TESTING A LINEAGE EXCLUSION STRATEGY FOR BREEDING RICE RESISTANT TO BLAST DISEASE

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ABSTRACT

Tapiero-Ortiz, Anibal L. Ph.D., Purdue University, May 2001. Testing a Lineage Exclusion Strategy for Breeding Rice Resistant to Blast Disease. Major Professors: Morris Levy and Gregory Shaner.

Rice blast, the disease caused by the fungus *Pyricularia grisea* Sacc. (teleomorph *Magnaporthe grisea* (Hebert) Barr.), has challenged plant breeding programs in their search for durable resistance. Rapid resistance breakdowns are commonly observed and are attributed to the frequent appearance of new pathotypes or to poor screening of the pathogen population prior to release. Recent population studies of the rice blast pathosystem have shown that *P. grisea* in a given region typically expresses a phylogenetic organization of distinct lineages (genetic families as defined by MGR-586 DNA fingerprinting). Each lineage exhibits a definable virulence spectrum and the potential for developing new pathotypes appears to be constrained by lineage-specific avirulences. Pyramiding resistance (R) genes in combinations that exclude the observed virulence spectra of each lineage can provide durable resistance to rice blast. The lineage exclusion strategy was tested on pyramids at three ecologically different sites in a primary rice growing region in Colombia, from 1996 to 1998. The pyramids were selected for resistance using isolates of representative Colombian lineages. The presence and homozygosity of the R-genes in the pyramid lines was confirmed with molecular markers. The pyramids proved to be highly resistant in commercial fields and no major changes in lineage composition or virulence spectrum were observed. However, some moderately virulent isolates were transiently observed in 1996 and 1997 but not in 1998 at a disease 'hot-spot' nursery. These isolates were all members of lineage SRL-6, the only lineage known previously to express virulence to both pyramided R-genes, although

differentially by individual isolates. The new aggressiveness of lineage SRL-6 seemed to compromise only a sub-population of the pyramids and no mixture of isolates from other lineages, expressing a similar complement of differential virulences, was capable of infecting the pyramids. Therefore, resistance breakdown (vulnerability) may depend on within-lineage rather than between-lineage distributions of virulence. For breeding purposes, the virulence potentials of *P. grisea* populations appear to be more predictable when viewed in terms of phylogenetic pathotype spectra rather than as a series of physiological races. The lineage exclusion strategy in Colombia proved to be an authentic breeding tool for managing rice blast disease.

INTRODUCTION

Rice blast is the disease caused by the filamentous fungus *Pyricularia grisea* Sacc. (teleomorph *Magnaporthe grisea* (Hebert) Barr.) (Rossman et al., 1990). This pathogen has challenged rice breeding programs in their search for durable resistance (Ou, 1985). The fungus colonizes leaves, nodes and inflorescences and infects the crop in most regions where rice (*Oryza sativa*) is planted. Spindle-shaped lesions, usually with a grayish center and brown margins, form on the leaves and later expand and coalesce, causing the infected leaves to die. On stems and nodes the disease causes neck rot and on inflorescences it causes panicle blast. Long dew periods, low soil moisture, and the use of high-nitrogen fertilizers on poor soils favor disease development. Primarily, genetic resistance and the time of the initial infection determine the extent of losses caused by leaf blast (Ou, 1985).

In regions where conditions remain favorable, rice blast can kill susceptible cultivars when crops are infected at the seedling or early tillering stages (Ou, 1985). Resistant cultivars under similar conducive environments may show only minute brown specks. Yield losses due to leaf blast are difficult to calculate but could approach values comparable to the percentage of diseased leaf area, depending on crop management (Tsai, 1988). Disease progress diminishes noticeably at maximum tillering stage, but it develops rapidly at the reproductive stages. Losses caused by node and panicle infections are substantial because the plant organs above the infected node usually die.

The use of genetic resistance is the most effective and economical way to control the disease in commercial fields (Correa-Victoria et al., 1994). However, resistance to rice blast has repeatedly proven to be of short duration, commonly failing in newly bred cultivars within a few years after their release. Genetic resistance failure has

been attributed to several causes, such as the frequent appearance of new pathotypes (Ou, 1985), or to the limited pre-release challenge of the breeding lines by the pathogen population in the region targeted (Zeigler et al., 1994). The latter is particularly important in regard to low-frequency virulences within the fungal population.

Although new pathotypes do emerge in the field, the claim of frequent appearance of new virulences (Ou, 1985) has been challenged by several studies (Latterell, 1971; Bonman et al., 1986). These indicate the occurrence of a certain level of virulence mutability, but not to the extent of generating distinct pathotypes from monoconidial isolates from single lesions, as previously suggested. Sensitivity of the cultivars to infection assay techniques, differences in environmental conditions within the tests, and the inherent subjectivity of scoring disease response, have been regarded as the most likely sources of the apparent variability. More recently (Valent and Chumley, 1994), molecular and genetic analyses have shown that instability can be a cytogenetic characteristic of specific loci in particular fungal strains. That is, stable genetic loci in some strains may be unstable in others depending on the karyotype, but not correlated with selection for virulence.

Rice blast has several features to make it a model system for plant pathology. Among these is strong evidence that, while *P. grisea* includes pathogens of many grasses, individual isolates have a limited host range (Valent, 1990). Further, strains pathogenic to rice can be subdivided into physiological races, or pathotypes, depending on the rice cultivars that they successfully infect. Most importantly for disease management, rice blast pathotype diversity conforms to a classical gene-for-gene model in several genetic studies (Silue et al., 1992; Valent, 1990). These features make the design of breeding strategies based on the deployment of vertical (major R-gene) resistance plausible.

Combining major R-genes, which exclude the commonly observed pathotypes of a pathogen in a region into a single cultivar (a pyramid), is a conventional resistance breeding strategy used to increase resistance effectiveness (Agrios, 1997). The strategy is based on the assumption that the diversity of pathogen virulence is constrained in some important ways, e.g., there is strong cultivar-specific selection, broadly virulent

pathotypes have reduced fitness, migration of virulent strains is minimal, and virulent pathotypes do not evolve easily by recombination. Thus, the effective deployment of pyramided R-genes depends on the pathogen population structure and the genetic organization of the pathotype diversity.

Levy et al. (1991) first developed a DNA fingerprinting (multilocus RFLP) technique to analyze the genetic diversity of the rice blast fungus by using a repetitive sequence probe called MGR586, after *Magnaporthe grisea* repeats probe 586 (Hamer et al., 1989). MGR-DNA fingerprinting analyses have shown that the population of *P. grisea* infecting rice in a given region typically expresses a phylogenetic organization of distinct lineages or genetic families, which have been maintained predominantly or exclusively by asexual reproduction. Fingerprint analysis successfully identified eight major lineages and their component pathotypes in the United States from a sample of isolates collected over a 30-year span. The temporal stability of MGR-defined lineages over a decade or more has also been reported for sites and/or regions of Colombia (Levy et al., 1993; Correa-Victoria et al., 1994), The Philippines (Zeigler et al., 1995), Europe (Roumen et al., 1997), southern China (Shen et al., 1998) and southern India (Sivaraj et al., 2000).

In the United States, each lineage expressed a predominant pathotype or a few similar pathotypes on a set of eight international differentials; some lineages expressed the same (convergent) pathotypes but several expressed certain pathotypes exclusively. At a comparatively pathotype-rich site at Santa Rosa, Colombia, six distinct and indigenous MGR lineages were defined (Levy et al., 1993). Each lineage at Santa Rosa was compatible with a specific subset of Colombian cultivars and expressed a subset of multiple pathotypes on rice blast international differentials. Typically, the pathotypes within a lineage had closely related virulences, indicating that the virulence spectrum expressed by each lineage had evolved from a common ancestry. This conclusion was reinforced by further evidence indicating that members of a lineage share a common limit to virulence, i.e., a particular avirulence (Correa-Victoria and Zeigler, 1993; Correa-Victoria et al., 2000). Consequently, while pathogenic changes can occur in any given lineage not all changes appear equally likely. Commonly, no member of a given lineage

can overcome the resistance expressed by a particular cultivar, and cultivars that are highly susceptible to a given lineage might be completely resistant to others (Zeigler et al., 1994).

Zeigler et al. (1995) first demonstrated that MGR lineage-specific resistance could be conferred by single R-genes, analyzing the response of a set of near-isogenic lines (NILs) to a broad sample of isolates from The Philippines. The NILs were recurrently developed in the genome of the highly susceptible *indica* rice cultivar, CO39, and were reported to carry distinct major R-genes (Mackill and Bonman, 1992). Resistance to blast in NILs C101LAC, C101A51, and C104PKT was conferred by independent dominant genes designated *Pi-1(t)*, *Pi-2(t)*, and *Pi-3(t)*, respectively. The R-gene designation was made in conformity with rules for rice as follows: *Pi* stands for *Pyricularia*; the number was assigned according to the resistance group of the NILs in that study; and *(t)* indicates a tentative designation. Resistance in C101PKT and C105TTP-4 was conferred by other dominant alleles at an additional locus, designated $Pi\text{-}4_{(a)}\text{-}(t)$ and $Pi\text{-}4_{(b)}\text{-}(t)$, but see (Inukai et al., 1994) for other interpretations. Zeigler et al.'s inoculation assays of broadly sampled Philippine lineages demonstrated that each lineage (i.e., all isolates within the lineage) was specifically incompatible with one or more of the NILs, with C101A51, bearing *Pi-2(t)*, giving the highest frequency of incompatible interctions. Each NIL was infected by isolates from at least one lineage and no universally effective R-gene was identified.

The specific relationships revealed among MGR lineages and the distributions of virulences/avirulences in the local pathogen populations have given rise to a new resistance breeding strategy called ‘lineage exclusion’ (Zeigler et al., 1994). The strategy assumes that the potential for developing new pathotypes is constrained by lineage-specific virulence limits (or fitness-enhancing avirulence gene functions). When lineage composition is stable in time, pyramids with combination of R-genes that exclude the observed virulence spectra of the prevalent lineages can provide durable resistance to rice blast. The strategy contrast with conventional R-gene pyramiding that combines resistances based solely on observed host susceptibilities or detected pathotypes. The major operational difference is that lineage exclusion does not advocate complements of

R-genes that are differentially susceptible to isolates within a single lineage, even though that compatibility for the combination of R-genes within the lineage has not yet been observed. Rather than breeding against pathotypes *per se*, lineage exclusion predicts that breeding against avirulences shared by all lineage members offers a phylogenetically more stable incompatibility to be exploited by breeders.

The research reported here was designed to test the lineage-exclusion hypothesis for breeding rice resistant to blast, in a major rice-growing region of Colombia where blast disease is chronic and severe. More than 400,000 ha of rice are cultivated yearly in Colombia, one-third of them in the eastern plains (Llanos Orientales). Most (70%) rice crops are irrigated, while the remainder depends on rainfall (upland). Grain yields are in the region of 5,800 kg/ha for irrigated rice and 4,800 kg/ha for upland. Two major landscapes, the upland plains and the foothills, characterize the eastern plains. The upland plains are basically grasslands with an extensive network of gallery forests on soils with high content of Fe^{3+} and Al^{4+}. The foothills are richer soils, the remains of tropical forests logged to clear land for pastures or cereal crops. Rice is grown in the foothills regularly, but a rotating cultivation is performed in the upland plains with a variety of commodity crops and forages.

The entire region is characterized by a dry season from December to March and a rainy season (>20 cm average monthly rainfall) lasting from April to November, with a short break from mid July-August. During the wettest months (April, May, June and October) rainfall can accumulate more than 40 cm per month. Yearly average temperature is 26ºC, average differences between daily maximum and minimum temperatures are 14ºC (higher during the dry season than the wet season). Effective daily sunshine is usually less than 5h, particularly during the rainy season. Extended dew periods are common in this region, where the average relative humidity is 65% during the dry season to over 85% during the rainy season, making this environment highly favorable for infections by *P. grisea* (Tapiero-Ortiz, 1991).

