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ORIGINAL PAPER

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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.)

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Abstract Ryegrass blast, also called gray leaf spot, is caused by the fungus *Pyricularia* sp. It is one of the most serious diseases of Italian ryegrass (*Lolium multiflorum* Lam.) in Japan. We analyzed segregation of resistance in an F_1 population from a cross between a resistant and a susceptible cultivar. The disease severity distribution in the F_1 population suggested that resistance was controlled by a major gene (*LmPi1*). Analysis of amplified fragment length polymorphisms with bulked segregant analysis identified several markers tightly linked to *LmPi1*. To identify other markers linked to *LmPi1*, we used expressed sequence tag-cleaved amplified polymorphic sequence (EST-CAPS) markers mapped in a reference population of Italian ryegrass. Of the 30 EST-CAPS markers screened, one marker, p56, flanking the *LmPi1* locus was found. The restriction pattern of p56 amplification showed a unique fragment corresponding to the resistant allele at the *LmPi1* locus. A linkage map constructed from the reference population showed that the *LmPi1* locus was located in linkage group 5 of Italian ryegrass. Genotype results obtained from resistant and susceptible cultivars indicate that the p56 marker is useful for introduction of the *LmPi1* gene into susceptible germplasm in order to develop ryegrass cultivars with enhanced resistance to ryegrass blast.

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Introduction

Ryegrass blast, caused by the fungus *Pyricularia* sp., is one of the most serious diseases of Italian ryegrass (*Lolium multiflorum* Lam.). It was first reported in Kochi Prefecture in 1969 and spread rapidly to southwestern Japan (Sumida et al. 2003). Italian ryegrass seedlings are attacked at the beginning of autumn, especially under hot, humid conditions. In cases of heavy infection, the disease often causes low forage quality and serious yield losses. However, no resistant cultivars of Italian ryegrass are found in Japan except for the new cultivar 'Sachiaoba' (Mizuno et al. 2003). For these reasons, improving the disease resistance of Italian ryegrass is one of the most important goals of breeding programs.

In recent years, molecular genetic maps using several types of markers, such as amplified fragment length polymorphisms (AFLPs), randomly amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), and simple sequence repeats (SSRs), have been developed in perennial ryegrass (*L. perenne* L.) (Bert et al. 1999; Jones et al. 2002a), Italian ryegrass (Hirata et al. 2000; Inoue et al. 2004), and a hybrid of these two species (Hayward et al. 1998). The linkage maps provide a powerful approach for identifying genetic markers linked to

major genes and quantitative trait loci (QTLs) associated with agronomically important characteristics, such as disease resistance. Studies of the genetics of disease resistance have made possible the identification of genetic markers linked to genes and QTLs for resistance to crown rust and gray leaf spot in perennial ryegrass (Dumsday et al. 2003; Curley et al. 2004) and for resistance to crown rust in Italian ryegrass (Fujimori et al. 2003). The identified markers can be used as tools for the introduction of the disease resistance into susceptible germplasm.

The use of amplified fragment length polymorphism (AFLP) markers permits the rapid detection of a large number of polymorphic loci. This makes AFLP analysis a powerful and reliable tool for the development of genetic linkage maps (Bert et al. 1999). Bulked segregant analysis (BSA; Michelmore et al. 1991) in combination with AFLP analysis is a proven method for identifying markers tightly linked to, or cosegregating with, genes underlying monogenic traits (Xu et al. 1999).

Ikeda et al. (2004) generated expressed sequence tags (ESTs) from cDNA libraries of Italian ryegrass and characterized them by comparing with sequences in a public database. The ESTs were then converted to cleaved amplified polymorphic sequence (CAPS) markers for genetic mapping. In all, 30 EST-derived CAPS markers could be mapped in a reference population of Italian ryegrass (our unpublished data). Seven linkage groups constructed by using the reference population (LG1–LG7) represent the seven haploid chromosomes of Italian ryegrass (M. Hirata et al., in preparation). Each of the linkage groups constructed in Italian ryegrass corresponds to one constructed in perennial ryegrass (Forster et al. 2001; Jones et al. 2002b; Inoue et al. 2004).

The aim of this study was to develop genetic markers linked tightly to resistance genes in order to facilitate breeding for resistance to ryegrass blast in Italian ryegrass.

