGGTGAGGATCTCGAGGC	CACTCCTCCTGCGTCATGTGC	435	–	M
C0873	40.7	ATTCTCAATAGCATAACGCAA	ACCCGGCAGATATGAC	782	–	NC
E0435B	42.5	GAGAGACAATTTGCGTACATC	TGGCTAAATTCGTACACTTGC	421	–	NC
S4677	45.2	ACCTCATGCGGCAGTATCTGA	GCCGGATCATCCCAAGTTATC	456	–	NC
R10143	45.2	GCCACCTCTCACCGTTCAGC	GGCACGAGTCGACGAAGTAGC	440	15	lm × ll
R1751	49.3	GATATCCGTGAAGACACTCAA	CCCTGATGAATGAGCAT	476	–	NC
S11615	55.9	ACGCAGCCTATGCTCTGACAG	CCGGTGCATGATGACAAGT	410	5	lm × ll
C30017	55.9	GCTTTCAGGGCCTCCGATTA	GCTTGGGAAGGGCTTGATTGC	543	15	nn × np
C12862	58.3	GAAAGAGAACGAGGAATCCAT	CCCTGGACTCTCCGTTGT	452	–	NC
S13972	60.8	GGTGCTAGCGGCGTTGA	TAGAAGCAGGGCCTTGTAGGA	467	–	NC
E61594	63	GGAGCCAATGCCTTATTC	TCCTCTCCAATTCCTTGTTCA	499	–	NC
E2005	65.1	TGCGCCTCCAGTTCCACGACT	GGGCGGCCATGACGATGATG	403	15	nn × np

Table 1 (continued)

EST clone	Genetic distance (cM)[a]	Primer sequences		Product length (bp)[b]	Electrophoresis temperature (°C)	Segregation types[c]
		Forward primer	Reverse primer			
C0745	68.2	GTTCGGCGGGGAGGCGGTCT	GGGGCCGGTGCCGTCGCAGAT	181	15	ef × eg
E60851	72.1	CGTGTGTCTCCGTTATGGAA	ATTCTGGTTCTTGGCAACAAT	541	15	ab × cd
E0421B	74.7	CTTGTCCTCAGGGCGGTACTT	CACCCGCACCCTCGACAAGA	624	15	ef × eg
C60150	76.9	CCTTCTACCGTGGGTTAACTG	AGAGGACCGTCGGTGATTCT	277	5	NS
C11140	79.1	TGCGAGATAGATGGTACATCA	CGGGTCTCATATTAAGATCAA	398	5	nn × np
E10960	79.1	GTGGTACCTTGTGCTGTGCTA	CCCAGTGTGCACAGCTAAGA	282	15	ef × eg
E50073	82.1	AACATCCCTGCTTTCATATGG	GCTTCTGGCATGTAACTTCC	607	–	NC
R10290	84.3	GCCTGCCGTGCACTTTACATA	TTACCGACAACCCGTGCTG	421	15	lm × ll
C12357	88.2	CGGTGCTGCGGGAGCTAC	ATGTGGGGGTTCATGACCGTA	464	–	M
C0506	90.4	GGCGAGGTCGAGGTCC	GGGGTGCTTCTCCTTGTAGTC	344	15	nn × np
S2116	91.5	ATTACAACATCCGGAACACCA	CAACATGTAATTTGCCGTTCA	563	–	NC
E0487	91.8	GAGTGGTCTGGTTCTGCGTAT	AAGCGATTCATTGGTACAGG	489	15	NS

[a] Position of EST markers on rice chromosome 9 (Wu et al. 2002)

[b] Expected product size from rice genomic DNA

[c] Parental genotypes were coded in accordance with JoinMap 4. *NC* Non-clear banding was observed in both parents of the mapping population. *NS* Non-segregated banding pattern in mapping population. *U* Segregation of unexpected banding pattern in mapping population. *M* Monomorphic between parents of mapping population