There has been considerable effort to reduce losses caused by rice blast in Colombia. Breeding has improved and different strategies such as the frequent

introduction of individual resistant cultivars, the use of cultivar mixtures and the selection of multilines have been attempted. Although forecast-based crop management was proposed to extend the duration of the new acquired resistances, all employed strategies have failed to provide a satisfactory control. Between 1972-1990, 11 of 20 apparently blast-resistant cultivars released had become susceptible within a few years, while the others were abandoned for different reasons (noticeably for susceptibility to hoja blanca virus). Strikingly, three of the last four cultivars released between 1985-1988 became susceptible to blast within less than a year of their release. Chemical control is an effective alternative but it is expensive. In the Eastern plains (40,000 ha), fungicide application for one seasonal crop alone can amount to 12% of the total production costs (Tapiero-Ortiz, 1991).

The Colombian rice-breeding program (ICA), in conjunction with the Centro Internacional de Agricultura Tropical (CIAT), introduced several changes to its breeding strategies by 1985. Following three decades of frustration to obtain durable resistance to rice blast, pre-release screening conditions were improved by increasing the pathotype diversity to challenge the breeding lines. To include those pathotypes expressed in lower frequencies in the field, a 'hot spot' blast nursery was developed in the eastern plains at Santa Rosa (Correa-Victoria et al., 1994). Known susceptible commercial cultivars and blast differentials are grown year round to generate an ample and diverse inoculum. Planting spreader rows prior to the breeding lines and inducing them to get infected guarantees a uniform distribution of the disease among the lines to test. This nursery has been regularly used by rice programs from around the world for screening against rice blast. As a result of the improvements, the release of a cultivar like Oryzica Llanos 5 was made possible by 1989. This cultivar has remained resistant to blast after being planted yearly in more than 50,000 ha where the disease is chronically severe, clearly demonstrating the possibility of durable blast resistance by major R-gene combinations in rice (Correa-Victoria et al., 1994).

After Levy et al. (1993) showed that the population of *P. grisea* at Santa Rosa was phylogenetically organized into a set of six discrete lineages with specific pathotype and cultivar associations, a clue for the basis of the durability of resistance in Oryzica

Llanos 5 emerged (Correa-Victoria and Zeigler, 1993). This cultivar is a descendant of a breeding scheme involving a known resistance donor (Colombia1) combined to unknown resistances from five other cultivars: CICA9, CICA7, IR36, IR22 and 5685 (Figure 1). Each of these cultivars, including the resistance donor, is susceptible to *P. grisea* at Santa Rosa. However, CICA9 is exclusively, although very heavily, infected by isolates in only two closely related lineages (SRL-1 and SRL-2) and completely resistant to isolates in the other lineages (Table 1). The other ancestors were susceptible to one or more of the other lineages but were completely resistant to isolates that attacked CICA9. This suggested that a combination of resistance genes matched by avirulence genes in each of the lineages would provide durable resistance to blast.

Furthermore, with the recently developed set of NILs bred in the background of CO39, all but one of the Colombian lineages expressed a lineage-specific avirulence to one or more of the R-genes involved (Levy, unpublished data). However, isolates in lineage SRL-6, the most abundant in Colombia, can defeat these two R-genes separately. Among the genes identified, the most common incompatibilities were for R-genes *Pi-2(t)* and *Pi-1(t)* (Table 2). Isolates in SRL-6 were additionally subdivided into three pathotypically differential subsets. The SRL-6a subset comprises isolates avirulent to cultivars with either *Pi-1(t)* or *Pi-2(t)* R-genes. Isolates in the SRL-6b group are virulent to cultivars with *Pi-1(t)* but not to cultivars with *Pi-2 (t)*. And the isolates grouped as SRL-6c are virulent to *Pi-2(t)* but not to *Pi-1(t)*. Prior to the current study no single isolate from Colombia had been found virulent to both the *Pi-1 (t)* and *Pi-2 (t)* R-genes.

The rice blast pathosystem in the eastern plains of Colombia provides an excellent regime for testing the efficacy of resistance breeding strategies based on lineage exclusion. To perform the tests, resistance gene pyramids were selected from a cross between the near-isogenic lines C101A51 x C101LAC using inoculation assays with archived isolates from all Colombian lineages. The R-gene composition of the pyramids was secondarily assessed with linked molecular markers. Once the presence of *Pi-1(t)* and *Pi-2(t)* in the pyramids was established, the following questions were addressed:

1. Does the R-gene complement provide lineage-exclusive resistance?

2. If so, how durable will this resistance be?

   a. What is the lineage-specific rate of new virulence mutations?

   b. To what degree are virulence mutation rates also isolate specific?

   c. What is the overall fitness and stability of new virulence mutations?

To answer these questions, the selected pyramids were tested in the rice blast nursery at Santa Rosa under high disease pressure. The NILs and other reliable cultivars from the Colombian breeding germplasm served as checks in the field tests to distinguish the lineage and pathotype structure in the various experiments. Isolates that successfully infected any pyramids in the initial survey at Santa Rosa in 1996 also were retested for virulence on the pyramids in inoculation assays conducted at Purdue University.

Assuming that the durability of resistance will be challenged by the pathogen's ability to overcome the new genetic barriers, a series of specific inoculation experiments was performed to evaluate lineage-specific rates of virulence mutations. Rare lesions that developed in the doubly resistant pyramids were collected and cultured from single spores. The resultant isolates were characterized by MGR-DNA fingerprint analysis and virulence patterns. The putative mutants were thereafter inoculated to a selected set of pyramids depending on their host source of isolation and sequentially reisolated and reinoculated to establish their virulence stability and fitness. The fingerprints of the putative newly re-selected isolates served to confirm the origins of suspected virulence changes.

Given that no major changes in lineage composition have been reported from Colombia since the population structure was initially identified, few modifications were expected in pathotype diversity in the field that had not been observed already. However, a final question remained to be addressed:

3. Is resistance that is based on lineage exclusion durable under field conditions or to what extent is it ecologically dependent?

To answer this question and to confirm the resistance of the pyramids, tests also were conducted at two ecologically different sites in commercial rice growing areas.

Relying on local assistance, serial plantings at each site provided ample testing over a broad spectrum of environmental conditions. Isolates collected from lesions in the test plots were then characterized for lineage composition and virulence features.

Table 1. Proportion of isolates of *Pyricularia grisea* by MGR lineages in Colombia compatible to the resistance spectrum of the rice cultivar Oryzica Llanos 5 and six of its ancestors.

	Lineage [1]								
Cultivar	1	2	3	4	5	6	7	10	13
CICA 7	0 [3]	0	0.14	0.19	0	0.57	0.06	0	0.3
CICA 9	1.00	1.00	0	0	0	0	0	0	0
IR 36	0	0	0	0	0.23	0.20	0	0	0
IR22	0.13	0	0	0.10	0.14	>0.50 [4]	0	0	0
Colombia 1	0	0	0	0.12	0	0	0	0	0
5685	0	0	0	0	0	0.16	0.45	0	0
Llanos 5	0	0	0	0	0	0	0	0	0
Isolates [2]	16	10	7	26	22	61	47	13	5

[1] Lineages as determined by MGR-DNA fingerprinting analysis.

[2] Number of isolates per lineage tested.

[3] Modified from (Correa-Victoria et al., 1994).

[4] The actual number of compatible isolates was not available.

Table 2. Resistance (R) of two near-isogenic lines to isolates from commonly identified lineages of *Pyricularia grisea* in Colombia.

		Lineage [1]											
Line	Gene [2]	1	2	3	4	5	6a	6b	6c	7	9	10	13
C101A51	*Pi-2(t)*	S [3]	S	R	R	R	R	R	S	R	R	R	R
C101LAC	*Pi-1(t)*	R	R	R	R	S	R	S	R	R	S	S	S
Isolates/lineage tested		11	11	5	19	30	22	5	8	17	7	13	2

[1] Lineages as determined by MGR-DNA fingerprinting.

[2] Tentative gene assignment (Mackill and Bonman, 1992)

[3] S = susceptibility, as determined by the presence of typical spindle-shaped lesions on at least 2% of the leaf area by one or more isolates; R= no susceptibility detected (based on 8 plants/cultivar/isolate).

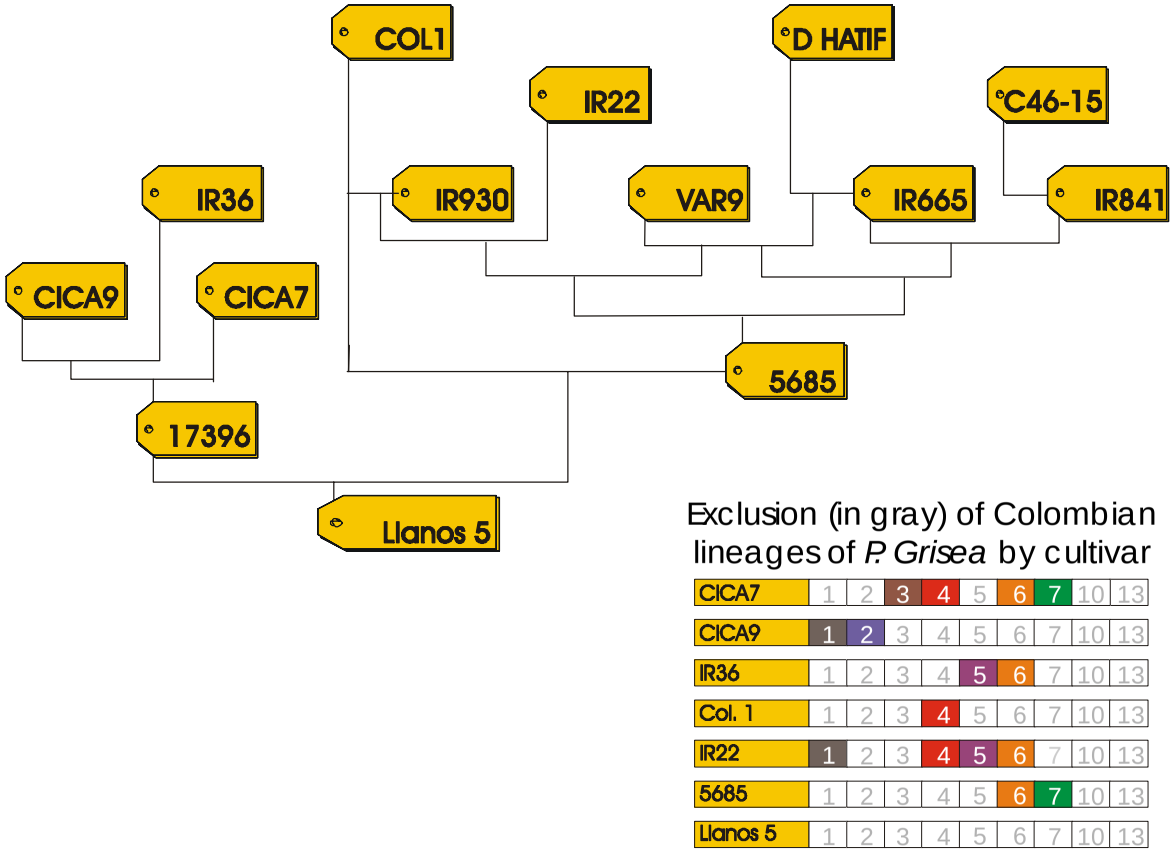

Figure 1. Schematic representation of the genealogical tree of the rice cultivar Oryzica Llanos 5. This resistant cultivar resulted from the cross of the line 17396 to an unnamed line from the cross of Colombia 1 to the line 5685. Highlighted boxes in the insert indicate Colombian MGR-DNA lineages to which selected cultivars are susceptible.