Materials and methods

Plant materials

An F_1 two-way pseudo-testcross mapping population (Grattapaglia and Sederoff 1994) was generated from a cross between two heterozygous individuals: a resistant individual of cv. 'Sachiaoba' as the female parent and a susceptible individual of cv. 'Minamiaoba' as the male parent. The resistant parent was previously identified by inoculation of *Pyricularia* sp. These procedures were carried out at the Yamaguchi Prefecture Agricultural Experiment Station, Yamaguchi, Japan. For the inoculation test, the F_1 population consisting of 161 individuals was grown in 15 cm×5 cm×10 cm seedling cases, each containing six plants, in a greenhouse at the Forage Crop Research Institute, Japan Grassland Agriculture and Forage Seed Association (GAFSA), Tochigi, Japan. To determine the linkage group of genes of interest, we used a cytoplasmic male-sterile (CMS) mapping population as a reference population for Italian ryegrass, described by Fujimori et al. (2000).

Inoculation test and scoring of disease severity

A field isolate of *Pyricularia* sp. was obtained from a natural infection of Italian ryegrass at the Yamaguchi Prefecture Agricultural Experiment Station. A mycelial plug of the isolate was placed on oatmeal agar (50 g oatmeal, 20 g sucrose, and 35 g agar in 1 l of water) in a petri dish and incubated at 25°C in the dark for 2 weeks. Aerial mycelia were washed off by rubbing the mycelial surfaces with a brush. The colonies were exposed to near-ultraviolet light at 25°C for 10 days to induce sporulation. The conidial suspension was filtered through a metal mesh (aperture 300 μm) and adjusted to a final concentration of 5×10^4 spores/ml. Three-week-old plants (four- to five-leaf stage) were inoculated by spraying the suspension at 1 ml per seedling case. The inoculated plants were placed in the dark at 25°C and 100% humidity for 24 h and subsequently transferred to a growth chamber equipped with fluorescent lights of 100 $\mu E/m^2s^s$, where they were maintained on a 16-h light/8-h dark cycle at 25°C. Disease severity was scored at 7 days after inoculation using the scoring system described in Table 1.

DNA preparation for marker analyses

Total genomic DNA was extracted from the leaves of the inoculated plants by the modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). For the BSA, genomic DNAs from ten resistant (score 1) and ten susceptible (score 3) individuals of the F_1 population were mixed in equal proportions to construct the resistant and susceptible bulks, respectively.

Table 1 Scoring of phenotypes after inoculation with ryegrass blast fungus (*Pyricularia* sp.)

Phenotype	Score	Symptoms
Resistant	0	Plants with no symptoms on their leaves
	1	Plants with brown spotted or brown spindle-shaped lesions on their leaves
Susceptible	2	Plants with a few white or gray lesions on their leaves
	3	Plants with their leaves covered with lesions that have joined up

Disease severity was scored 7 days after inoculation

Bulked segregant analysis–amplified fragment length polymorphism analysis

AFLPs were generated by using the protocol of Vos et al. (1995) with modifications by Myburg et al. (2001). Selective amplification was carried out using various combinations of primers with three selective nucleotides (*Eco*RI + 3 and *Mse*I + 3). Using the two bulks and their parents, we analyzed a total of 512 AFLP primer combinations: 256 primer combinations from *Eco*RI + AXX/*Mse*I + AXX and 256 primer combinations from *Eco*RI + TXX/*Mse*I + TXX (X = A, C, G or T). The polymerase chain reaction (PCR) products were then separated by electrophoresis in 6% denaturing acrylamide gels in a LI-COR DNA analyzer (LI-COR, Lincoln, NE, USA), following the manufacturer's instructions.

Screening of EST-CAPS markers

To screen ESTs linked to resistance genes, we used 30 EST-CAPS markers mapped on the CMS population. The markers were initially used to amplify the genomic DNA from each of ten resistant and ten susceptible individuals of the two bulks and their parents. The PCR products were digested with appropriate endonucleases to detect restriction-site polymorphisms of the marker. The appearance of the PCR products and their digested products was checked by electrophoresis in 1.5% agarose gels followed by staining with ethidium bromide. The PCR amplifications with an identified EST-CAPS marker (see Results) were performed in a total volume of 10 µl containing 100 ng of genomic DNA, 0.2 µM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 U of *Taq* DNA polymerase. The reaction conditions for the amplification were initial denaturation at 94°C for 5 min; 40 cycles at 94°C for 2 min, 60°C for 1 min, and 72°C for 2 min; and final extension at 72°C for 5 min. The PCR products were subsequently digested with the endonuclease *Hha*I in a total volume of 10 µl containing 5 µl of the PCR products, to detect restriction-site polymorphisms of the EST-CAPS marker.

