Chapter 8

Genomic Approaches to Developing Molecular Markers Linked to Grey Leaf Spot Resistance Loci in Ryegrasses

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Additional information is available at the end of the chapter

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Abstract

Ryegrass grey leaf spot (GLS), which is also called ryegrass blast, is caused by *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*). It is a serious disease in ryegrasses including perennial ryegrass (*Lolium perenne* L.) and Italian ryegrass (*L. multiflorum* Lam.). Heavily infected young seedlings die within days, and grass stands can be seriously damaged by the disease. Thus, the development of GLS-resistant cultivars has become one of the most important objectives in ryegrass breeding. This chapter provides an overview of the current information regarding molecular marker development in the breeding of GLS-resistant ryegrass cultivars. It focuses on the pathology of GLS, heritability and breeding of GLS resistance, and development of molecular markers linked to a major ryegrass GLS resistance gene.

Keywords: Comparative genomics, Forage grasses, *Lolium*, Molecular breeding, Resistance gene

1. Introduction

Perennial ryegrass (*Lolium perenne* L.) and Italian ryegrass (*L. multiflorum* Lam.) are taxonomically related cool-season grasses and are the most cultivated species in the genus *Lolium* in temperate regions. Perennial ryegrass is mainly used as turf and for grazing, whereas Italian ryegrass is primarily grown for hay and silage.

Ryegrass grey leaf spot (GLS), also called ryegrass blast, is a major disease of perennial ryegrass in the United States [1] and Italian ryegrass in Japan [2-4]. Rice blast and ryegrass GLS are caused by a common pathogenic fungal species, *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) [5]. Severely infected young seedlings die within days, and infected ryegrass stands can cause widespread damage and losses.

Effective GLS management strategies in ryegrass turf include the use of chemical fungicides. However, the high cost of fungicide application is an important limitation for growers managing large turf areas [1]. Additionally, overreliance on fungicides may lead to the development of fungicide-resistant fungal strains [6] and adversely affect nontarget organisms [7], ultimately resulting in adverse ecological consequences. Furthermore, the bioaccumulation of fungicides in domesticated animals (e.g., cattle) and its possible effects on the safety of dairy products are potential problems associated with fungicide use. There are currently no labeled fungicides effective against GLS in the United States [8] and Japan [3]. Therefore, there are a limited number of disease management options.

In this context, cultural management practices such as minimizing drought stress, reducing leaf wetness, avoiding excessive applications of nitrogen, and soil compaction may help to reduce disease severity [9]. However, these practices often do not work efficiently because the disease develops rapidly in susceptible ryegrass cultivars [1]. Thus, integrated management including the use of GLS-resistant cultivars is necessary to establish productive ryegrass cultural systems.

This chapter focuses on ryegrass breeding for the development of GLS-resistant cultivars. The main topics covered herein include pathology of ryegrass GLS, diversity and conventional breeding of GLS-resistant ryegrasses, and development of molecular markers linked to GLS resistance loci.

2. Pathology of ryegrass GLS

2.1. Taxonomy

In 2002, the causal pathogen of GLS of grass species including ryegrasses (*Lolium* species) and rice blast was identified as a new species, *M. oryzae* (anamorph *P. oryzae*). This new species was considered distinct from *Magnaporthe grisea* (anamorph *P. grisea*), which is associated with the grass genus *Digitaria*. The distinction was based on phylogenetic analyses and laboratory mating experiments that showed the two species were not interfertile, although there were no morphological differences between them [5].

In this chapter, the term "*M. oryzae*" is used. However, it is important to note that a formal change from *M. grisea* to *M. oryzae* has not yet occurred. A proposal for changing the name based on the results of [5] is allowed under the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code). A proposal will be submitted to and discussed by the Nomenclature Committee for Fungi of the International Association for Plant Taxonomy [10]. A final decision on a name change will be made during the Nomenclature Session of the International Botanical Congress in 2017 [10].

2.2. Population structure and host specificity

Analysis of genomic DNA using molecular markers is the most powerful method for determining the population structures of the *Magnaporthe* species. Repetitive DNA elements such

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as transposons and retrotransposons are often used to generate probes for Southern blotting experiments during DNA fingerprinting [11-15]. This is because of the diversity in copy numbers of elements and the richness of polymorphisms around, within, or among the elements, which might be caused by base substitutions or insertions and deletions. The use of internal transcribed spacer regions between ribosomal DNAs as probes for DNA fingerprinting is also common [12, 13]. Similarly, the internal transcribed spacer regions have been sequenced for population structure analyses [14]. Table 1 lists the repetitive sequences that have been used to analyze the population structure of *Magnaporthe* species associated with grass weeds, turf grasses, and/or forage grasses in addition to major crops such as rice and wheat (*Triticum aestivum*) [11-15].