MATERIALS AND METHODS

Selection of the Pyramids

The general protocol for selecting doubly resistant pyramids bearing the R-genes *Pi-1(t)* and *Pi-2(t)* is schematically represented in Figure 2. The F_1 from the cross of C101LAC (*Pi-1(t)*) to C101A51 (*Pi-2(t)*) was acquired from CIAT and inoculated at Purdue University with isolates virulent to these R-genes, representing current lineages in Colombia. Resistant F_1 plants were allowed to self-pollinate and a single-seed descendant (plant 12) was chosen for further screening. Ultimately, several non-segregating, doubly resistant F_3 families were isolated. Pyramids selected during the test were chosen for R-gene marker analysis. DNA was extracted from the leaf tissue of seedlings at the 5th leaf stage for performing RFLP analyses to confirm the presence of loci linked to *Pi-1(t)* and *Pi-2(t)* in the selected plants. After digesting the DNA with different enzymes, the probes *RG-64* for *Pi-2(t)*, and *RG-303*, *RZ-536* (gifts from the Cornell Research Foundation, Inc.) and *G-181* (a gift from the Japanese Rice Genome Research Program of the National Institute of Agrobiological Resources) for *Pi-1(t)* were used to confirm the homozygous condition of the associated loci in the pyramids.

Screening for Doubly Resistant Pyramids

Pathotype identification was performed following procedures reported elsewhere (Levy et al., 1993). Selected diseased tissues with discrete, typical lesions were placed in Petri dish humidity chambers to induce sporulation. Conidia from single lesions were streaked and spread on a thin layer of 1% water agar to germinate. Single

germinated spores were transferred to prune agar and allowed to grow for 5 days at 25ºC under low light to induce mycelial growth. For storage purposes, small pieces (7 mm diameter) of sterilized filter paper (Whatman no. 2) were placed on the colony borders and left there until completely covered by the mycelium (usually 3-5 days). Afterwards, the cultures were placed under continous fluorescent light for 7 days at 25ºC to induce sporulation. The filter papers were then aseptically transferred to plastic isolated cells and kept in a dryer cabinet (Samplatec Corp.). The dried isolates were stored at 4ºC inside glassine stamp envelopes.

For inoculation purposes, conidia were scraped from prune agar cultures and suspended into 20–25 ml of a sterile 0.2% gelatin solution, immediately prior to use. The suspensions were then filtered through Miracloth and adjusted to 8 x 10^4 conidia/ml, as customarily recommended, with the aid of a hemacytometer.

Plants for inoculation were grown in potting soil in 175 cm^3 plastic pots (4 plants/pot), for approximately 3 weeks. Two weeks after germination a complete (20:20:20 NPK, 3-4 g/l water) fertilizer was applied to the 1 to 2-leaf seedlings. Plants were inoculated at the 1.4-leaf stage after being transferred from the greenhouse to a high humidity (>85%) chamber and low light conditions for 24 h. Each test was conducted on 16 plants per replication per cultivar, and all replications were kept inside the same growth chamber. A De Vilbiss apparatus connected to a pneumatic pump (Gast Mfg. Corp.) at 4.5 kg/cm^2 pressure was used to uniformly spray the conidial suspension onto the seedlings. Inoculated plants were kept for another 24 h under darkness and 100% humidity before they were transferred to a misting room at >60% RH and 12 h photoperiod.

Plants were scored 5-7 days after the inoculation using a three category scale, and including the leaf area infected whenever susceptible lesions were recorded, as follows: an initial 0-2 score was assigned for no visible lesions to small rounded lesions without further discrimination; 3 indicated rounded lesions to isolated spindle-shaped lesions covering less than 2% of the leaf area and 4+ was given to typical spindle-shaped lesions covering more than 2% of the leaf area. Score 4+ was interpreted as susceptible,

or moderately susceptible if less than 5% diseased area was observed where the susceptible check was severely infected. Ambiguous scores were re-evaluated 10 d after inoculation. Fungal strains forming rare or unusual leaf and sheath lesions on presumably resistant hosts were re-isolated for further fingerprinting and pathotype confirmation.

The F_2 generation of the C101LACxC101A51-12 progeny was assayed initially for resistance to SRL-5 and SRL-2 isolates, individually as well as in combination. This served as a preliminary screening for *Pi-1(t)* + *Pi-2(t)* double-resistant pyramids. A χ^2 test was also performed to check the efficiency of serially inoculating as compared to the use of inoculum mixtures. From 132 plants tested, 65 were found resistant after the second inoculation in the serial test as compared to 73 resistant from 133 total, when using the mixture. Given the statistical goodness-of-fit of the mixture test to the 9:7 expected ratio ($\chi^2 = 0.009$; $\boldsymbol{P} > 0.95$), the use of mixtures was chosen as the customary technique for testing complementary lineages.

A total of 138 plants out of 265 was found resistant in the F_2 (Table 4). The F_3 pyramids of the resistant F_2 available were tested again with isolates from lineages SRL-2 and SRL-5. Two consecutive tests were performed on the 22 resistant F_3 pyramids obtained. Isolates belonging to lineages SRL-6 and isolates from the recently characterized Altillanura region in Colombia, ALL-9, ALL-10 and ALL-13, were also included because of their ability to overcome *Pi-1(t)* (Manry, 1995). A total of eight resistant and apparently non-segregating F_3 pyramid families, and nine derivative F_4 families, were finally chosen for further field and greenhouse testing.

Resistance-Gene Marker Analysis

Several restriction fragment length polymorphism (RFLP) markers have been linked to blast resistance loci in rice (Table 5). Specifically, the genomic clone *RG*-64 has been found tightly linked to the *Pi-2(t)* blast resistance locus on chromosome 6, at a map distance of 2.8 ± 1.4(SE) centiMorgans (cM) (Hittalmani et al., 1995). After sequencing *RG-64*, specific primers were determined for generating a polymerase chain

reaction (PCR)-based polymorphic marker. Although the amplified products (sequenced-tagged-sites) using these primers do not show any polymorphism, further cleavage with the restriction enzyme *Hae*III generated a polymorphic fragment that differentiated resistant and susceptible phenotypes. An accuracy of more than 95% was found in distinguishing F_2 resistant plants by this approach. A similar accuracy was obtained with *RG64* as an RFLP probe when testing for disease response in the F_3.

The single-copy rice cDNA clone *RZ-536* has been linked to the *Pi-1(t)* locus at a map distance of 14.0 ± 4.5 (SE) cM; clone *RZ-424* is also linked to this gene at 19.6 ± 5.3 (SE) cM in chromosome 11 (Yu et al., 1996). The clones *G-181* (Kurata et al., 1994) and *RG-303* (McCouch et al., 1998) are also linked to *Pi-1 (t)*. According to the consensus (current) genomic map, these markers are flanking the locus with *RG-303* on the opposite side to the others. Restriction site polymorphisms between resistant and susceptible phenotypes have been identified for these markers.

A modified CTAB DNA extraction procedure similar to that described for *Musa* and *Ipomoea* (Gawel and Jarret, 1991) was followed for extracting rice plant genomic DNA. The extraction buffer was made by mixing and autoclaving 1.5% (w/v) CTAB, 100 mM Tris-HCl at pH 8.0, 1.4 M NaCl and 20 mM EDTA, and adding mercaptoethanol to 1.0% (v/v) immediately prior to use. Healthy lyophilized leaves were ground with a mortar and pestle in liquid nitrogen from 5-leaf rice seedlings (1.5 growth stage). Pre-heated extraction buffer (10 ml) was added to the ground tissue (500 mg), mixed gently and incubated at 65ºC for 30 min. Chloroform:isoamyl alcohol (24:1, v/v) was then added (7.5 ml) to the buffered tissue and mixed by inversion at room temperature for 15 min. After centrifuging the mixture at 5,000 rpm for 5 min, the aqueous phase was filtered through Miracloth and an equal volume of ice-cold isopropanol was added and mixed gently until the DNA precipitated. The precipitated DNA was subsequently hooked with a bent Pasteur pipette, rinsed with 70% ethanol, and transferred to a micro centrifuge tube. After centrifugation at 12,000 rpm for 15 sec, the DNA was blotted dry (3h to overnight) and then dissolved in 250 µL TE (10 mM Tris, 1.0 mM EDTA, pH 8). RNAse (final concentration 10 µg/mL) was added to the dissolved DNA and incubated at room temperature for 30 min. A 1/10 volume of 3 M

NaOAc (pH 6.8) and two volumes of 95% ethanol were added to the suspension. The precipitated DNA was then hooked out and rinsed in 70% ethanol, blotted dry and finally dissolved in 50 µL TE for storage (4ºC).

Two oligonucleotide primers, 5'gttgtttgagctctccaatgcctgttc3' and 5'ctgcagtgcaatgtacggccagg3', were used to amplify the *RG-64* locus from genomic DNA from the pyramids, the NILs and selected cultivars, according to the following protocol modified from Zheng et al. (1995). Ten ng of template DNA, 0.5 mM primers, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1X Taq DNA polymerase buffer and 1.25 U of *Taq* DNA polymerase (Promega) were mixed in a 25 µL final suspension. Denaturation was achieved at 94ºC for 2 min, annealing at 56ºC for 1 min, and synthesis at 72ºC for 1 min. The cycle was repeated 34 times with 30 sec denaturation each in a MJ Research thermocycler. The amplified products were digested with *Hae*III and separated by electrophoresis at 44 V in a 1.2% agarose gel for 6 h.

Southern blot hybridizations with the clones *RZ536*, *RG303* and *G181* were performed to screen for *Pi-1(t)* in the prospective pyramids. Genomic DNA from the susceptible check, the NILs, and the R-gene donors was digested with several restriction endonucleases (*Eco*RI, *Eco*RV, *Dra*I, *Hind*III, *Taq*I and *Sca*I), separated on 0.8% agarose gels by electrophoresis, and transferred onto Hybond N^+ membrane according to specifications of the manufacturer. The digestions indicating polymorphism between the R-gene donors versus the susceptible check for each marker were chosen for hybridizations with radioactively labeled probes.

Field Test of Lineage Exclusion

Field experiments to test for durability of resistance were conducted at three sites in the eastern plains from 1996-1998: Puerto Lopez in the upland plains and Santa Rosa and Granada in the foothills. The sites are approximately 150 km apart at an area located within 04:8º N 72:50º W - 03:35º N 73:44º W and lay at 300 – 400 meters above sea level and (Figure 3). Prevailing winds run from the upland plains towards the hills with ENE-WSW direction. Rainfall was 3.1 m during 1996 and 2.44 m during 1997 with

approximately 2/3 of the total rain accumulating from April through August. Average temperature was 26ºC for both years; maximum temperatures were 31ºC (1996) and 34ºC (1997) and minimum temperatures were 21ºC and 20ºC, respectively (Edgar Almanza, Corpoica La Libertad, personal communication).

A nursery trap composed of cultivars popular in the region during the last 30 years (Table 6) was planted together with the selected pyramids in 1 m densely sown rows separated by 20 cm. Fertilization and crop practices were performed following standard procedures established by the Colombian National Rice Program. Each site was planted 2-4 times a year from 1996-1998, depending on resources available. Seeds for the 1996-1997 experiments were amplified at CIAT in a rice blast-free environment from an initial shipment from Purdue University. Seeds for the 1998 experiments were harvested from mature, uninfected pyramids, in the 1997 field trials.

Lesion type and infected leaf area were scored several times from the later seedling stages (approximately 25d after germination) until the maximum tillering growth stage according to the 0–9 Standard Evaluation System (SES), International Rice Research Institute (IRRI). This scoring system differentiates lesion type from minute brown specks (grade 1) up to typical lesions with 2%-5% leaf area infected (LAI) in grade 4. Subsequent scores in the scale are pre-established increments in LAI as follows: 5%-10% (grade 5), 10%-25% (grade 6), 25%-50% (grade 7), 50%-75% (grade 8), and 75% to nearly complete leaf coverage (grade 9). Plant reactions to infections in the field are regarded as resistant up to grade 3, moderately resistant at grade 4, moderately susceptible at grade 5 and susceptible from grade 6 upwards. Adjustments are usually made at the moderate levels, accordingly to the severity reached in the susceptible check in a given test.

DNA fingerprint analysis of monoconidial isolates obtained from the pyramids and selected cultivars in the field, as well as from the Colombian collection at Purdue University were performed following methods described previously (Levy et al., 1993). DNA was extracted from mycelia grown in liquid medium, digested with *Eco*RI restriction enzyme, and separated by electrophoresis (2 μg) in 0.8% agarose gels for 48 h

at 44 V. The DNA was blotted on Hybond N^+ nylon membranes according to suggestions from the manufacturer and probed with radioactively labeled MGR586.