To confirm the linkage group of genes of interest, we used a resistance gene analog (RGA) marker from Italian ryegrass, RG036G04. The PCR was performed under conditions similar to those for the EST-CAPS marker except that the annealing step was 65°C for 1 min. Primers used for the PCR amplification were RG036G04-L1 (5′-GTGGAACCATGGAGTATGGG-3′) and RG036G04-R1 (5′-AAAAGTTGATGTCC-TTTAAGACACG-3′). The PCR products were digested with the endonuclease *Hae*III to detect the CAPS polymorphism.

Genotypic analysis and map construction

For genotypic analysis, each polymorphic marker was scored as a dominant marker (present:absent) in each individual of the F_1 population. Linkage analysis was carried out using the CP algorithm in JoinMap 3.0 (Van Ooijen and Voorrips 2001) on the segregation data obtained from the polymorphic markers. The segregating markers were grouped to construct the linkage groups at a LOD score of 4.0. The grouped markers were mapped at a LOD score of 3.0 and a recombination fraction of 0.25. The Kosambi mapping function was used to convert recombination frequencies to map distance (Kosambi 1944). The location of the resistance gene was determined from the marker order in the linkage group and the phenotypic data of the F_1 population by interval mapping in MapQTL (Van Ooijen and Maliepaard 1996). The position of the gene was estimated at the maximum LOD score with a 1-LOD support interval.

Cloning and sequencing of the EST marker

PCR products from genomic DNA amplifications with the single identified EST-CAPS marker were cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA). Nucleotide sequences of ten clones from each PCR product were determined with a Thermo Sequenase Cycle Sequencing Kit (Amersham Biosciences, Freiburg, Germany) and a LI-COR DNA analyzer, following the manufacturers' instructions. Sequence analyses were conducted with Sequencher 4.02 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Genotyping test of p56

To test the usefulness of marker p56, we checked genotypes showing p56 amplification and blast phenotypes in 'Sachiaoba'. To check blast phenotypes, we inoculated 59 'Sachiaoba' plants with a spore suspension of *Pyricularia* sp.

Results

Evaluation of blast resistance in the F_1 population

The F_1 population consisted of 161 plants. The frequency distribution of resistant and susceptible phenotypes is shown in Fig. 1. The resistant parent scored 1 after inoculation of *Pyricularia* sp. The F_1 plants were segregated into 82 resistant plants and 79 susceptible plants (Table 2). The segregating ratio was 1:1, indicating the presence of a single dominant gene for resistance to blast ($\chi^2 = 0.056$, $0.7 < P < 0.9$).

Screening of AFLP markers and map construction

To screen the markers efficiently, we used BSA with the AFLP analysis. From the first set of 256 AFLP primer combinations (*Eco*RI-AXX and *Mse*I-AXX), we

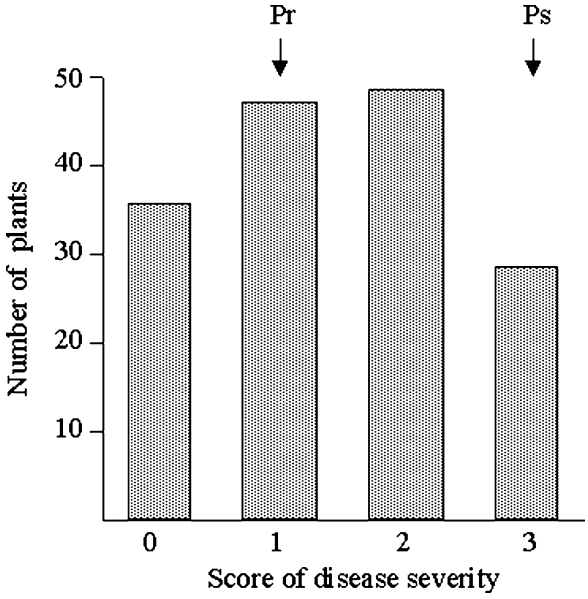

Fig. 1 Frequency distribution of disease severity to ryegrass blast in the F_1 population derived from a cross between resistant (Pr) and susceptible (Ps) parents. The parental means are indicated by *arrows*