Target	Feature	Reference	
		Sequence	Result of application
MAGGY	Retrotransposon	[16]	[11, 12]
MGLR-3	Retrotransposon	[17]	[13]
MGR583	Retrotransposon	[18, 19]	[12, 14]
MGR586	Transposon	[18, 20]	[11-14]
Pot2	Transposon	[21]	[11-15]
rDNA	Ribosomal DNA	[22, 23]	[12-14]
RETRO5	Retroelement	[24]	[12]

Table 1. Repetitive DNA sequences for DNA fingerprinting of *Magnaporthe* species associated with grass weeds, turf grasses, and/or forage grasses

In some cases, probes derived from these repetitive DNA sequences cannot clearly distinguish between isolates from different hosts. Restriction fragment length polymorphisms (RFLPs) with single-copy probes derived from long insert-cosmid clones (35–40 kb) are appropriate for the initial comparison of poorly characterized isolates from different hosts [12]. In addition to the repetitive DNA sequences, amplified fragment length polymorphisms (AFLPs) can produce many markers and provide a higher resolution for population structure analyses even within the same *Magnaporthe* lineage [25, 26].

Population structures can be determined in dendrograms constructed by analyzing genetic distances among isolates, which are reflected by differences in the banding patterns obtained during molecular marker analyses. Dendrograms of ryegrass isolates have often revealed genetic similarities between ryegrass isolates and isolates from wheat [12-14, 25] and tall fescue (*Schedonorus arundinaceus*) [12, 25].

In artificial inoculation conditions, isolates from ryegrasses, wheat, and tall fescue can cause serious infections in all hosts. Table 2 summarizes the data from six studies on the pathogenicity of *Magnaporthe* isolates from ryegrasses, tall fescue, wheat, rice, and/or crabgrass [13-15, 25, 27, 28]. The isolates from ryegrasses are generally avirulent, but can be virulent to rice [13,

14]. Conversely, although the rice isolates are thought to be unable to cause serious infections in ryegrasses [13, 14], they are occasionally highly virulent to the plant species [27]. The wheat isolates are avirulent to rice [14, 27], although the rice isolates are virulent to wheat [13, 27]. Some isolates from crabgrass (*Digitariasanguinalis*) are virulent to tall fescue [25] and ryegrasses [25, 28], highly virulent to Italian ryegrass [25] but are avirulent to wheat [14, 25]. Additionally, isolates from perennial ryegrass, wheat, and rice can infect crabgrass, but these are generally not highly virulent to crabgrass [14]. Many isolates from tall fescue are avirulent to crabgrass [25].

Original host[a]	**Inoculated host**[b]						**Reference**
	PR	IR	TF	W	R	CG	
Perennial ryegrass (PR)	++		++	++	-		[13]
	++	++		+-	+-	+-	[14]
							[15]
							[25]
							[27]
							[28]
Italian ryegrass (IR)							[13]
							[14]
	++	++	++				[15]
							[25]
							[27]
		++					[28]
Tall fescue (TF)							[13]
							[14]
							[15]
	++	++	++	++-		-	[25]
							[27]
							[28]
Wheat (W)							[13]
	++	++		++	-	+	[14]
							[15]
	++	++	++	++-		-	[25]
	++	++	++	++	-	-	[27]
							[28]
Rice (R)	-		-	+	++		[13]

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Original host[a]	Inoculated host[b]						Reference
	PR	IR	TF	W	R	CG	
	-	+		-	++	+-	[14]
							[15]
							[25]
	++-	++	++	+-	++	-	[27]
							[28]
Crabgrass (CG)							[13]
	-	-		-	-	++	[14]
							[15]
	+-	++-	+-	-		++	[25]
							[27]
		+-					[28]

[a]According to [5], the crabgrass isolate might be *M. grisea* and the others might be *M. oryzae*.

[b]+: virulent; ++: highly virulent; -: avirulent; +-: virulent but sometimes fails to infect; ++-: highly virulent but sometimes fails to infect.

Table 2. Pathogenicity and host specificity of *Magnaporthe* species during artificial inoculations

In addition to the isolates listed in Table 2, during artificial inoculations, ryegrasses are highly susceptible to isolates from weeping lovegrass (*Eragrostis curvula*) [25], and susceptible to isolates from finger millet (*Eleusine coracana*) [14], St. Augstinegrass (*Stenotaphrum secundatum*) [25, 28], Alexandergrass (*Brachiaria plantaginea*) [27], Pennsylvania smartweed (*Polygonum pensylvanicum*) [28], and soybean (*Glycine max*) [28].

The cross-infections observed during artificial inoculations suggest that "opportunistic" cross-infections may occur in nature [12]. However, population structure analyses based on molecular marker analyses have revealed that although there are genetic differences even in isolates from the same host species, the population structures are generally associated with host differences. This indicates that the host species is a major selective factor for constructing isolate populations, and cross-infections among hosts might not be detectable in nature [25]. Nevertheless, ryegrasses might be infected by tall fescue isolates because these hosts are congeneric [29-31]. Therefore, the isolates from ryegrasses and tall fescue are genetically quite similar [12] or belong to the same lineage in some cases [25]. Additionally, wheat isolates are genetically similar to the ryegrass and tall fescue isolates, and all can cause serious infections in wheat, ryegrass, and tall fescue in artificial inoculation conditions (Table 2). However, the wheat isolates are clearly genetically distinct [12, 25]. This might explain why no epidemics of wheat blast caused by the cross-infection of ryegrass isolates and *vice versa*, have been reported [12]. This may also be the case for weeping lovegrass, in which there are genetic similarities and cross-pathogenicity among hosts [25]. Therefore, isolates from wheat and/or weeping

lovegrass may be progenitors of isolates of ryegrasses and tall fescue rather than being directly responsible for GLS in ryegrasses or tall fescue [12, 25].