A Southern blot was performed with representative archived isolates of Colombian lineages to be used as a reference master for visual lineage assignments of the field isolates (Figure 4). To facilitate the comparison, the duration of electrophoresis was the same for newly acquired as well as for the reference master isolates. After lineage assignments, Southern blots of representative haplotypes were constructed for phenetic cluster analysis. The unweighted pair group with arithmetic average (UPGMA) method was used for the cluster analyses. This method analyzes similarities and dissimilarities from comparisons of molecular profiles (Levy et al., 1993). The rule for clustering is such that, on average, all members in the same cluster are closer to each other than any member is to the next most similar cluster. In the case of the MGR-DNA fragments, the data are treated as dominant characters of binary alternatives (presence or absence), and the profiles are clustered after calculating all pair-wise comparisons. A hierarchical pattern of relative relationships among profiles was expressed in a phenogram. The analysis was performed using the SAS program (Statistical Analysis System), version 6.12.

Table 3. Features of the isolates used for testing the pyramids, by MGR lineages.

Lineage		Isolate	Serial no. [2]	Lineage	Isolate	Serial no.
a) [1]	SRL 1	CICA9-7	SR 13018	SRL 6c	I 3-12-2	SR 29005
	SRL 2	CICA9-37-1 [3]	AL13014		I 3-2-2	SR 29004
		CICA9-38-1	AL13015		I 3-4-1	SR 29050
		CICA9-36-1	AL13013		I 5-3-1	SR 29008
		Linea2-15	SR38005		I 5-5-3	SR 29057
		Linea2-20	SR38007		FN-28-2-1 [3]	AL 18027
		OLL5-25	SR55014		I1 -2-1	SR 29046
		Linea23-9-1	AL44021		I5-10-2	SR 29009
		PAPERU2-1	SR195001			
		MTU9-1-2	SR191000			
b)	SRL 5	FN-47-1	SR 18036	SRL 6b	DULAR16-1	AL 16006
		I 6-5-2[3]	SR 29010		FN-26-5	AL 18023
		I 9-1-1	SR 29016		FN-45-1	AL 18034
		I 9-9-2	SR 29017		CICA8-104-1[3]	SR 12002
		I 14-6-2	SR 29080		CEYSV27-1	SR 6068
		I 16-4-1	SR 29084		CEYSV27-2	SR 6038
		I 18-1-2	SR 29088		ORY2-19-2 [3]	SR 52001
		I 20-3-2	SR 29038	ALL 9	TSU2-1	AL 70001
		IR42-4-4	SR 26009	ALL 10	CT-80000	AL 150000
		IR42-5-2	SR 26011		K1-18-1	LL 30008
				ALL 13	K51-9-1	AL 33008
					K51-1-3	AL 33002
c)	SRL4	I15-7-2	SR 29029	ALL 7	USEN3-1	AL 71002

[1] Compatibility (+ = virulence) of lineages to the R-genes: a) *Pi-1* (-) *Pi-2* (+); b) *Pi-1* (+) *Pi-2* (-); c) *Pi-1* (-) *Pi-2* (-).

[2] Identification number given at Purdue University.

[3] Isolates used for mixtures in double compatibility tests.

Table 4. Resistance of the progeny of plant C101A51xC101LAC-12 to representative isolates of *Pyricularia grisea* by MGR lineages from Colombia.

		Plants (F_2)/Plant Families (F_3 and F_4)		
Progeny	Lineage	Resistant [1]	Susceptible [2]	Total
F_2	2 + 5 [3]	73	60	133
	2 / 5 [4]	65	67	132
F_3 [5]	2 + 5	22	57	79
	5 + 6b + 6c + 9	5	13	18
	6c + 9 + 10 + 13	8	1	9
F_4 [5]	6c + 9 + 10 + 13	9	0	9

[1] Resistant plants in the F_2; non-segregating resistant families in the F_3 and F_4.

[2] F_3 plants showing typical spindle-shaped lesions and at least 2% of leaf area infected; segregation for susceptibility in the F_3 and F_4.

[3] Inoculations with both isolates mixed together

[4] Serial inoculations with either isolate in a 5-day span. Mixture vs serial inoculations: $\chi^2 = 1.7$ ($P > 0.2$)

[5] Tests performed on 16 plants/plant family.

Table 5. Features of Mackill and Bonman's near-isogenic lines (NILs) bearing single blast resistance genes bred into the genetic background of rice cultivar CO39.

NIL	Resistance gene	Chromosome	RFLP Marker
C101A51	*Pi-2(t)* [1]	6	*RG-64* [2]
C101LAC	*Pi-1(t)*	11	*RZ-424* [3], *RG-303* [4] and *G-181* [5]
C101PKT	$Pi\text{-}4^{a}(t)$	12	*RG-869* [2]
C104PKT	*Pi-3(t)*	?	-
C105TTP-4L23	$Pi\text{-}4^{b}(t)$	12	*RG-869*

[1] Tentative denomination for *Pyricularia grisea* resistance genes (Mackill and Bonman, 1992).

[2] (Hittalmani et al., 1995).

[3] (Yu et al., 1996).

[4] (McCouch et al., 1998).

[5] (Kurata et al., 1994).

Table 6. Rice commercial cultivars, blast differentials and *Pi-1(t)* + *Pi-2(t)* pyramids assembled in the nursery traps, and advanced lines at field tests in Colombia.

Cultivars and differentials	Pyramids		
	Purdue's F_3 - F_4 C101A51 x C101LAC-12-		CIAT's F_6 (CT13432-) [1]
5685	F_3	F_4	1. PL2-1-1-M-M-M
A5173	P100	P100-01 to 08	2. PL2-4-2-M-M-M
BLUEBONNET	P101	P101-01 to 07	3. PL2-11-1-M-M-M
Caribe 8	P106	P101-18	4. PL4-2-1-1-M-M-M
CEYSVONI	P119	P266-01 to 08	5. PL4-2-2-M-M-M
CICA4	P266	P281-01 to 16	6. PL4-14-1-M-M-M
CICA8	P278		7. PL5-1-1-M-M-M
CICA9	P281		8. PL5-1-2-M-M-M
CO39			9. PL7-5-2-M-M-M
FUKUNISHIKI			10. PL7-7-1-M-M-M
IR8			11. PL7-10-1-M-M-M
IRAT13			12. PL8-7-2-M-M-M
LAC23			13. PL8-9-1-M-M-M
Linea 2			14. PL8-10-2-M-M-M
METICA1			15. PL8-15-3-M-M-M
ORYZICA1			
ORYZICA3			
ORYZICA Llanos 5			
PETA			
Sabana 6			

[1] Pyramids from the same C101A51 x C101LAC cross as the Purdue's lines, selected by the Centro Internacional de Agricultura Tropical (CIAT) at Santa Rosa.

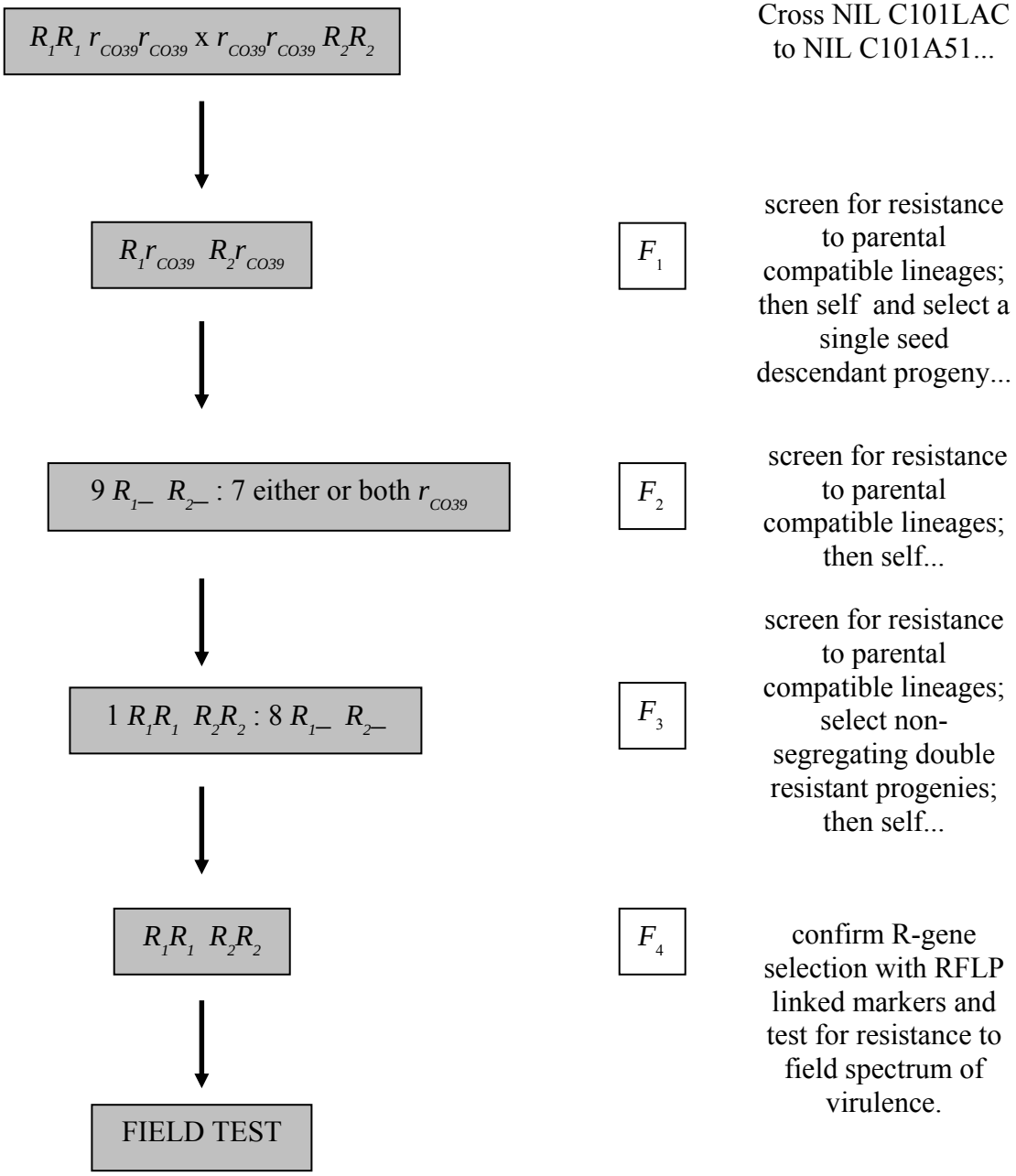

Figure 2. Diagram for selecting R-gene pyramids to test a lineage exclusion strategy for breeding rice resistant to blast disease.

Figure 3. Map of Colombia indicating the sites for the field testing in the eastern plains as follows: 1: Granada. 2: Santa Rosa. 3: Puerto Lopez.