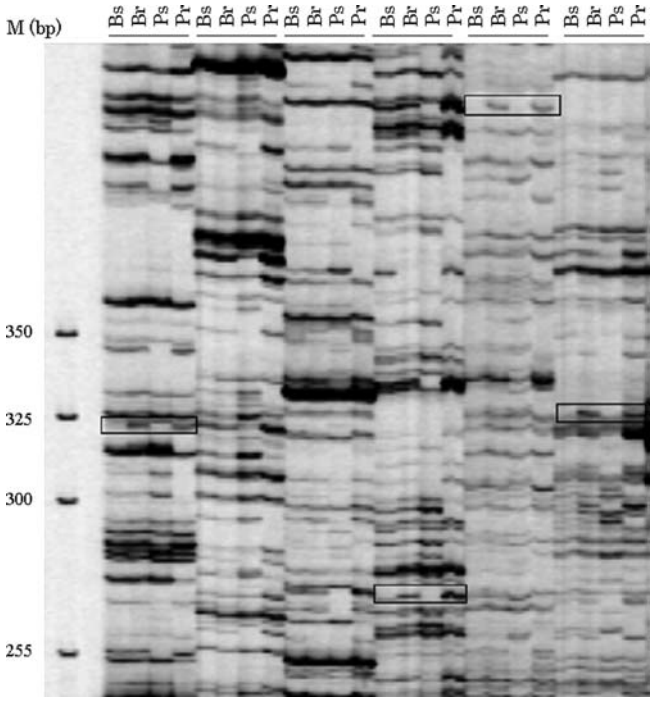

Fig. 2 Screening of *Eco*RI/*Mse*I primer combinations by bulked segregant analysis–amplified fragment length polymorphism (BSA–AFLP) analysis. Each set of four lanes shows, from right to left, bands amplified from resistant parent (Pr), susceptible parent (Ps), resistant bulk (Br), and susceptible bulk (Bs), using the same primer combination. Polymorphisms between the two bulks are *boxed*. *M* size marker

Table 2 Segregation of the p56 marker in the F_1 population

Phenotype	Score	No. of plants	Detection of 289-bp fragment		
			+[a]	−[a]	Unknown
Resistant	0	35	22	13	0
	1	47	39	8	0
Susceptible	2	50	15	34	1
	3	29	3	25	1
Total		161	79	80	2

Phenotype and lesion type of each plant were scored in accordance with Table 1
[a]Presence (+) or absence (−) of the 289-bp fragment

screened 12 combinations with polymorphic bands that appeared in both the resistant parent and resistant bulk but not in the susceptible parent and susceptible bulk (Fig. 2). We then screened 13 additional primer combinations from the second set of 256 combinations (*Eco*RI-TXX and *Mse*I-TXX). One clear polymorphic band was used from every primer combination. Thus, a total of 25 polymorphic bands (range, 150–700 bp) were used for map construction. To construct the linkage map, we investigated polymorphic loci of the 25 markers in the segregating F_1 population. All of the screened AFLP markers were heterozygous in the resistant parent and absent in the susceptible parent; the markers segregated in a 1:1 (present:absent) ratio in the F_1 population (Fig. 3a). Linkage analysis revealed that all 25 markers formed a single linkage group with a single resistance gene from the resistant parent (Fig. 4). The location of the resistance gene was detected on the linkage map, and the gene was designated *LmPi1* (Fig. 4).

Screening of EST markers and mapping

Among the 30 EST-CAPS markers mapped in the CMS population, one marker, p56, was found to be linked to blast resistance in the F_1 population. The restriction patterns of p56 amplification with *Hha*I in the F_1 population showed two short fragments of 289 and 185 bp (Fig. 3b). The 289-bp fragment, derived from the resistant parent, segregated in a 1:1 (present:absent) ratio in the F_1 population (Table 2). The 185-bp fragment, derived from both parents, segregated in a 3:1 (present:absent) ratio in the F_1 population (data not shown). The fragments differed in length by 104 bp. Linkage analysis revealed that p56 was mapped on the linkage map with *LmPi1* (Fig. 4). In addition, one RGA marker, RG036G04, flanking the p56 marker on the CMS linkage map, was also mapped on the linkage map with *LmPi1*. In our previous study, both p56 and RG036G04 were mapped to linkage group 5 (LG5) (our unpublished data). Therefore, the *LmPi1* locus is also located in LG5 in Italian ryegrass.