3. Diversity and conventional breeding of GLS-resistant ryegrasses

3.1. Heritability and genetic effects of GLS resistance

To breed for GLS-resistant ryegrasses, genetic material conferring resistance to GLS must be identified. For this purpose, researchers have investigated the diversity among resistant phenotypes [32-37]. Although most commercial cultivars and experimental lines are susceptible to GLS, some resistant genotypes have been identified in cultivars and experimental lines of Italian ryegrass [32-34] and perennial ryegrass [32, 35, 36]. Perennial ryegrass might be the more GLS-resistant species as resistant phenotypes are more common than in Italian ryegrass [32]. Additionally, in Italian ryegrass, tetraploid lines were slightly more resistant than diploid lines [33]. This is also the case in perennial ryegrass.

The diversity in GLS resistance has encouraged breeders to continue to attempt to generate GLS-resistant cultivars. In outcrossing plants like ryegrasses, a phenotypic recurrent selection is often used to improve important agronomic traits mainly controlled by genes with an additive effect. The effects of recurrent selection have been observed in Italian ryegrass and GLS-resistant experimental lines have been selected [33, 34], indicating that GLS resistance can be conferred using recurrent selection and is possibly controlled by additive gene effects.

Recurrent selection has also been effective in perennial ryegrass [35, 37]. The broad-sense heritability estimates were very high at 0.92 [35] and 0.95 [37] without any interaction between cultivar and environment. These results suggest that GLS resistance is controlled by strong genetic effects [35, 37]. Further, the phenotypic means of populations composed of selected individuals were dramatically shifted toward the selected GLS resistance. Therefore, GLS resistance was thought to be controlled by a few genes and the frequency of the genes in the selected population rapidly increased during selection cycles [35, 37]. However, much of the additive gene effects cannot be obtained with only one cycle of selection. The genetic gain during the second selection cycle was higher than that of the first cycle in the GLS-resistant phenotype [37].

Narrow-sense heritability and the number of genes having additive effects in GLS resistance are among the most important considerations for breeders because the additive gene effects actually reflect the effect of selection. However, these have not been estimated by the studies mentioned above. Diallel cross analysis is a way to determine narrow-sense heritability, number of genes having additive effects, general combining ability (GCA), and specific combining ability (SCA) of parent plants [38-40]. In perennial ryegrass, diallel crosses involving six and eight parents have been analyzed to investigate the GCA, SCA, narrow-sense heritability, and the number of genes involved in GLS resistance [36]. The GCA and SCA were highly significant and accounted for 80–86% and 7–17% of the total genotypic variance, respectively [36]. The significant SCA values suggest that dominant genes or those that interact

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with related genes must have been involved in the parents. The considerably higher GCA values also suggest that GLS resistance is mainly controlled by additive gene effects as previously concluded [35, 37]. The narrow-sense heritability and number of genes having additive effects were estimated to range from 0.57 to 0.76 and 2.1 to 4.4, respectively [36]. Results of the diallel cross analysis were consistent with those of the abovementioned studies [35, 37]. Thus, phenotypic recurrent selection was very effective in improving GLS resistance in ryegrasses. Because of the quantitative additive gene effects, resistant phenotypes in the selected lines would be durable although the possibility that some genes with additive effects might be more important for GLS resistance cannot be ruled out. The gene most responsible for GLS resistance may be inherited by the next generation and act as a quasi-qualitative major partial resistance gene.

3.2. Available GLS-resistant ryegrass cultivars

Although almost all of the commercially available cultivars released before 2004 were very susceptible to GLS [9], many GLS-resistant perennial ryegrass cultivars are currently available in the United States [41]. In contrast, GLS-resistant Italian ryegrass cultivars are very rare, but the diploid cultivar "Sachiaoba" [2] in Japan and the tetraploid cultivar "Jumbo" [42] in the United States have been registered as GLS-resistant in 1998 and 2000, respectively. However, an article published in 2010 reported a lack of annual ryegrass cultivars resistant to *P. grisea* in the United States, which led to the belief that GLS resistance in Italian ryegrass was insufficient [8]. All of these resistant cultivars have partial resistance, and no completely resistant perennial ryegrass or Italian ryegrass cultivars have been released. Therefore, continued breeding for GLS resistance is necessary.

4. Development of molecular markers linked to GLS resistance loci

In addition to conventional breeding, researchers have used molecular breeding techniques involving molecular markers to develop disease-resistant cultivars of major crops. Developing resistance to rice blast is a major focus among plant pathologists, and many molecular markers relevant for the breeding of rice blast-resistant cultivars have been reported [43, 44]. Regarding ryegrasses, research groups in the United States and Japan have found genetic loci for GLS resistance and have identified molecular markers linked to the resistance loci in an Italian × perennial ryegrass hybrid [45-47] and Italian ryegrass [4, 48, 49].