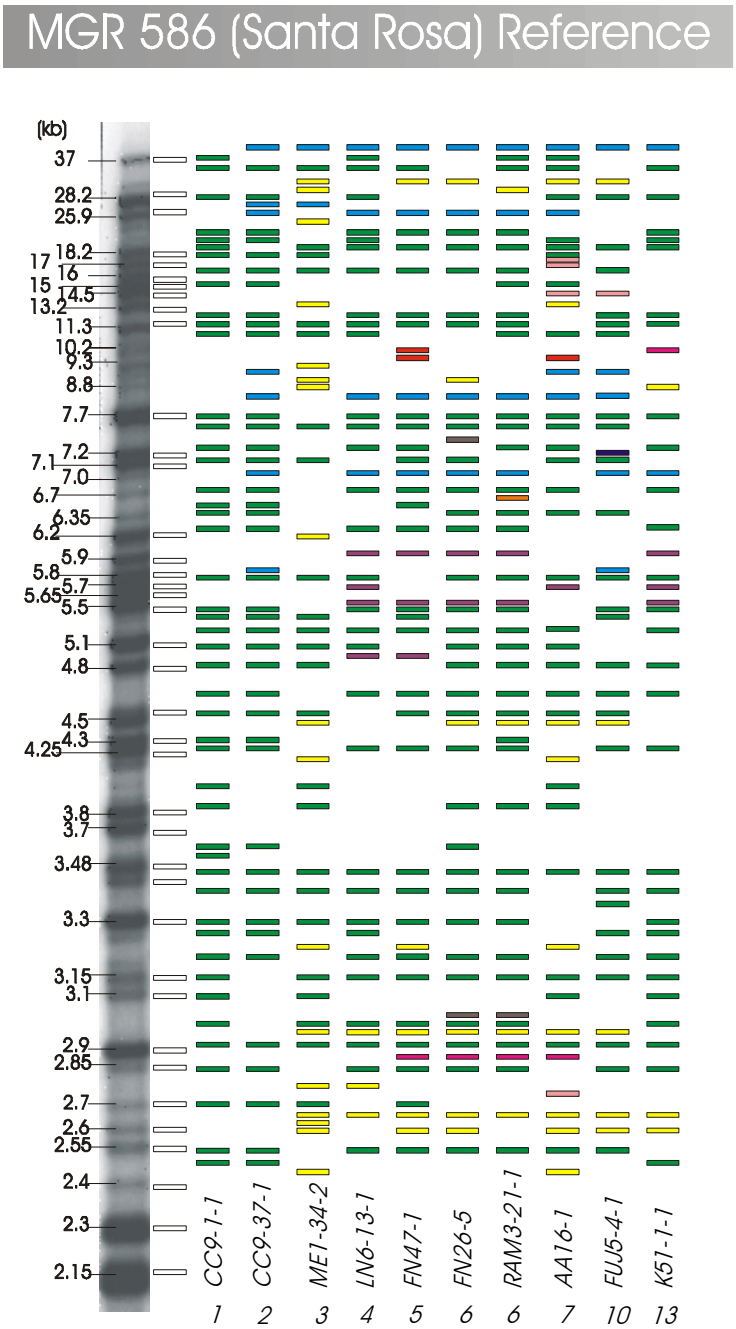

Figure 4. Schematic representation of MGR586 DNA fingerprint profiles of *Pyricularia grisea* in Colombian lineages (indicated by number below the name of the isolate). Bands representing RFLP fragments have been colored to better identify differences and similarities between lineages.

RESULTS

Selection of the Pyramids

Seed of seven of the single-seed descent plants selected from the cross C101A51 x C101LAC-12 as bearing *Pi-1(t)* + *Pi-2(t)* (hereafter named pyramids or P#) were successfully multiplied. The capability of the pyramids to exclude the spectrum of virulence of *P. grisea* in Colombia was tested in field surveys and greenhouse experiments and the homozygous condition of both R-genes in the pyramids was checked by molecular markers. This further served to clarify the appearance of new or previously undetected virulences.

Resistance-Gene Marker Analysis

The *Hae*III digestion of the PCR-amplified *RG-64* DNA showed a 0.75 kb restriction fragment marking the *Pi-2(t)*-linked locus on the probe itself (positive check) (Figure 5). This DNA fragment co-migrated with analogous fragments in C101A51 and pyramids P101, P266, P281. The F_4 pyramid descendants P101-04, P101-07, P266-06, P266-07, P281-10 and P281-10-M also had the 0.75 kb band, indicating the presence and homozygosity of the *Pi-2(t)*-linked locus in these progenies, as well. In contrast, the recurrent susceptible parent CO39 and the *Pi-1(t)* donor C101LAC were marked by a contrasting 0.65 kb band.

While the *RG-64* marker unambiguously indicated the presence of the *Pi-2(t)* locus, the RFLP markers linked to the *Pi-1(t)* locus gave less straightforward results. *RG-303* hybridizations in *Taq*I digestions of DNA from the *Pi-2(t)* donor (C101A51) and

CO39 showed a 2.0 kb hybridizing fragment in contrast to a 2.4 kb fragment in the *Pi-1(t)* donor C101LAC and LAC23 (Figure 6). However, the pyramids P101, P266, P281 and their progenies P101-04, P101-07, P266-06, P266-07, P281-10 and P281-10-M also were marked by a 2.0 kb hybridizing fragment, similarly to Co39. A second hybridizing element about 4.0 kb in size differentiated some pyramids from the recurrent susceptible donor. However, this fragment was not observed in the *Pi-1(t)* donor nor in the NIL.

Additional genetic differences were observed between C101LAC and the F_3 pyramids P100, P101 and P266 with RFLP probes *G-181* and *RG-303* on *Eco*RI-digested DNA (Figure 7). The Southern blots showed a 4.5 kb band for C101LAC compared to a 4.0 kb band in CO39, two accessions of C101A51, and A5173. However, the *Pi-1 (t)* donor, LAC23, and one of the accessions of C101LAC (7) also were marked by a 4.0 kb band.

Hybridizations with the probe *RZ-536* on *Dra*I-digested DNA marked the *Pi-1(t)*–linked locus with an 11 kb band in C101LAC and LAC23 (Figure 8). The pyramids P100, P101, P106, P119, P266, P281 and their F_4 progenies P100-03, P100-01-M, P101-03, P101–M, P-106-05, P106–M, P119-01 and P-119-M, together with the pyramid check HIT, which has been reported as homozygous for the *Pi-1(t)* locus as tested with *RZ-424* (Dr. S. Hittalmani, personal communication), also were marked by the 11 kb fragment. The susceptible check CO39 was marked by a 10 kb restriction fragment, which also was present in the F_3 pyramid P278.

Other *RZ-536* hybridizations on *Eco*RI-digested DNA similarly distinguished CO39 from the *Pi-1(t)*-bearing C101LAC, LAC23, the pyramids P100, P106, P119, P266, P281 and their descendants P106-05, P106-07, P281-10 and P281-10-M. The *Pi-1(t)*-linked locus was marked by a 9 kb RFLP observed from the R-gene donor and the pyramids (Figure 9). In contrast, CO39 and several accessions of C101A51 were marked by a 6 kb RFLP. A possible heterozygous condition of the F_3 pyramid P278 was indicated by the display of both bands and its F_4 descendants P278-02 and P278-06 were marked by a single and both bands, respectively. This pyramid seems to be still

segregating for *Pi-1(t)*. A set of F_6 descendants from the cross C101A51 x C101LAC that were selected at Santa Rosa by CIAT (Dr. F. Correa, personal communication) was also examined for *RZ*-536 RFLPs in *Eco*RI digestions. The pyramids CTF6-01, CTF6-02, CTF6-04, CTF6-07 and CTF6-14 were found to be homozygous for the 9 kb *Pi-1(t)* marker. In similar fashion, the homozygous condition of the *RZ-536* locus was confirmed in all 15 field-resistant pyramids from CIAT, simultaneously with several accessions of C101LAC and HIT (Figure 10).

To clarify the mismatching identification of the *Pi-1(t)*-linked loci on different accessions of C101LAC, *Dra*I-digested DNA from several accessions obtained at IRRI, CIAT and Purdue were Southern blotted and hybridized with *RZ536* (Figure 11). The seed accession from CIAT, three locally reproduced lots from this accession, and a greenhouse-reproduced lot (GH-04-1) acquired originally from IRRI, all contained the 11 kb restriction fragment present in LAC23 and HIT. However, the DNA from one seed lot from IRRI, several from the greenhouse (multiplied from the same seed acquired from IRRI) and CO39, were marked by a 10 kb band. These results indicated the occurrence of genetic impurities among different accessions of C101LAC.

Summarizing, the F_3 pyramids P101 and P266 were marked as positive and homozygous by all of the RFLP markers linked to both the *Pi-1(t)* and *Pi-2(t)* loci. Additionally, the F_3 pyramids P100, P106, P278 and P281 were positive for the *Pi-2(t)* marker and at least one of the probes linked to the *Pi-1(t)* locus. Several F_4 pyramids (particularly P101-04, P101-07, P266-06, P266-07 and 281-10) as well as F_5 pyramid P281-10-M also were marked as positive by restriction markers linked to both R-genes (Table 7).

Resistance Test of the Pyramids

The comparative reaction of both C101A51 and C101LAC, the susceptible check (CO39) and the *Pi-1(t)* + *Pi-2(t)*-bearing pyramids (P101, P101-04, P101-07, P266-02, P266-06, P266-07, P281-10 and P281-10-M) to artificial inoculations with

representative isolates from Colombian lineages SRL-2 (n= 8), SRL-5 (n= 13), SRL-6b (n= 8) and SRL-6c (n= 8) and the mixtures SRL-2+5 (n= 3) and SRL-6b+6c (n= 4) confirmed the capability of the pyramids to exclude complementary *Pi-1(t)* + *Pi-2(t)* virulences (Figure 12). Whereas the parents were severely damaged, neither single isolates nor mixtures of isolates (cultured together in the same Petri dish to permit potential parasexual recombination) were virulent on any member of the selected pyramids.

SRL-2 isolates severely infected (80% LAI) the susceptible cultivars CO39 and C101A51, but caused little disease (small flecks and less than 3% LAI) to the *Pi-1(t)*-bearing C101LAC and did not affect the pyramids. Lineage SRL-5 isolates severely infected CO39 and C101LAC (70% and 60% LAI, respectively) but did not infect the *Pi-2(t)*-bearing C101A51 at susceptible levels (4% LAI) or the pyramids. The combination of SRL-2 + SRL-5 isolates severely infected C101A51 and C101LAC as well as CO39 (70% LAI). No isolate from lineages SRL-2 or SRL-5, individually or in combination, affected the pyramids. The maximum LAI recorded on any pyramid during the entire experiment was 4%, which is a high resistance level as compared to the leaf area affected under the same conditions on check cultivars.

SRL6b isolates severely infected CO39 (80% LAI) and C101LAC (60% LAI) but caused little disease on C101A51 or the pyramids (4% and 1% LAI, respectively). Likewise, the isolates from the SRL-6c lineage severely infected C101A51 (70% LAI) and CO39 (80% LAI) but caused small, rounded, yet resistant, lesions on C101LAC (12% LAI) and the pyramids (3% LAI). The combination of SRL-6b + SRL-6c isolates severely infected CO39, C101A51 and C101LAC (75%, 60% and 40% LAI, respectively) but produced only small rounded lesions on the pyramids (4% LAI average but up to 9% LAI on P266 and 8% LAI on P281-10-M). Additional pathogenicity tests indicated the absence of virulence of the selected isolates from lineages SRL-1, SRL-4, ALL-7, ALL-10 and ALL-13 towards the pyramids.

The SRL-6c lineage isolates that produced moderate resistant reactions on some of the pyramids were tested again at Purdue and at CIAT. These isolates were not

virulent to the *Pi-1(t)*-bearing NIL C101LAC nor to the pyramids in these tests (Dr. Fernando Correa, personal communication).

Results from the artificial inoculation assays indicated the achievement of highly favorable conditions for the infection process at Purdue. Consequently, it was concluded that the best way to evaluate virulence or susceptibility from this assay was by comparing the levels of disease between the tested lines and the susceptible checks.

Isolates from rare and atypical lesions developed on the pyramids at the selection and screening processes were subsequently cultured to clarify the occurrence of mutations. No significant changes in pathogenicity, other than the loss of virulence, were recorded in these tests, even though some of the nine original re-isolates were serially re-tested up to three times (Table 8). None of the re-isolations from lineages SRL-2, SRL-5, SRL-6b and SRL-2 + SRL-5 apparently affecting the pyramids gave indications of increased virulence towards the pyramided genes. The occurrence of rare or unusual lesions on the pyramids did not indicate shifts in virulence but only the capability of the fungus to survive and reproduce on resistant hosts, although at a minimal extent, under test conditions.

The MGR-DNA haplotypes of the re-isolated strains were identical to those of the respective isolates initially inoculated (Figure 13). The conservation of original haplotypes, even after inoculations with combined lineages, indicated the absence of inter-lineage parasexual events for *P. grisea*, during the limited extent of the experiments performed.