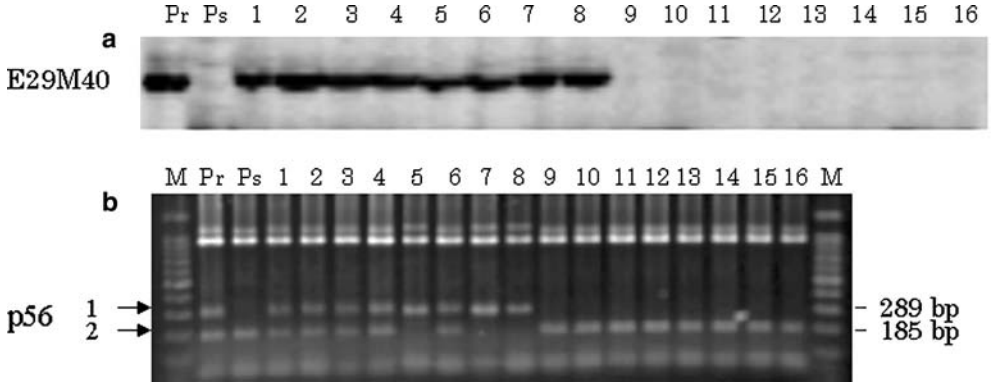

Fig. 3 Segregation pattern of markers linked tightly to the resistance gene *LmPi1* in the F_1 population. **a** AFLP marker E29M40, which was screened with the BSA method, segregated in a 1:1 (present:absent) ratio in the F_1 population. **b** Restriction pattern of EST marker p56. Polymerase chain reaction (PCR) products amplified with the primers p56F1 and p56R1 (boxed in Fig. 5) were digested with *Hha*I to detect cleaved amplified polymorphic sequence (CAPS) markers. One fragment of 289 bp (*arrow 1*) derived from the resistant parent segregated in a 1:1 (present:absent) ratio in the F_1 population. The other fragment of 185 bp (*arrow 2*) derived from both parents segregated in a 3:1 (present:absent) ratio in the F_1 population. The *first two lanes* are the resistant (Pr) and susceptible (Ps) parents, followed by eight resistant (score 1; *lanes 1* to *8*) and eight susceptible (score 3; *lanes 9 to 16*) individuals, which were used for the construction of the two bulks. Fragment sizes of the digests were calculated on the basis of sequence data (Fig. 5)