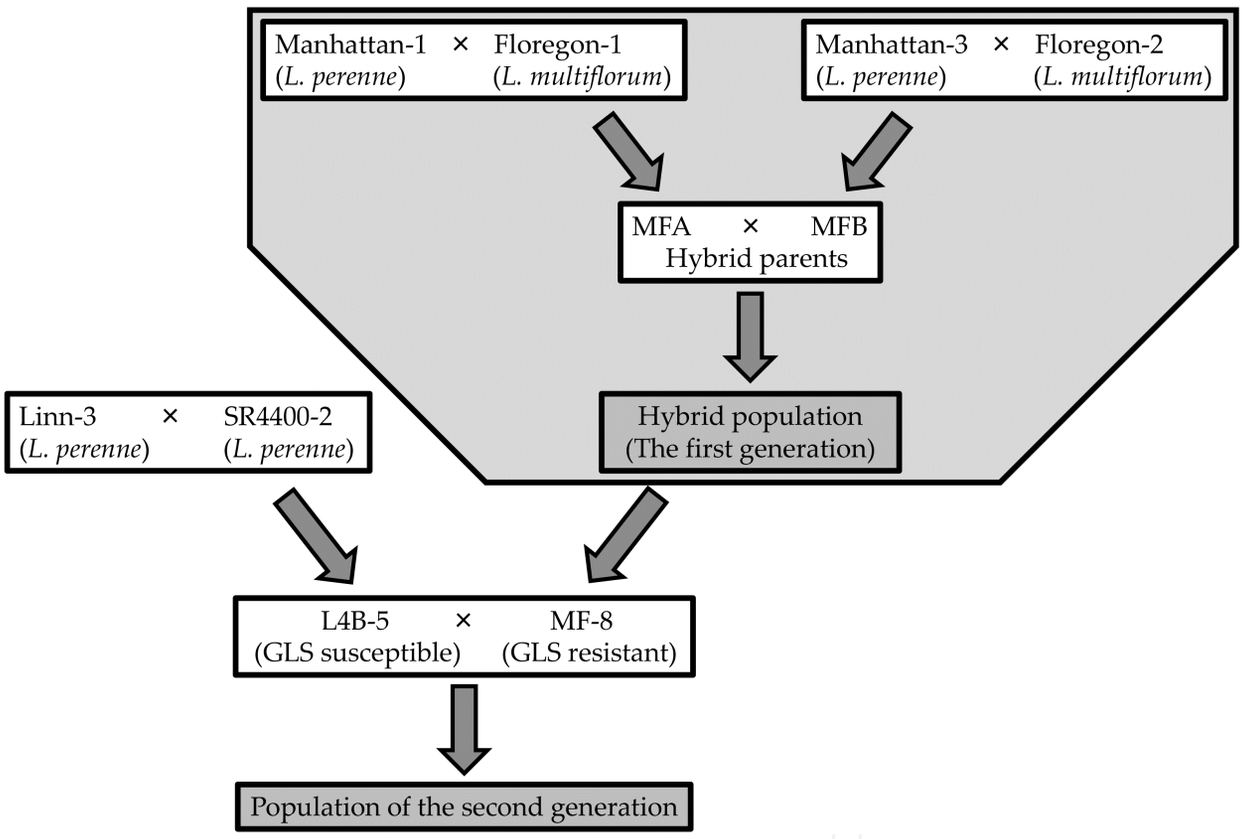
4.1. Molecular marker development for GLS resistance in an Italian × perennial ryegrass hybrid

4.1.1. Mapping population derived from Italian × perennial ryegrass hybrid parents

A research group in the United States developed a mapping population consisting of progeny individuals derived from a cross between Italian × perennial ryegrass hybrid heterozygous parental clones MFA and MFB [45, 46]. The parental clones were obtained in separate crosses

between two different grandparental clones of the perennial ryegrass cultivar "Manhattan" and two different grandparental clones of the Italian ryegrass cultivar "Floregon" (Figure 1). A second-generation mapping population [47] was then developed. The GLS-resistant MF-8 was selected from the first mapping population and crossed with the GLS-susceptible L4B-5 obtained in a cross between a clonal individual of the forage-type perennial ryegrass cultivar "Linn" and a clonal individual of the turf-type perennial ryegrass cultivar "SR4400" (Figure 1).

The grandparental clones and parents of the mapping populations could be asexually maintained and propagated. However, the grandparental clones of the Italian ryegrass cultivar "Floregon" could not be maintained because of the annuality of this species [46]. Similarly, the two mapping populations exhibited perenniality, with each individual capable of being clonally maintained and propagated to produce clonal replicates for multiple experiments [45-47].



Modified and combined from [45, 47].

Figure 1. Diagram of crosses for the development of mapping populations over two generations.

4.1.2. Phenotyping of GLS resistance/susceptibility in an Italian × perennial ryegrass hybrid

In two previous studies, seven perennial ryegrass isolates obtained from diseased perennial ryegrass fairways and one rice lab strain capable of infecting rice and ryegrass were used in inoculation tests of the parents and grandparents of the first-generation mapping population

[45, 46]. Of these, one of the perennial ryegrass isolates, GG9 [45, 46], and the rice lab strain 6082 [46] were chosen and used for quantitative trait locus (QTL) analyses because of their high sporulation capacity in culture and high virulence [46].