Field Tests

Field tests of the selected F_3 and F_4 pyramids were conducted for the first time at Santa Rosa in July 1996, although the resistance of a progeny from the same C101A51 x C101LAC cross has been routinely screened at this site by CIAT, since 1995. During the earliest 1996 planting, blast disease severely affected more than 75% of the leaf area of C101A51, followed by the susceptible check CO39 with between 25-50% LAI (Figure 14). LAC23, Llanos 5 and C101LAC were barely affected (less than 2% LAI), while the

disease compromised the pyramids P101 and P266 at susceptible levels (25-50%). During the second planting at Santa Rosa (November 1996), the severity of the disease was below susceptible levels in the NIL and the pyramids but at a moderately susceptible level on CO39 (grade 5). The only trial conducted at Granada in 1996 showed disease severity on the pyramids, C101LAC, Llanos 5 and CO39 within the threshold of resistance levels (grade 4). Severity at susceptible levels (grade 5) was recorded only on C101A51 in this trial.

Four field trials were conducted with monthly planting intervals at three sites from April-July in 1997. During 1997, the warmest and driest year of the three tested, rainfall diminished noticeably (more than 600 mm per year), and differences between daily maximum and minimum temperatures were higher (14ºC as compared to 10ºC in 1996). These weather conditions, regarded as highly favorable for rice blast disease incidence, compromised plant growth and caused severe rice blast epidemics at all sites.

Again, the resistance of the pyramids was compromised only at the Santa Rosa disease nursery, where during the second and third planting dates in 1997 the disease severity reached grade 6 (Figure 15). Rice blast killed CO39 (grade 9) during the first and second planting dates, and C101A51 at the second, at Santa Rosa. These cultivars also were severely infected at this site throughout the year. C101LAC, Llanos 5 and the pyramids were barely affected at Santa Rosa during the first and fourth planting dates in 1997. The severity of the disease reached levels lower than grade 5 on these cultivars but was only up to grade 4 on the pyramids.

In Puerto Lopez, one or both NILs were severely infected throughout 1997. In contrast, for Llanos 5 and the pyramids the disease was always below grade 4.

In Granada, the severity of the disease rose to susceptible levels during the fourth planting of 1997 on CO39, both on the NILs and Llanos 5 (Figure 15). The pyramids were unaffected by rice blast in Granada during 1997.

During 1998, disease was recorded again at susceptible levels in CO39 and C101A51 at Santa Rosa (July and September) and Puerto Lopez (July) (Figure 16).

C101LAC, Llanos 5 and the pyramids were never affected during 1998. The maximum level of disease recorded in the pyramids at Santa Rosa was lower than grade 4 (July).

However, there was an additional variable in the 1998 field trials. The pyramids planted in 1998 were derived from seeds collected in mass from uninfected individual pyramids from the 1997 trials; the original seed stocks were exhausted during the first two years of trials. Consequently, there may have been a secondary selection for enhanced resistance prior to the 1998 experiments.

In summary, the field tests demonstrated that the *Pi-1(t)* + *Pi-2(t)* pyramids were consistently and highly resistant to blast infection at the two commercial field sites tested, even when both R-genes (in NILs) were individually defeated at the sites. However, there were transient breakdowns of the pyramids at the Santa Rosa blast "hot spot" during certain 1996 and 1997 experiments. The disease severity of the infected pyramids was, almost always, less than that of one or both of the NIL parents. The disappearance of susceptible levels of pyramid infection at Santa Rosa in 1998 accompanied a change in pyramid seed source.

Population Structure of Field Isolates

A total of 376 isolates, 165 from the pyramids, were characterized by MGR-DNA fingerprinting analysis (Table 9). The fingerprints showed the isolates collected from the field experiments to be members of three previously characterized lineages SRL-2, SRL-4 and SRL-6. The vast majority of isolates (241) were of lineage SRL-6, 134 of them isolated from lesions developing on the pyramids. The second-most abundant lineage was SRL-4 (104 isolates), but only 27 of them were recorded as infecting the pyramids in the field. Twenty-six isolates were identified as in lineage SRL-2 but only two of them were from lesions on the pyramids during the field experiments.

UPGMA analysis of representative haplotypes (H) from the Santa Rosa experiments and selected archival isolates illustrated the limited haplotype diversity encountered during the experiments (Figure 17). Field isolates from lineage SRL-6

clustered in two branches with 15% average distance. Two very similar (4% distance) haplotypes found on the pyramids (H-P100 and H-P106) clustered closely with archived isolate I3-2-2; the latter was collected in Santa Rosa during 1991 and is compatible with *Pi-2(t)*. Other archival SRL-6 isolates from Santa Rosa that were not compatible with *Pi-2(t)*, F28-2-1 and C8-104-1, were more distantly (16-20%) related to the pyramid-infecting SRL-6 isolates. However, the total SRL-6 haplotype distance shown is within that previously observed (Levy et al., 1993; Manry, 1995).

Isolates assigned to lineage SRL-4 averaged 28% distance from the SRL-6 cluster and had 18% average distance among themselves. The between-lineage distance is slightly less (vs. 35%) and the distance within lineage SRL-4 is more (vs. 8%) than that detected at Santa Rosa in 1993 in a more extensive sampling (Levy et al., 1993). The average distance between SRL-2 isolates from the experiments was comparable to that observed previously and the SRL-2 haplotypes were very similar (94%) to archived SRL-2 isolate C9-37-1. The distinctiveness of the SRL-2 cluster from those of the other lineages was also as observed previously.

The relative abundance of lineages varied among sites and years. Most isolates collected at Granada in 1997 were identified as lineage SRL-4 (Figure 18). Isolates in other lineages were very rare at this site. In contrast, the SRL-6 lineage predominated at Puerto Lopez in 1997, when only a third of the sample was identified as lineage SRL-4, and this was limited to a single planting date. Isolates in lineage SRL-2, although at a ten-fold lesser frequency, were also found in Puerto Lopez during 1997. A more even distribution of lineages SRL-4 and SRL-6 was found at this site during 1998, while isolates of lineage SRL-2 were rare. The widest representation of Colombian lineages was collected at Santa Rosa, although the predominance of SRL-6 isolates was very noticeable. The large majority of the isolates collected at Santa Rosa during 1997 was lineage SRL-6. Isolates in lineage SRL-4 had a limited presence at this site that year. A more balanced distribution of lineages SRL-6 and SRL-4 was observed at Santa Rosa during 1998.

Four distinct haplotype profiles within the SRL-6 lineage were found at Santa Rosa in 1996, although most of the isolates from the pyramids were grouped into only two of them (Table 10). The same or very similar (1-2 bands different) haplotypes were identified from lesions developed on the pyramids during the following years, particularly those of the two most abundant 1996 profiles. The continuing haplotype similarities in the field suggest that the principal haplotypes infecting the pyramids were resident at Santa Rosa throughout the three years of the study. By example, the stable tester (originally wild-type) GUY11 shows small but still perceptible haplotype differences from cultures maintained at different laboratories in Africa, India and France (Figure 19). The fingerprint differences detected by the presence or absence of a few fragments in GUY11 haplotypes (shown by the arrows) were comparable in proportion to the haplotype differences found in lineage SRL-6 isolates infecting the pyramids at Santa Rosa from 1996-98.

Because the severity recorded during the field tests on the pyramids suggested a probable emergence of new virulence towards the combination of the selected genes or, alternatively, a lack of penetrance of the R-genes on the selected pyramids, several pathogenicity assays were conducted. The virulence of the single-spore cultures from lesions on the pyramids was tested by artificially inoculating their original hosts of isolation. These tests showed that only isolates from lineage SRL-6 were able to reinfect their original pyramid host (Table 11). The two SRL-2 isolates from the pyramids at Santa Rosa (1996), were not virulent to the pyramids from which they were collected or to C101LAC, as expected. Apparently, these SRL-2 isolates were collected from opportunistic infections or from unknown contaminant plants in the field plots. The SRL-4 isolate collected from P-278 was partially virulent to this pyramid and also virulent towards C101A51, a feature not previously documented in lineage SRL-4 (Table 1). However, given that this isolate did not infect C101LAC, further suspicions emerged about the probable segregation of *Pi-1(t)* in pyramid P278.

An additional set of isolates from newly reported infections on Oryzica Llanos 5 in Colombia was acquired to clarify any recent virulence shifts for lineage SRL-4. Lineage SRL-4 isolates from the Colombian cultivar Oryzica Llanos 5 were found not to

be reinfective in artificial inoculations at CIAT (Dr. Fernando Correa, personal communication). Because the resistance in Llanos 5 has been maintained for a prolonged time not only in Colombia but also at different environments around the world, the isolates were also tested against the NILs and different pyramid accessions. These isolates were virulent to C101A51 and CO39 only, confirming the reported emergence of new virulence towards *Pi-2(t)* in the SRL-4 lineage (Table 12). However, these isolates were not capable of infecting the pyramids. The lineage SRL-6 isolate from P-266-01, included for comparison in this test, maintained pathogenicity towards the pyramids selected at Purdue but was not virulent on F_6 pyramids selected at CIAT or those from Dr. S. Hittalmani. The severity on the pyramids from Purdue was as high as that observed on the susceptible cultivar CO39 and C101A51. Notably, however, the level of susceptibility on C101LAC was marginal, which again raised the issue of the level of penetrance of the *Pi-1(t)* gene in some pyramids selected at Purdue.

The blast population diversity detected on the pyramids, the parent NILs and selected cultivars included only three MGR lineages, SRL-2, SRL-4 and SRL-6. Interestingly, these are the principal lineages in the testing area that exclusively show *Pi-2(t)* compatibility. However, only isolates belonging to SRL-6, the only lineage among these three having some compatibility with *Pi-1(t)*, were re-infective on the pyramids.

Pyramid infection was restricted to the unique ecology of the Santa Rosa "hot spot", but only occasionally so during 1996-1997 and not at all during 1998 following a secondary selection of seed from resistant pyramids in the 1997 trials. Advanced generation, field-selected pyramids from CIAT also were resistant to inoculation with all SRL-6 isolates tested.

Table 7. Linkage to the *Pi-1(t)* and *Pi-2(t)* genes in pyramids from a cross between the near-isogenic lines C101A51xC101LAC, as determined by RFLP markers.

	Pi-2(t) marker	*Pi-1(t)* marker		
Progeny	*RG-64*	*RG-303*	*G-181*	*RZ-536*
F_3	P100		P100	P100
	P101	P101	P101	P101
	P106			P106
				P119
	P266	P266	P266	P266
	P278			P278
	P281	P281		P281
F_4	P101-04	P101-04		P100-03
	P101-07	P266-06		P101-03
	P266-06	P266-07		P101-M
	P266-07			P106-05
	P281-10			P106-07
				P106-M
				P119-01
				P119-M
				P266-21
				P266-M
				P278-01
				P278-02
				P278-06
				P281-10
F_5	P281-10-M			P281-10-M

Table 8. Pathogenicity of a sample of strains of *Pyricularia grisea* isolated from atypical and rare lesions on several pyramids and C101A51, after inoculations with isolates from MGR lineages in Colombia.

Inoculum			Reaction [1]		
Lineage	Isolate (s)	Isolated from	C101A51	C101LAC	Pyramids
SRL2	MTU9-1-2	P281-10-M	0.4	-	-
			0.8	-	-
			0.1	-	-
	MTU9-1-2	P281-10-M [2]	0.2	-	-
SRL5	I 6-5-2	P266-06	0.1	0.7	-
			-	-	-
SRL6b+c	ORY2-19-2 + FN-28-2-1	C101A51	0.1	-	-
SRL2 + SRL5	CICA9-37-1 + I6-5-2	P101-07	-	0.7	-
			-	-	-
			-	0.3	-
		P266-06	-	0.4	-
			-	-	-
		P266-07	0.2	-	-
		P281-10	0.6	-	-

[1] Proportion of leaf area infected or negative (–) when less than 0.05 (16 plants/cultivar).

[2] Re-isolated from a first inoculation of P281.