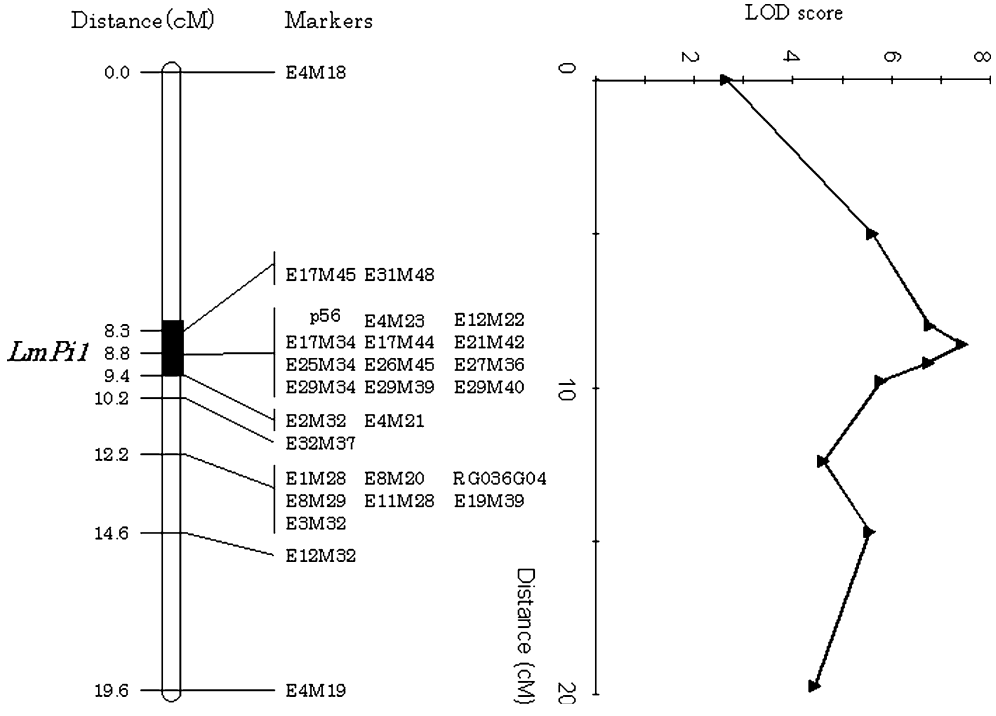

Fig. 4 Linkage map showing the position of the resistance gene *LmPi1*. The graph to the right of the linkage map indicates the position of *LmPi1* with a 1-LOD support interval. The markers are indicated to the *right of the map*. Two markers located on LG5 in the cytoplasmic male-sterile (CMS) population (data not shown); p56 and RG036G04 are an expressed sequence tag (EST)-CAPS and a resistance gene analog (RGA)-CAPS marker, respectively. The others are AFLP markers

To obtain detailed information on the p56 marker, we cloned and sequenced the PCR amplification products of genomic DNA of the resistant parent (Pr) with p56. Two fragments with different sequences were obtained as PCR products amplified with the primers p56F1 and p56R1 (boxed in Fig. 5). The total sizes of the two fragments were 1,243 and 1,139 bp (differing by 104 bp), corresponding to the resistant and susceptible alleles, respectively, at the *LmPi1* locus. Alignment of these two sequences showed one insert region of 104 bp (nucleotides 1093 to 1196) in the resistant allele. From the sequences obtained, one PCR primer, p56F2 (Fig. 5), was newly designated for the genotyping test described in the next section. With the p56F2 and p56R1 primers, a 516-bp fragment was amplified from the resistant allele, and a 412-bp fragment from the susceptible allele (again differing by 104 bp).

Genotyping test of p56

To test the usefulness of marker p56, we checked genotypes and blast phenotypes in Sachiaoba. Seven