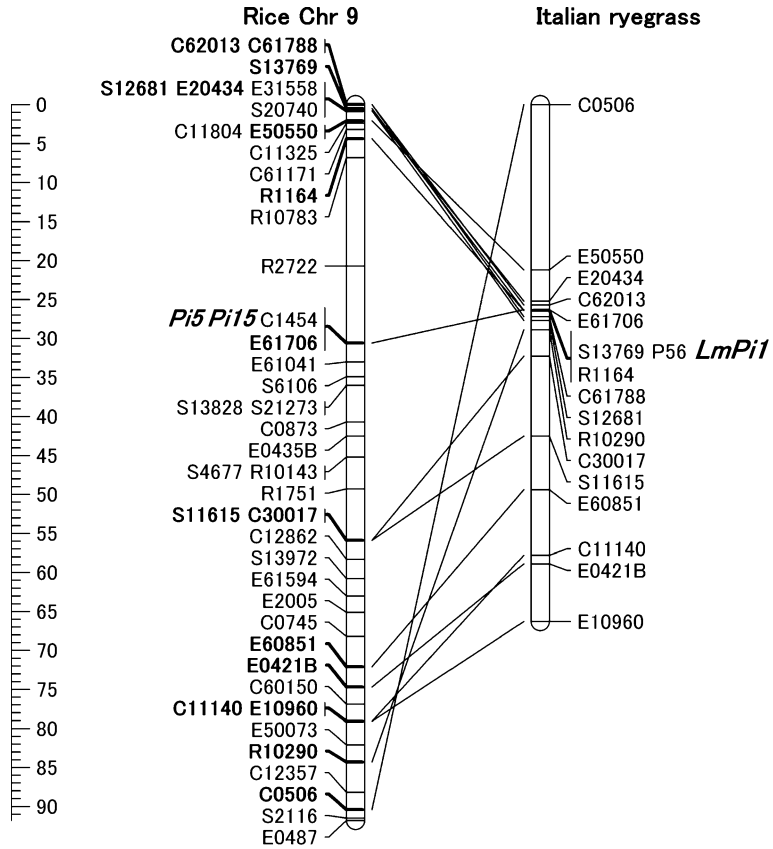

Fig. 1 Comparative rice Chr 9—Italian ryegrass partial LG5 genetic maps. Markers on the rice Chr 9 genetic map were selected from the rice EST map (Wu et al. 2002). Comparative loci between rice and Italian ryegrass are shown in *bold* on the rice Chr 9 genetic map and are connected with *solid lines*. Locations of the *Pi5* and *Pi15* loci in rice and the *LmPi1* locus in Italian ryegrass are shown in *italics*. Genetic distances are measured in centimorgans against the rule at the *left side* of the figure

Project 2005) has revealed the genetic and physical map positions of their ESTs and has concomitantly clarified the exon/intron structures of their genes. Recent comparative genomics thus uses the PCR-based marker system composed of the following procedures: (1) the ESTs of the non-model plant are aligned with the genomic sequences of the model plant; (2) intron-scanning primers are designed from conserved predicted exon sequences; and (3) polymorphisms in intron sequences, such as insertion–deletion (indel) and single nucleotide polymorphism (SNP), are detected by agarose gel electrophoresis, cleaved amplified polymorphic sequences (CAPS) analysis, or SSCP analysis (Bertin et al. 2005; Feltus et al. 2006; Tamura et al. 2009). Thanks to a high frequency of polymorphism in the intron sequences and high levels of conservation of the exon sequences and exon/intron structures of genes among plant species, this strategy has proved successful and should be adaptable to many non-model plants.

Here, we utilised the strategy to design rice EST-derived intron-scanning primers (Table 1) and succeeded in PCR amplification from the female and/or male parent of an F_1 mapping population with the success rate of 64.3%. Bertin et al. (2005) designed 299 pearl millet (*Pennisetum glaucum*) EST-derived intron-scanning primer pairs in which the exon/intron structure was predicted by alignment with rice genome sequences; 77% of the primer pairs worked well for PCR amplification across a genotype panel composed of eight inbred lines. Direct genome amplification with 15 primer pairs preliminarily screened from among the 299 primer pairs resulted in 50% success in other cereals, including rice, wheat (*Triticum aestivum*), barley, and finger millet (*Eleusine coracana*) (Bertin et al. 2005). By a similar strategy utilising rice genome

sequences, Feltus et al. (2006) designed sorghum EST-derived intron-scanning primers and succeeded in genome amplification, with success rates of 55.4% in pearl millet and 57.3% in *Cynodon* spp. Our results and these previous reports suggest that the success rate of inter-species transferability of intron-scanning primers in the Gramineae ranges from about 50% to 65%.