Table 9. MGR lineage distributions for isolates of *Pyricularia grisea* from current cultivars and pyramids in field tests at three sites in Colombia.

			MGR Lineage [1]			
Location	Year	Tests [2]	SRL-2	SRL-4	SRL-6	Unassigned
Santa Rosa	1996	2	9 (2) [3]	4 (1)	8 (6)	3
	1997	4	5	9 (4)	99 (62)	2
	1998	2	1 (1)	27 (12)	45 (22)	0
Puerto Lopez	1997	3	8	18 (2)	66 (40)	0
	1998	2	1	19 (8)	21 (3)	0
Granada	1997	2	2	27 (1)	2 (1)	0
			26 (3)	104 (28)	241 (134)	5

[1] Lineages as established by MGR-DNA fingerprinting at Santa Rosa (Levy et al., 1993)

[2] No. of tests per site.

[3] No. of isolates and (No. of isolates from C101A51xC101LAC pyramids) assigned to the lineage.

Table 10. Lineages assigned to isolates of *Pyricularia grisea* from current cultivars and pyramids from the cross C101A51xC101LAC–12 in Santa Rosa, 1996.

MGR Lineage						
SRL-2 [1]	SRL-4 [1]	SRL-6A [2]	SRL-6B [2]	SRL-6C [2]	SRL-6D [2]	Unassigned
A5173	BB50	IR8	CARIBE8	BB50	P100-05	CICA4
C101A51	CICA7	ORY1	METICA1	ORYZ3		C101LAC
LAC23	CO39	P100[4]	P101	5685		
LINEA2	FUKUN	P106	P119	P266-07		
LLANOS5	P2782	P100-03	P100-03			
PETA		P100-06	P100-07			
P100-01[3]		P100-08	P266-02			
P101-03		P101-01	P266-04			
		P101-04	P266-05			
		P101-05	P266-06			
		P101-07	P266-08			
		P101-18				
		P266-01				
		P266-03				

[1] Isolates from lineages SRL 2 and SRL4 collected on the pyramids were not pathogenic in artificial inoculations to the host from which they were isolated.

[2] Four haplotypes were identified within the lineage SRL-6 as indicated by the given letter following the number.

[3] P followed by a number means F_3 of the cross C101A51xC101LAC-12.

[4] P followed by two numbers means F_4 of the same cross.

Table 11. Ratio of susceptible/total plants in pathogenicity tests of isolates of *Pyricularia grisea* from lesions on the pyramids at Santa Rosa during 1996.

	Isolated from C101A51xC101LAC-12-							
	100	101	106	119	266	278	100-01	101-3
Lineage [1]	6	6	6	6	6	4	2	2
Pyramid of origin	1.0 [2]	1.0	1.0	1.0	1.0	0.7	0.2	0.1
C101A51	1.0	1.0	1.0	1.0	1.0	1.0	0.9	0.3
C101LAC	0.8	0.8	1.0	1.0	1.0	0.3	0.3	0.2
Co 39	1.0	1.0	1.0	1.0	1.0	1.0	0.8	1.0

[1] Colombian lineages as determined by MGR-DNA fingerprinting (Levy et al., 1993).

[2] Reactions based on the presence of sporulating lesions covering at least 5% of the leaf area on 16 plants/cultivar.

Table 12. Percentage leaf area infected on different accessions of the pyramids from a cross of near-isogenic lines C101A51xC101LAC and their parental ancestors, in a pathogenicity test with strains of *Pyricularia grisea* from P266-01 and Oryzica Llanos 5.

					Pyramids		
Lineage	Isolate	CO39	C101A51	C101LAC	CIAT	HIT	Purdue
SRL6	P266-01 [1]	35 [3]	35	8	2	2	30
SRL4	55035-40 [2]	35	65	0	0	0	0

[1] Isolated from C101A51xC101LAC-12-266-01 at Santa Rosa, Colombia, 1996.

[2] Six isolates partially compatible to Oryzica Llanos 5 (a gift from Centro Internacional de Agricultura Tropical, CIAT) (Correa-Victoria et al., 2000).

[3] Reaction based on average reaction of 16 plants/cultivar.

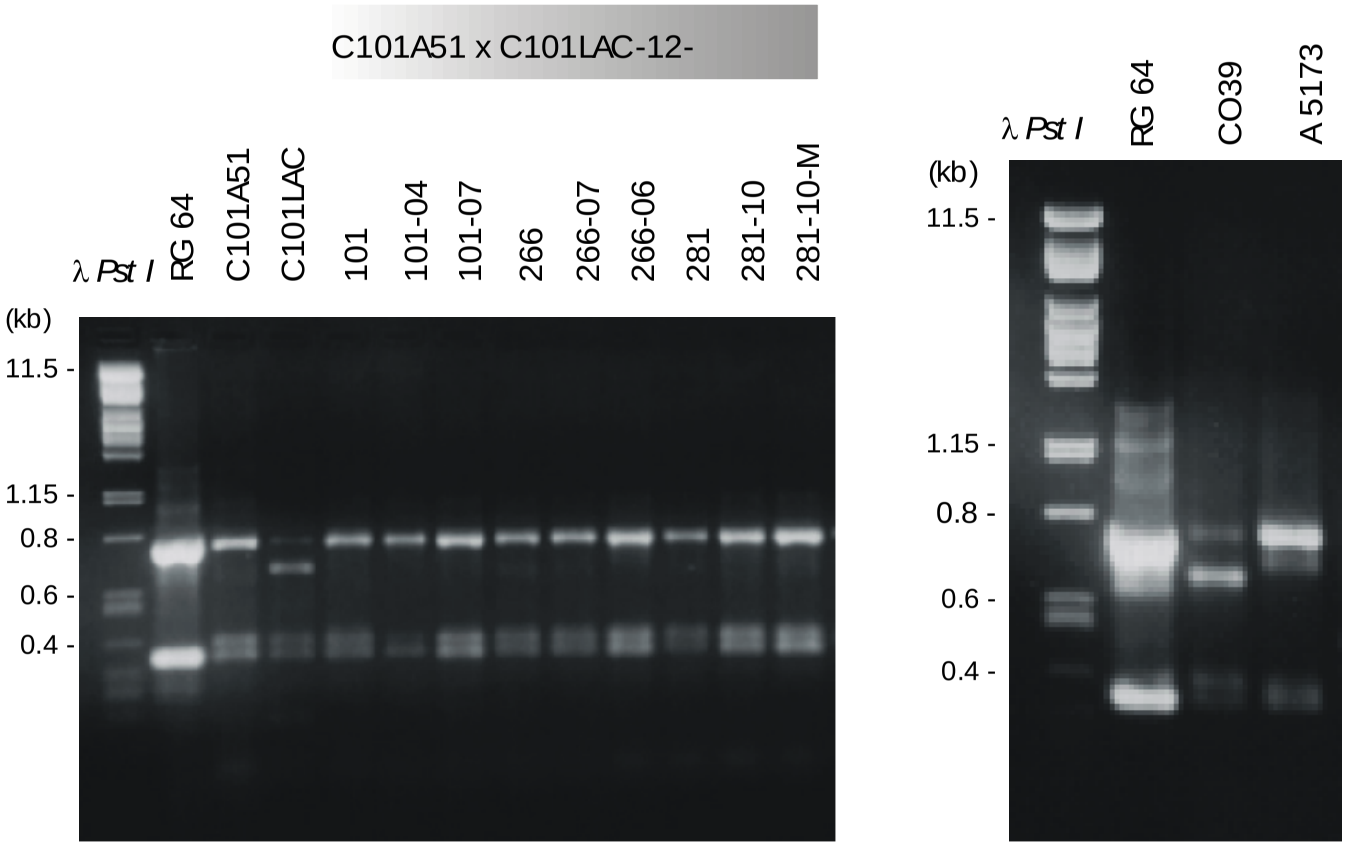

Figure 5. *Hae*III digestion of *RG-64* amplified DNA from the near-isogenic lines C101LAC (*Pi-2(t)*) and C101A51 (*Pi-1(t)*), the pyramids from the cross (C101A51xC101LAC-12-) and the susceptible check CO39. The 0.75 kb band indicates the homozygosity of the *RG64* locus, linked to the *Pi-2(t)* gene.

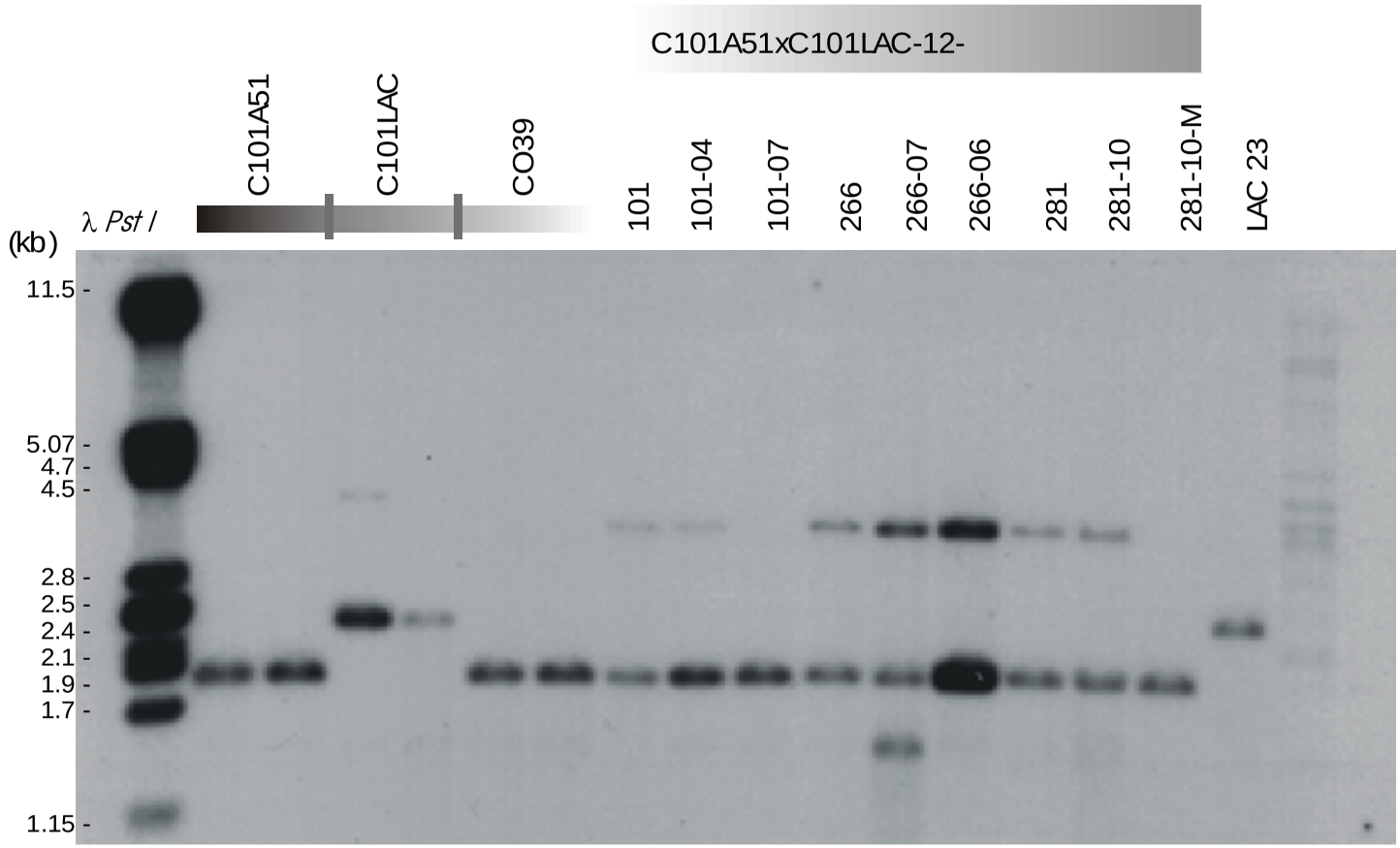

Figure 6. RFLPs of *Taq*I digested DNA from the near-isogenic lines C101A51 and C101LAC bearing *Pi-2(t)* and *Pi-1(t)*, respectively, the pyramids from their cross and the susceptible check CO39, with the *Pi-1(t)* linked probe *RG-303*. The 2.4 kb fragment marked the homozygosity of the locus on C101LAC and LAC23. A second band (4.0 kb) is observed differentiating several members of the pyramids from CO39.