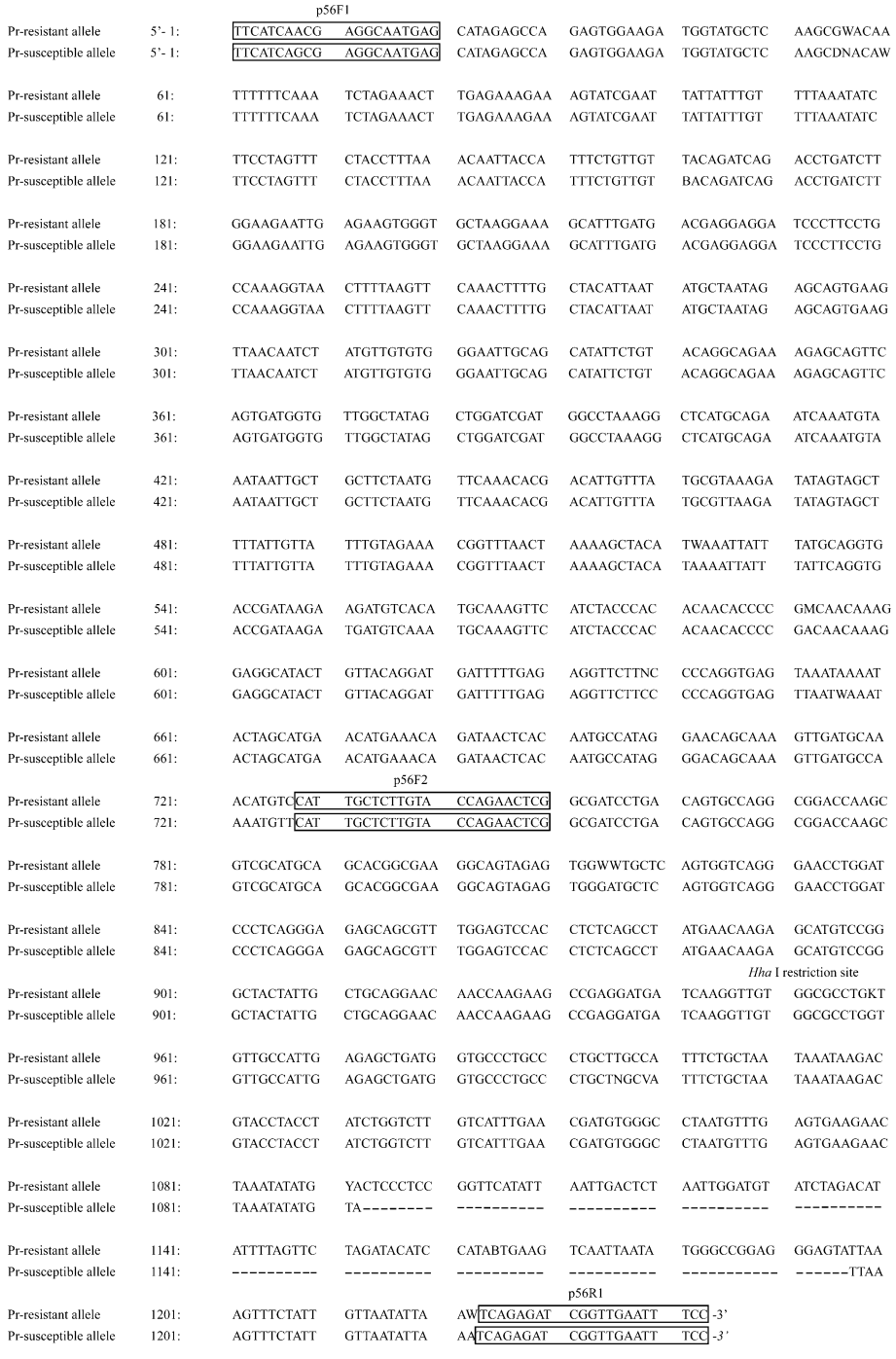

Fig. 5 Nucleotide sequences of the p56 locus. The p56 PCR product amplified from genomic DNA of the resistant parent was cloned and sequenced. Two sequence clones with different total sizes, 1,243 and 1,139 bp, were obtained (GenBank accession numbers AB195690 and AB195691, respectively). *Underscores* indicate the insert region in the resistant allele (nucleotides 1093–1196). Two primers (p56F1 and p56R1) designed for generation of the EST and one primer (p56F2) newly designed for the genotyping test are *boxed*. The *Hha*I restriction site for the detection of CAPS markers is present in the sequence of each allele (nucleotides 952–955, *shaded*)

(39%) of 18 'Sachiaoba' plants with the 516-bp fragment showed resistance, and 38 (93%) of 41 plants without the fragment showed susceptibility (Table 3).

Discussion

We constructed a genetic linkage map of the region incorporating the *LmPi1* locus in the resistant parent. Primer combinations for AFLPs were screened by BSA. The combined BSA–AFLP technique proved to be effective for the identification of the region harboring the gene of interest.

Among ESTs generated from cDNA libraries of Italian ryegrass, we found marker p56 flanking *LmPi1*. The CAPS profile of PCR products amplified with p56 showed two fragments corresponding to the resistant and susceptible alleles at the *LmPi1* locus (Fig. 3b). The p56 marker was useful for the genotyping test, because it is a co-dominant marker and easy to assay.

A BLASTX search of amino acid sequences in the NCBI database (http://www.ncbi.nlm.nih.gov) showed

Table 3 Relationship between p56 genotypes and blast phenotypes in 'Sachiaoba'

Phenotype	Score	No. of plants	Detection of 516-bp fragment	
			+[a]	−[a]
Resistant	0	0		
	1	10	7	3
Susceptible	2	10	4	6
	3	39	7	32
Total		59	18	41

Phenotype and lesion type of each plant were scored in accordance with Table 1

[a]Presence (+) or absence (−) of the 516-bp fragment corresponding to the resistant allele at the *LmPil* locus

high similarity between p56 cDNA and parts of *HvAS1* and *HvAS2*, which encode asparagine synthetase in barley (Møller et al. 2003).