Because the mapping population could be asexually propagated, two inoculation experiments were independently conducted with three or four replicates in one study [45] and four inoculation experiments were completed with four replicates in another [46]. The inoculation experiments were conducted in growth chambers or mist chambers. The GLS resistance/susceptibility phenotypes of the mapping population were scored based on the rating scale provided in Table 3. In one study, lesion numbers and proportions of resistant lesions were recorded because inoculated individuals often had both resistant and susceptible lesions [45]. In another study, the youngest leaves of each plant were used because symptoms were most severe in these leaves when mixed lesion types occurred on the same plant [46].

Phenotype	Score	Symptoms
Resistant	0	No visible symptoms
	1	Dark brown, non-sporulating 2–3 mm lesions
	2	Dark brown, non-sporulating lesions with a small central necrotic area
Susceptible	3	Circular or small diamond-shaped lesions with prominent dark brown borders and grey or white central sporulating areas
	4	Large, expanding, completely unbordered sporulating lesions, often with chlorotic halos

From [45, 46]

Table 3. Rating scale for grey leaf spot severity in an Italian × perennial ryegrass hybrid

Similar disease reactions and phenotypic segregation patterns were observed in the mapping population inoculated with the perennial ryegrass isolate GG9, but the results were different from those of experiments involving the rice lab strain 6082 [45, 46]. In another study, where the second-generation mapping population was developed, two perennial ryegrass isolates, including GG9, were used. Each isolate was included in two experiments involving four clonal replicates of the mapping population [47]. Similar disease reactions and phenotype segregation patterns were reported for the second-generation mapping population [47]. No symptom-free individuals were observed throughout these studies [45-47]. The results from these three independent studies indicate the existence of different factors regulating the host–pathogen interactions involving perennial ryegrass isolates and a rice lab strain. This is relevant for determining the *Magnaporthe* species population structure based on the host specificities mentioned in Section 2.2.

Similar to the studies mentioned in Section 3.1, the broad-sense heritability for GLS-resistant/susceptible phenotypes was high in the experiments with the perennial ryegrass isolates with

values of 0.895–0.932 [46] and 0.88 [47]. These results indicate that the GLS resistance of the mapping populations was mainly controlled by genetic effects.

4.1.3. Detection and mapping of GLS resistance loci in an Italian × perennial ryegrass hybrid

Phenotypic data related to GLS resistance/susceptibility have been analyzed to identify GLS resistance loci in mapping populations [45-47]. A genetic linkage map was constructed using RFLP, AFLP, simple sequence repeat (SSR), and random amplified polymorphic DNA markers [45-47]. Isozyme and morphological markers have also been used [47]. The genetic linkage map from [46] was described in detail in another study [50]. Probes for RFLP markers were derived from other well-studied crops such as barley, oat, and rice so that synteny-based comparative studies among different plant species could be conducted with the constructed map [51]. In these studies, two sets of genetic linkage maps composed of seven linkage groups (LGs) derived from both parents were constructed using a two-way pseudo-testcross mapping strategy [52].

In one study, although results were not shown in detail, QTL analysis detected two genomic regions for GLS resistance against the perennial ryegrass isolate GG9 [45]. The identified QTLs were on LG 2 (for proportions of resistant lesions) and LG 4 (for lesion numbers) [45]. The logarithm of odds (LOD) obtained by interval mapping [53] ranged from about 2.0 to 6.0, although the LOD scores were not always significant [45]. In addition to these QTL regions, some regions were noted on LGs 1, 3, and 5, but these were not consistently detected [45].

Isolate GG9 and rice lab strain 6082 were used to inoculate the same population used in [46]. Significant QTLs were detected on LGs 3 and 6 and LGs 2 and 4 for GG9 and 6082, respectively, indicating that GLS resistance against the different isolates was controlled by different genetic effects [46]. Percentages of phenotypic variance explained by the QTLs at the highest LOD scores were 20.1–37.9% for LG 3 and 9.2–10.7% for LG 6 for resistance against GG9, and 8.9–10.0% for LG 2, and 9.9% for LG 4 for resistance against 6082 [46]. The QTL differences between the two isolates were expected because the disease reaction and phenotype segregation of the mapping population were different between the isolates [46] (see Section 4.1.2). Nevertheless, significant QTLs were detected on LGs 2 and 4 for GLS resistance against GG9 and 6082 [45, 46]. However, the QTL relationships between the two studies cannot be confirmed by their location on genetic linkage maps because no marker information linked to the QTLs was provided in [45]. Additionally, the locations of the QTLs for GLS resistance against GG9 differed between the two studies even though the same mapping population was used. This inconsistency was not explained [46], but differences in the phenotype segregation of the mapping population during the GG9 inoculation experiments may have been a factor. That is, in one study, the phenotypic distribution of the mapping population seemed skewed toward resistance in the first experiment, but there was a trend toward susceptibility in the second experiment [45]. In the other study, the patterns of phenotype segregation in the mapping population were consistent and showed a trend toward susceptibility over three experiments [46]. These differences in the same mapping population may have been caused by unknown environmental factors that affected the expression of certain genes in the plant hosts and/or pathogens. Irrespective of the high broad-sense heritability, the values for the phenotypic

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variance explained by the QTLs are considered quite low, indicating there might be undetected genetic factors with minor effects on GLS resistance/susceptibility [46].