In general, as mentioned above, introns contain higher frequencies of polymorphisms, such as SNPs and indel polymorphisms, than do exons. For example, the average rates of polymorphism on a per-locus basis in eight rice genotypes were 12.1/kb in the intron and 3.6/kb in the exon (Feltus et al. 2006). On the other hand, SSCP analysis can detect polymorphism of a DNA fragment by both its size and its conformation, as derived from base substitution and/or indels. Therefore, the use of a combination of SSCP analysis and intron-scanning primers is convenient for high-throughput detection of polymorphisms in introns. Indeed, here, of the 27 primer pairs that amplified PCR fragments from the parents of the F_1 mapping population, 92.6% (25/27) could detect SSCPs between the parents. This frequency is much higher than that of a CAPS analysis of Italian ryegrass ESTs (28.5%) in which the primers were designed without consideration of intron scanning (Miura et al. 2007).

We constructed a genetic linkage map composed of 16 rice EST-derived markers covering 66.3 cM (Fig. 1). It was clear that the constructed map corresponded to part of LG5 of Italian ryegrass, since marker P56, which is tightly linked to the *LmPi1* locus on LG5, was also integrated into the map (Fig. 1). The comparative genomics shown in Fig. 1 supports the previous reports of Jones et al. (2002) and Sim et al. (2005), who found synteny between ryegrass LG5 and rice Chr 9, although this was not clearly observed in our previous mapping with Italian ryegrass ESTs (Miura et al. 2007).

Disruption of synteny between cereals is often revealed at resistance gene loci. For example, the barley stem rust resistance gene *Rpg1* was not cloned by a synteny-based approach, but was cloned by a positional cloning approach, because the gene was not found in the syntenous position in rice (Brueggeman et al. 2002). Also, syntenies with rice at loci of the barley leaf rust resistance genes *Rph5* and *Rph16* were found to be broken (Perovic et al. 2004; Mammadov et al. 2005). Here, however, putative counterparts of the *LmPi1* locus were found in rice. That is, the *Pi5* and *Pi15* loci for rice blast resistance are co-located in a resistance gene cluster on rice Chr 9 (Pan et al. 2003) and are tightly linked to marker C1454 (Jeon et al. 2003; Lin et al. 2007; Lee et al. 2009). Marker C1454 is linked to marker E61706 in rice at a genetic distance of 0 cM, indicating that marker E61706 is also linked to *Pi5* and *Pi15* (Fig. 1). On the other hand, although we could not integrate marker C1454 into the partial genetic linkage map of Italian ryegrass, we could integrate E61706 (Fig. 1). Interestingly, E61706 is genetically very close to marker P56, which is tightly linked to the *LmPi1* locus (Fig. 1). These results accordingly suggest that the *LmPi1* locus is a candidate for orthology with the *Pi5* locus or the *Pi15* locus.

Because the *Pi5* and *Pi15* loci are associated with broad-spectrum resistance to rice blast, the corresponding genomic regions have been well studied by the construction of physical maps (Jeon et al. 2003; Pan et al. 2003; Lin et al. 2007), and high-precision markers have been developed for marker-assisted selection (MAS) to screen for cultivars harbouring the *Pi5* locus (Yi et al. 2004). Furthermore, the *Pi5* locus has more recently been cloned, and its features have been elucidated: *Pi5*-mediated blast resistance is controlled by both *Pi5-1* and *Pi5-2*, which are co-located within a 130-kb interval (Lee et al. 2009). Thus, although *LmPi1*-mediated GLS resistance is controlled by a single genetic locus (Miura et al. 2005), it is probable that it is regulated by more than one gene at the locus, in additional to *Pi5*-mediated resistance. Unlike in rice, to our knowledge there is no published report that clearly delineates the fungal races of GLS, since differential varieties for race identification have never been developed in ryegrasses, although some research groups have investigated the genetic diversity and pathogenicity of *Lolium* isolates of *P. oryzae* (Viji et al. 2001; Farman 2002; Tosa et al. 2007). However, as well as the *Pi5* and *Pi15* loci, it is possible that the *LmPi1* locus is associated with durable resistance to a wide range of unknown races that have not yet been identified. Thus, further understanding of the mechanism of *LmPi1*-mediated resistance should contribute to the breeding of cultivars with durable resistance to GLS. Nevertheless, the comprehensive information that we have on the *Pi5* and *Pi15* loci encourages us to conduct high-resolution mapping and detailed

analysis of the genomic structure of the *LmPi1* locus, through which we can develop high-precision markers for MAS and synteny-based cloning.

Acknowledgements We thank Ms K. Akimoto (Japan Grassland Agriculture and Forage Seed Association) for her superior assistance throughout this study. This work was funded by a research grant from the Japan Racing Association (JRA) and supported by National Agriculture and Food Research Organisation (NARO), Japan.