Disease severity in the F_1 population segregated in a 1:1 (resistant:susceptible) ratio, and the linkage analysis revealed all of the screened AFLP markers formed a single linkage group. The results suggest that the resistance is controlled by one major gene. Although the p56 marker flanks *LmPi1*, the phenotypes of disease severity were inconsistent with the p56 genotypes in some plants of the F_1 population (Table 2). This result differs from that obtained for *Pc1*, a resistance gene for crown rust, in the consistency of flanking markers with the genotype (Fujimori et al. 2003). For example, 61 F_1 plants with the 289-bp fragment showed resistance, whereas 18 with the 289-bp fragment were judged susceptible. This discrepancy between phenotype and genotype probably occurred because the expression of resistance was sensitive to environmental factors, though this might be also caused, in a few of the plants, by genetic recombination event between p56 and *LmPi1* locus. We support this explanation as follows: (1) almost all the 18 susceptible plants with the 289-bp fragment showed a score of 2, indicating moderate susceptibility, indicating that the infection was not severe (Table 2). (2) Most plants (93%) without the 516-bp fragment showed susceptibility, whereas among plants with the fragment, 39% showed resistance and 61% showed susceptibility (Table 3). Although some resistant individuals scored 0 in the F_1 population (Table 2), all 10 resistant 'Sachiaoba' plants had a score of 1 (Table 3). We suggest that some resistant individuals in the F_1 population are due to additional genetic effects, which enhance resistance, derived from the susceptible parent, as Dumsday et al. (2003) reported in perennial ryegrass. The susceptible parent was heterozygous for two alleles (185 bp and null) at the p56 locus (Fig. 3b); however, we did not obtain sufficient evidence to prove such effects. Mizuno et al. (2003) reported that resistant 'Sachiaoba' plants showed a blast phenotype score of either 0 or 1 in an artificial inoculation test. Therefore, it is also possible that this difference results from a difference in the particular inoculation conditions.

When the various results are taken into account, it is likely that the *LmPi1* gene leads to moderate resistance, in which symptoms of infected plants are characterized by spindle-shaped lesions that are sensitive to environmental factors, as seen in partial resistance to rice blast (Tabien et al. 2002).

Italian ryegrass is a self-incompatible, out-crossing plant. Genetic diversity must be maintained within each cultivar in order to prevent inbreeding depression. Therefore, many genotypes are present in each cultivar. The selection of elite genotypes to use as parents of synthetic cultivars requires that superior genotypes be identified on the basis of their phenotype (Hayward et al. 1994). Of the ten resistant 'Sachiaoba' plants, six were heterozygous, having both fragments, corresponding to the two alleles, at *LmPi1* (Fig. 6). The resistance could be fixed genetically by the selection of individuals homozygous for the resistant allele using the p56 co-dominant marker. In Italian ryegrass, elite parents are necessary to breed synthetic cultivars. Crosses with individuals homozygous for the resistant allele could be used to introgress the gene into each elite parent. During varietal development, although segregation of the resistant allele would be observed, the introgression would have a sufficient effect to enhance resistance to ryegrass blast.

The resistant allele was detected with high frequency (approximately 30%) in 'Sachiaoba' (Table 3), but the allele was not detected in the susceptible cultivars 'Minamiaoba', 'Waseyutaka', or 'Shiwasuaoba' (data not shown). In addition, since the *LmPi1* locus was mapped to LG5 in *Lolium*, it is different from the two QTLs for gray leaf spot resistance in perennial ryegrass (Curley et al. 2004). Our results indicate that the p56 marker could be used for introgression of the *LmPi1* resistance allele into susceptible germplasm in Italian ryegrass, as well as into that in perennial ryegrass, which can be crossed with Italian ryegrass.

In summary, we developed EST and AFLP markers flanking *LmPi1*, a gene for resistance to ryegrass blast.

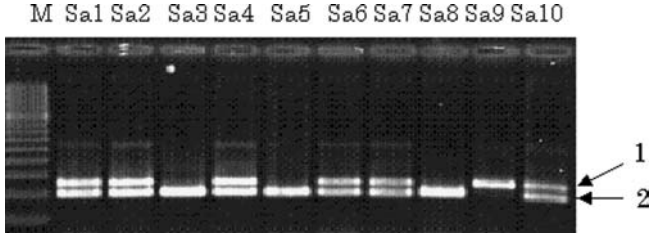

Fig. 6 Genotypic analysis of ten resistant 'Sachiaoba' plants (Sa1–10) using the p56 marker. Each PCR product amplified with the primers p56F2 and p56R1 (boxed in Fig. 5) has either one or both of the fragments (516 and 412 bp) corresponding to the resistant (*arrow 1*) and susceptible (*arrow 2*) alleles at the *LmPi1* locus. These ten plants showed resistance (score 1) on the inoculation test (Table 3). The sizes of the two fragments were estimated from the sequence data (Fig. 5). *M* 200-bp DNA ladder marker

To identify markers linked more tightly to the target gene, we need to analyze larger populations in order to increase the chance of detecting rare recombination events between each marker and the *LmPi1* locus. Having several AFLP markers linked to *LmPi1* would lay the groundwork for further fine mapping. The development of additional AFLP markers linked to each of the alleles at the *LmPi1* locus is also necessary for the construction of a more detailed map with the gene.

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