Although the QTLs for GLS resistance may be unstable and sometimes adversely influenced by environmental factors, the most significant QTL detected on LG 3 [46] might be detectable in the second generation mapping population developed in [47] (Figure 1). The percentage of phenotypic variance explained by the QTL on LG 3 at the highest LOD scores was 9.3–10.8%. Although this is lower than the values reported in [46], it suggests that the QTL is functional in a population with a different genetic background, which is promising for breeding programs focused on developing GLS-resistant ryegrass. However, the nearest RFLP marker (CDO460) closely linked to the major QTL on LG 3 [46] was not mapped in [47]. Therefore, it is necessary to confirm whether the QTL detected in [47] really corresponds to the QTL detected in [46].

4.2. Molecular marker development for GLS resistance in Italian ryegrass

4.2.1. Mapping population derived from a single cross in Italian ryegrass

Marker development studies involving Italian ryegrass have been completed with F_1 mapping populations obtained from a single cross between resistant and susceptible genotypes [4, 49]. Annuality is a more common characteristic among grass species than the perenniality of the previously mentioned Italian × perennial ryegrass hybrid (see Section 4.1). Therefore, it might be difficult to maintain and asexually propagate the Italian ryegrass population to produce clonal replicates like those used in the studies of hybrid populations [45-47]. Regardless, GLS-resistant genotypes, which can involve a resistant parent of the mapping population, are very rare because most Italian ryegrass commercial cultivars are susceptible to GLS, similar to perennial ryegrass. Thus, it would be ideal if the resistant genotypes could at least be maintained. An *in vitro* preservation method [54] can be used to maintain and clonally propagate rare genotypes [55].

4.2.2. Detection of a GLS resistance locus by bulked segregant analysis in Italian ryegrass

A major genetic locus in Italian ryegrass for crown rust resistance has been detected using bulked segregant analysis (BSA) [56], and AFLP markers tightly linked to the locus have been developed [57]. Researchers have attempted to detect a GLS resistance locus in Italian ryegrass [4]. An F_1 mapping population was generated from a single cross between a resistant individual from cultivar "Sachiaoba" [2] as the female parent and a susceptible individual from cultivar "Minamiaoba" as the male parent. The rating scale used for phenotyping the F_1 mapping population is provided in Table 4.

The inoculation test used during phenotyping was completed only once because of the annuality of the plant material. Nevertheless, disease severity in the mapping population segregated in a 1:1 ratio (resistant:susceptible) [4]. This result suggests that resistance is controlled by one genetic locus. Therefore, the resistance locus was considered a suitable target detectable by BSA. As predicted, AFLP markers specific for resistant phenotypes were screened by BSA, and a single genetic linkage map composed of 25 of the screened AFLP

Phenotype	Score	Symptoms
Resistant	0	Plants with no leaf symptoms
	1	Plants with brown spotted or brown spindle-shaped leaf lesions
Susceptible	2	Plants with a few white or grey leaf lesions
	3	Plants with leaves covered in lesions

From [4]

Table 4. Rating scale for grey leaf spot severity in Italian ryegrass

markers was constructed [4]. Additionally, the cleaved amplified polymorphic sequence (CAPS) markers derived from Italian ryegrass expressed sequence tags (ESTs) [58] were mapped. The LG associated with the constructed map could be identified because the CAPS markers had already been assigned to seven Italian ryegrass LGs [59]. As a result, the p56 CAPS marker located on LG 5 was mapped, indicating that the resistance locus was on LG 5. Additionally, a significant QTL was detected by interval mapping. The gene at the identified resistance locus was designated *LmPi1* [4]. Although the results of the QTL analysis, including LOD score and phenotypic variance, were not described in the study, the raw data were analyzed for this chapter. The highest LOD score obtained by interval mapping was 7.36, and the percentage of the phenotypic variance explained by the QTL at the highest LOD score was 19.0%. Although broad-sense heritability of the resistance is unknown, the percentage of the phenotypic variance was unexpectedly low because the strong effect of a major gene was expected based on phenotype segregation data. Similar to the results of the Italian × perennial ryegrass hybrid, the low proportion of the phenotypic variance indicates there might be undetected genetic factors in other genomic regions that have a minor effect on GLS resistance/susceptibility (see Section 4.1.3).

4.2.3. Targeted mapping of rice ESTs to the LmPi1 locus

The sequenced rice genome [60] and expanded EST datasets in various plant species enable comparative genomics studies of model and nonmodel plants, in which collinearity of molecular markers and genes in syntenic regions can be elucidated. Based on syntenic regions, high-resolution mapping of genetic loci associated with agronomic traits is possible. This is true even for nonmodel crops where EST-derived markers can be used to map landmarks and demonstrate synteny among different species [61-63]. Conserved intron-scanning primers (CISPs) can be easily developed and used to study nonmodel species [64]. For CISP development, polymerase chain reaction (PCR) primers are designed within relatively conserved exons nearby boundaries between an exon and a variation-rich intron. Target segments are generated by PCR where the introns are scanned during the extension step. Polymorphisms in the PCR products are detected as variations in the introns including base substitutions or insertions and deletions.