References

Armstead, I. P., Skøt, L., Turner, L. B., Skøt, K., Donnison, I. S., Humphreys, M. O., et al. (2005). Identification of perennial ryegrass (*Lolium perenne* (L.)) and meadow fescue (*Festuca pratensis* (Huds.)) candidate orthologous sequences to the rice *Hd1*(*Se1*) and barley *HvCO1 CONSTANS*-like genes through comparative mapping and microsynteny. *New Phytologist, 167*, 239–247.

Armstead, I. P., Turner, L. B., Marshall, A. H., Humphreys, M. O., King, I. P., & Thorogood, D. (2008). Identifying genetic components controlling fertility in the outcrossing grass species perennial ryegrass (*Lolium perenne*) by quantitative trait loci analysis and comparative genetics. *New Phytologist, 178*, 559–571.

Ballini, E., Morel, J. B., Droc, G., Price, A., Courtois, B., Notteghem, J. L., et al. (2008). A genome-wide meta-analysis of rice blast resistance genes and quantitative trait loci provides new insights into partial and complete resistance. *Molecular Plant-Microbe Interactions, 21*, 859–868.

Bertin, I., Zhu, J. H., & Gale, M. D. (2005). SSCP-SNP in pearl millet—a new marker system for comparative genetics. *Theoretical and Applied Genetics, 110*, 1467–1472.

Brueggeman, R., Rostoks, N., Kudrna, D., Kilian, A., Han, F., Chen, J., et al. (2002). The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 9328–9333.

Couch, B. C., & Kohn, L. M. (2002). A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. *Mycologia, 94*, 683–693.

Curley, J., Sim, S. C., Warnke, S., Leong, S., Barker, R., & Jung, G. (2005). QTL mapping of resistance to gray leaf spot in ryegrass. *Theoretical and Applied Genetics, 111*, 1107–1117.

Farman, M. L. (2002). *Pyricularia grisea* isolates causing gray leaf spot on perennial ryegrass (*Lolium perenne*) in the United States: relationship to *P. grisea* isolates from other host plants. *Phytopathology, 92*, 245–254.

Feltus, F. A., Singh, H. P., Lohithaswa, H. C., Schulze, S. R., Silva, T. D., & Paterson, A. H. (2006). A comparative genomics strategy for targeted discovery of single-nucleotide polymorphisms and conserved-noncoding sequences in orphan crops. *Plant Physiology, 140*, 1183–1191.

Han, Y., Bonos, S. A., Clarke, B. B., & Meyer, W. A. (2006). Inheritance of resistance to gray leaf spot disease in perennial ryegrass. *Crop Science, 46*, 1143–1148.

International Rice Genome Sequencing Project. (2005). The map-based sequence of the rice genome. *Nature, 436*, 793–800.

Jena, K. K., & Mackill, D. J. (2008). Molecular markers and their use in marker-assisted selection in rice. *Crop Science, 48*, 1266–1276.

Jeon, J.-S., Chen, D., Yi, G.-H., Wang, G. L., & Ronald, P. C. (2003). Genetic and physical mapping of *Pi5(t)*, a locus associated with broad-spectrum resistance to rice blast. *Molecular Genetics and Genomics, 269*, 280–289.

Jo, Y.-K., Barker, R., Pfender, W., Warnke, S., Sim, S.-C., & Jung, G. (2008). Comparative analysis of multiple disease resistance in ryegrass and cereal crops. *Theoretical and Applied Genetics, 117*, 531–543.

Jones, E. S., Mahoney, N. L., Hayward, M. D., Armstead, I. P., Jones, J. G., Humphreys, M. O., et al. (2002). An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other Poaceae genomes. *Genome, 45*, 282–295.

Le Cunff, L., Garsmeur, O., Raboin, L. M., Pauquet, J., Telismart, H., Selvi, A., et al. (2008). Diploid/polyploid syntenic shuttle mapping and haplotype-specific chromosome walking toward a rust resistance gene (*Bru1*) in highly polyploid sugarcane (2n ~ 12x ~ 115). *Genetics, 180*, 649–660.

Lee, S.-K., Song, M.-Y., Seo, Y.-S., Kim, H.-K., Ko, S., Cao, P.-J., et al. (2009). Rice *Pi5*-mediated resistance to *Magnaporthe oryzae* requires the presence of two coiled-coil-nucleotide-binding-leucine-rich repeat genes. *Genetics, 181*, 1627–1638.