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Synteny among ryegrasses, rice, and other grasses such as oat and Triticeae species has been revealed. Ryegrass LG 5, where the previously mentioned *LmPi1* is located, has been shown to be syntenic to rice chromosome (Chr) 9 [51, 65]. Thus, to enhance the single genetic linkage map of *LmPi1*, targeted mapping of rice ESTs to the *LmPi1* locus has been attempted using the F_1 mapping population DNA used to detect the *LmPi1* locus [48]. The CISPs were designed by aligning the rice genome sequence and ESTs on rice Chr 9. Polymorphic PCR products were detected by single-strand conformation polymorphism analysis [48]. Consequently, a single genetic linkage map spanning 66.3 cM composed of 17 CISP markers and the p56 marker tightly linked to *LmPi1* (see Section 4.2.2) was constructed. There was significant collinearity of marker orders between rice Chr 9 and the newly constructed map corresponding to ryegrass LG 5 [48].

Recently, the primer design method involving CISPs has been improved for temperate forage grasses including ryegrasses [66]. Primers were called Conserved Three-prime-End Region (COTER) primers. They were developed from EST sequences of tall fescue and wheat, and eight bases at the 3′ end of each primer were identical to rice orthologues, which provided high transferability in six temperate grasses [66]. The COTER primers have been used for targeted mapping of a locus for brittleness to a single genetic linkage map in a mutant Italian ryegrass line (unpublished data), thereby providing further evidence of the high transferability of these primers.

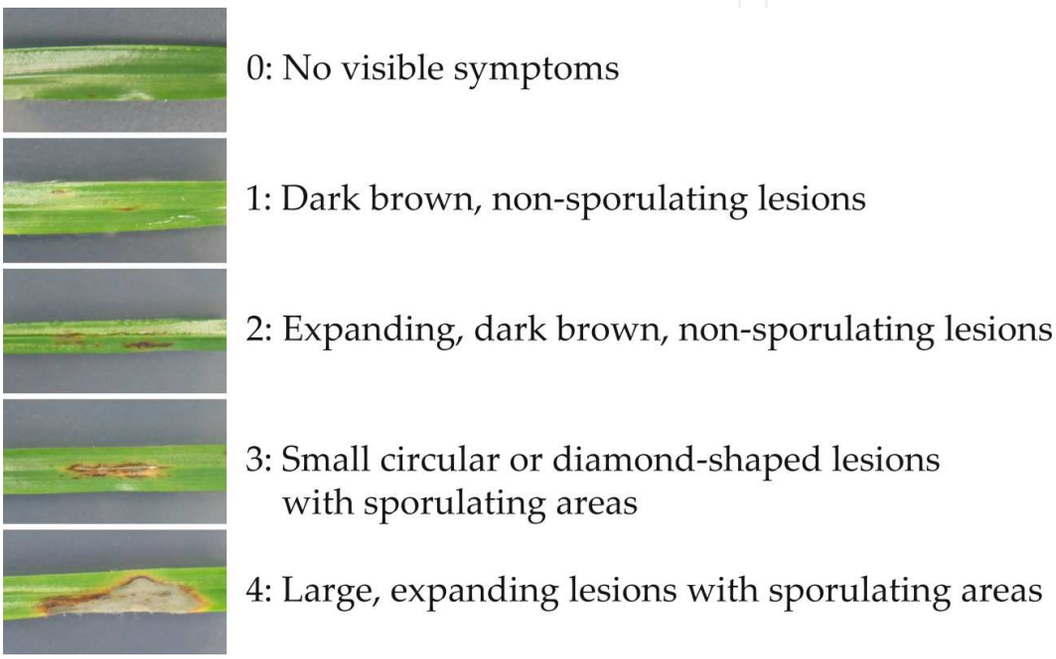
4.2.4. Detection of a novel major locus for GLS resistance in Italian ryegrass

There has been an attempt to identify a resistance locus using a similar approach to that used to identify *LmPi1* [49]. An F_1 mapping population was generated from a single cross between a resistant individual from the commercial cultivar "Surrey" [67] as the female parent and a susceptible individual from the cultivar "Minamiaoba" as the male parent. As described in Section 3.2, the tetraploid cultivar "Jumbo" [42] has been registered as a GLS-resistant cultivar in the United States. The cultivar was developed by doubling the chromosomes of the diploid "Surrey." Thus, it was reasonable to expect that resistance genotypes existed in "Surrey." However, different genetic factors were expected from the resistant parent because the source material was different from that used in the study of *LmPi1*, which explains why "Surrey" was chosen as the resistant female parent.

4.2.4.1. Artificial inoculation method using detached leaves

A high heritability of target traits enables very precise QTL analyses. However, the severity of GLS symptoms in ryegrasses is influenced by environmental factors such as temperature and humidity [1, 68, 69]. Fluctuations in these factors may prevent accurate phenotyping of GLS resistance/susceptibility of the mapping population, thereby decreasing the heritability of the disease reaction. Accordingly, phenotyping in stable environmental conditions may lead to increased heritability. Additionally, repeated phenotyping in stable environmental conditions can further moderate environmental effects and increase the accuracy of the phenotype evaluation.

Multiple phenotypic evaluations of the Italian ryegrass F_1 mapping population infected with GLS has not been conducted because of the annuality of Italian ryegrass and the fact that GLS is highly lethal to infected plants. Thus, a novel inoculation method, the filter-paper method, has been employed for the phenotypic evaluation of F_1 mapping populations [70]. This method can overcome the difficulties of working with Italian ryegrass because it only requires detached leaves from young seedlings. The rating scale for this method is provided in Figure 2. The scale is similar to those of other studies [45, 46] (Table 3) but differs because the score is based on lesion type and not size. More recently, the filter-paper method has been shown to be applicable to the evaluation of resistance to rice blast [71].



Modified from [70]

Figure 2. Rating scale for grey leaf spot severity used in the filter-paper method.

4.2.4.2. Detection of the LmPi2 locus

Based on the filter-paper method, GLS severity was evaluated twice in young, expanding leaves and fully expanded leaves under controlled inoculation conditions [49]. A significant correlation was observed for all GLS severity scores at different leaf ages, but higher correlation coefficients were found between results from the same leaf stage. Additionally, results of repeated-measures analysis of variance (ANOVA) indicated there were significant differences in GLS severity scores among genotypes for all inoculations, whereas the differences were not significant for inoculated leaves of the same age. This indicated that the results of the filter-paper method were highly reproducible [49]. Because of this method, high broad-sense heritability was determined from the results of the repeated-measures ANOVA, with values of 0.701, 0.779, and 0.665 for young leaves, expanded leaves, and all inoculations, respectively [49].

http://dx.doi.org/10.5772/61966

The ratios for phenotype segregation of the mapping population were 1:1 for young leaves and 3:1 for expanded leaves. Therefore, it was concluded that one or two genes controlled GLS resistance in the mapping population [49]. These results and the high broad-sense heritability mentioned earlier encouraged the use of BSA to identify the most important genes. Preliminary analysis with AFLP markers demonstrated that two markers specific to the resistant parent and resistant bulk were genetically linked. Thus, the two markers along with SSR markers from a reference map of Italian ryegrass [72] were further analyzed. Because the two SSR markers were located on LG 3 in the reference map, the resistance locus was predicted to be located on LG 3. A single genetic linkage map was constructed with the AFLP and SSR markers. Further, ESTs from rice Chr 1 were converted to CISP markers because LG 3 was syntenic to rice Chr 1. Grass anchor RFLP probes located on LG 3 [51, 65] were also converted to CISP markers. The enhanced single genetic linkage map covering 133.6 cM showed significant collinearity with rice Chr 1 in their marker orders [49]. A significant QTL was also detected by interval mapping. The highest LOD scores from interval mapping were 13.8, 15.2, and 17.9 for young leaves, expanded leaves, and total data from four inoculation experiments, respectively [49]. Percentages of phenotypic variance explained by the QTL at the highest LOD scores were 61.0, 68.1, and 69.5% for young leaves, expanded leaves, and total data from four inoculation experiments, respectively [49]. The most important point of this study was that, unlike for *LmPi1*, the broad-sense heritability score (0.665) and percentage of phenotypic variance explained by the QTL at the highest LOD score (69.5%) were very similar. In other words, although only a single genetic linkage map of LG 3 was constructed, most of the genetic factors for the GLS resistance phenotype in the mapping population can be explained by the functions of a single gene.

The detected locus is clearly distinguishable from *LmPi1* because it is located on a different LG. Conversely, the QTL detected in [46] with the highest percentages of phenotypic variance explained was located on the same LG as the detected locus. The two resistance loci could not be distinguished because there was no common marker around the locus that could be used as a landmark. However, there were markers close to both loci on LG 3 of the Italian ryegrass reference genetic linkage map [72]. The genetic distance between the two loci was estimated to be over 25 cM, suggesting the detected locus is probably not the QTL detected in [46]. The detected locus was designated *LmPi2* [49], which is the second identified GLS resistance locus in Italian ryegrass.

5. Conclusion

This chapter summarized the advances that have been made in the molecular breeding of GLS resistance in ryegrasses. Rice blast and GLS are caused by *M. oryzae*, but rice blast has been studied more extensively because of the importance of this staple food crop. Nevertheless, there are still incidences of rice blast leading to considerable yield losses, and numerous issues regarding this disease require further research. The breeding history of rice-blast-resistant cultivars is a major consideration during breeding of GLS-resistant ryegrasses. The breakdown of resistance regulated by a few genes is one of the most important factors related to the

development of rice-blast-resistant cultivars [44]. Similar concerns would apply to the breeding of GLS-resistant ryegrass cultivars if a small number of genes mediated the resistance. Although some genomic regions associated with GLS resistance have been identified, further studies are required in ryegrasses because our knowledge of GLS resistance is more limited than our understanding of rice blast resistance. To establish highly productive cultural system for ryegrasses, synchronized approaches between cultural disease management practices and breeding for GLS resistance, promoted by advances in plant genomics, are necessary.

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