Lin, F., Liu, Y., Wang, L., Liu, X., & Pan, Q. (2007). A high-resolution map of the rice blast resistance gene *Pi15* constructed by sequence-ready markers. *Plant Breeding, 126*, 287–290.

Mammadov, J. A., Steffenson, B. J., & Saghai Maroof, M. A. (2005). High-resolution mapping of the barley leaf rust resistance gene *Rph5* using barley expressed sequence tags (ESTs) and synteny with rice. *Theoretical and Applied Genetics, 111*, 1651–1660.

Miura, Y., Ding, C., Ozaki, R., Hirata, M., Fujimori, M., Takahashi, W., et al. (2005). Development of EST-derived CAPS and AFLP markers linked to a gene for resistance to ryegrass blast (*Pyricularia* sp.) in Italian ryegrass (*Lolium multiflorum* Lam.). *Theoretical and Applied Genetics, 111*, 811–818.

Miura, Y., Hirata, M., & Fujimori, M. (2007). Mapping of EST-derived CAPS markers in Italian ryegrass (*Lolium multiflorum* Lam.). *Plant Breeding, 126*, 353–360.

Pan, Q.-H., Hu, Z.-D., Tanisaka, T., & Wang, L. (2003). Fine mapping of the blast resistance gene *Pi15*, linked to *Pii*, on rice chromosome 9. *Acta Botanica Sinica, 45*, 871–877.

Perovic, D., Stein, N., Zhang, H., Drescher, A., Prasad, M., Kota, R., et al. (2004). An integrated approach for comparative mapping in rice and barley with special reference to the Rph16 resistance locus. *Functional and Integrative Genomics, 4*, 74–83.

Reith, P. E., Prine, G. M., & Blount, A. R. (2003). Selection for gray leaf spot disease resistance in annual ryegrass. *Soil and Crop Science Society of Florida Proceedings, 62*, 69–73.

Sim, S., Chang, T., Curley, J., Warnke, S. E., Barker, R. E., & Jung, G. (2005). Chromosomal rearrangements differentiating the ryegrass genome from the Triticeae, oat, and rice

genomes using common heterologous RFLP probes. *Theoretical and Applied Genetics, 110*, 1011–1019.

Sim, S., Diesburg, K., Casler, M., & Jung, G. (2007). Mapping and comparative analysis of QTL for crown rust resistance in an Italian x perennial ryegrass population. *Phytopathology, 97*, 767–776.

Takahashi, W., Miura, Y., & Sasaki, T. (2009). A novel inoculation method for evaluation of grey leaf spot resistance in Italian ryegrass. *Journal of Plant Pathology, 91*, 171–176.

Tamura, K., Yonemaru, J., Hisano, H., Kanamori, H., King, J., King, I. P., et al. (2009). Development of intron-flanking EST markers for the *Lolium*/*Festuca* complex using rice genomic information. *Theoretical and Applied Genetics, 118*, 1549–1560.

The Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature, 408*, 796–815.

Tosa, Y., Uddin, W., Viji, G., Kang, S., & Mayama, S. (2007). Comparative genetic analysis of *Magnaporthe oryzae* isolates causing gray leaf spot of perennial ryegrass turf in the United States and Japan. *Plant Disease, 91*, 517–524.

Trevathan, L. E. (1982). Response of ryegrass plant introductions to artificial inoculation with *Pyricularia grisea* under greenhouse conditions. *Plant Disease, 66*, 696–697.

Viji, G., Wu, B., Kang, S., & Uddin, W. (2001). *Pyricularia grisea* causing gray leaf spot of perennial ryegrass turf: population structure and host specificity. *Plant Disease, 85*, 817–826.

Voorrips, R. E. (2002). MapChart: software for the graphical presentation of linkage maps and QTLs. *The Journal of Heredity, 93*, 77–78.

Wu, J., Maehara, T., Shimokawa, T., Yamamoto, S., Harada, C., Takazaki, Y., et al. (2002). A comprehensive rice transcript map containing 6591 expressed sequence tag sites. *The Plant Cell, 14*, 525–535.

Yi, G., Lee, S.-K., Hong, Y.-K., Cho, Y.-C., Nam, M.-H., Kim, S.-C., et al. (2004). Use of *Pi5(t)* markers in marker-assisted selection to screen for cultivars with resistance to *Magnaporthe grisea*. *Theoretical and Applied Genetics, 109*, 978–985.