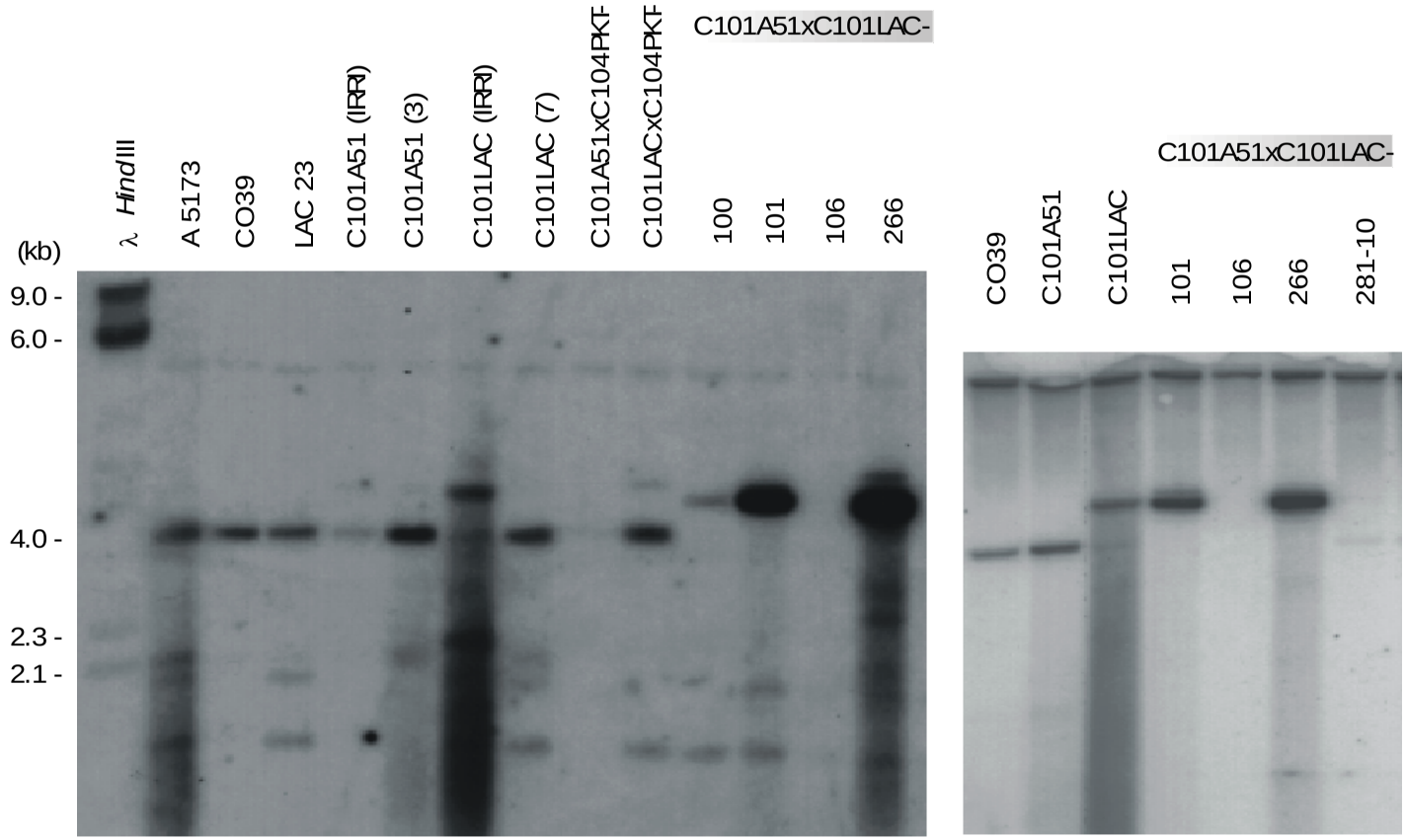

Figure 7. RFLPs of *Eco*RI digested DNA from the near-isogenic lines C101LAC (Pi-1(t)) and C101A51 (*Pi-2(t)*), the pyramids from their cross and the susceptible check CO39, with the probes *RG-303* (left) and *G-181* (right). The 4.5 kb band indicates the homozygosity of the *Pi-1(t)* linked locus in C101LAC and several members of the pyramids.

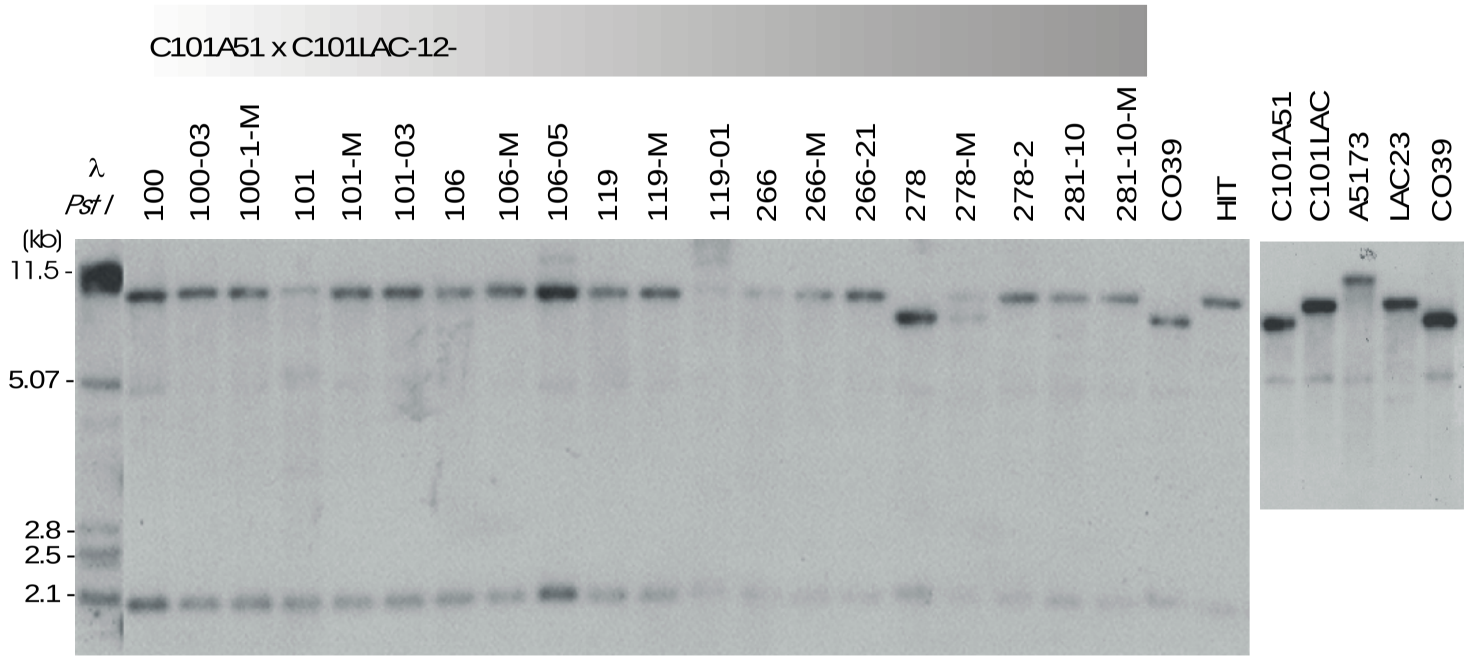

Figure 8. RFLPs of *Dra*I digested DNA from the near-isogenic lines C101LAC and C101A51 bearing *Pi-1(t)* and *Pi-2(t)*, respectively, the pyramids from their cross and the susceptible check CO39, hybridized with the *Pi-1(t)* linked probe *RZ-536*. The homozygosity of the *Pi-1(t)* linked locus is marked by the 11 kb hybridizing element in C101LAC, LAC23 and most members of the pyramids.

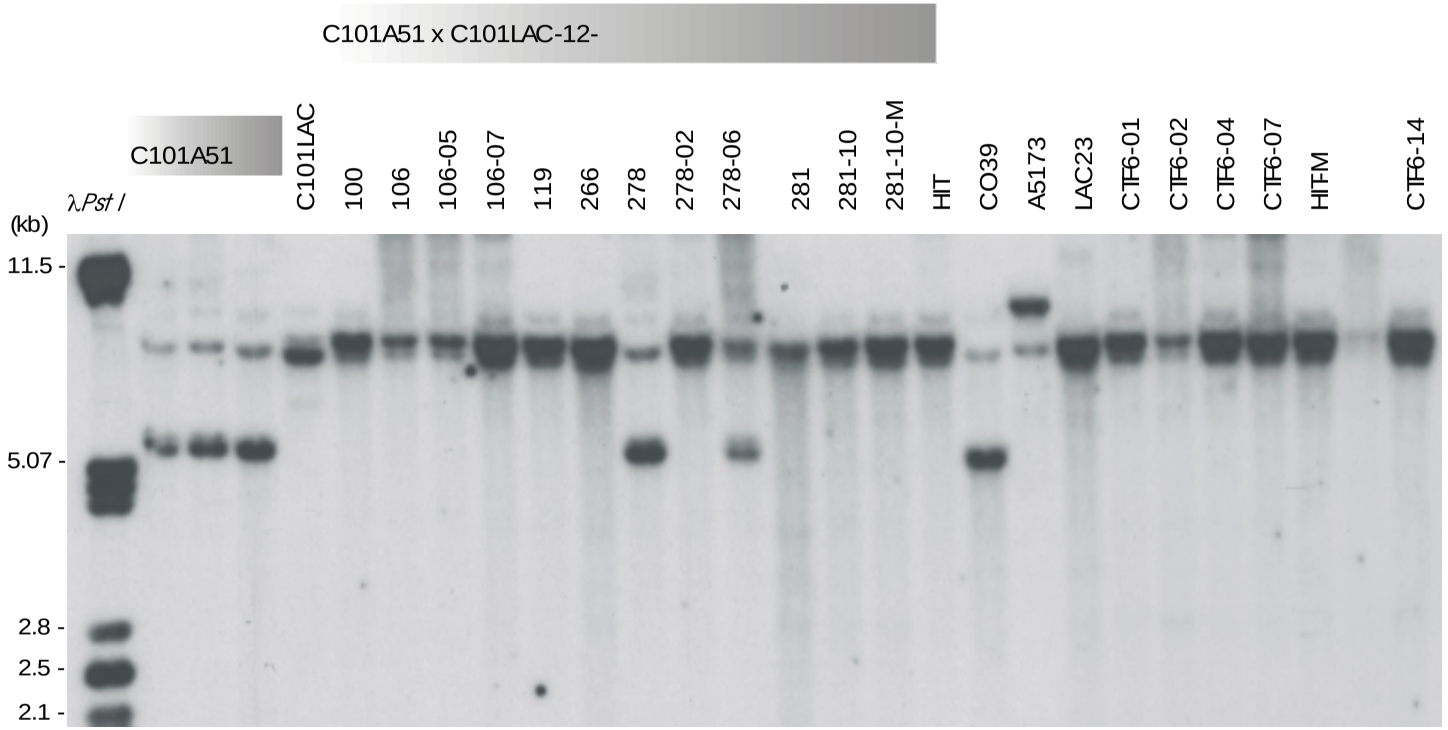

Figure 9. RFLPs of *Eco*RI digested DNA from the near-isogenic lines bearing *Pi-1(t)* (C101LAC) and *Pi-2(t)* (C101A51) with *RZ-536*. The pyramids selected by CIAT at Santa Rosa (CTF6-), at Purdue (C101A51xC101LAC-12-) and by S. Hittalmani (HIT) were marked by the 9.0 kb band indicating the homozygosity of the *Pi-1(t)*-linked locus, in contrast to C101A51 and the susceptible check CO39.

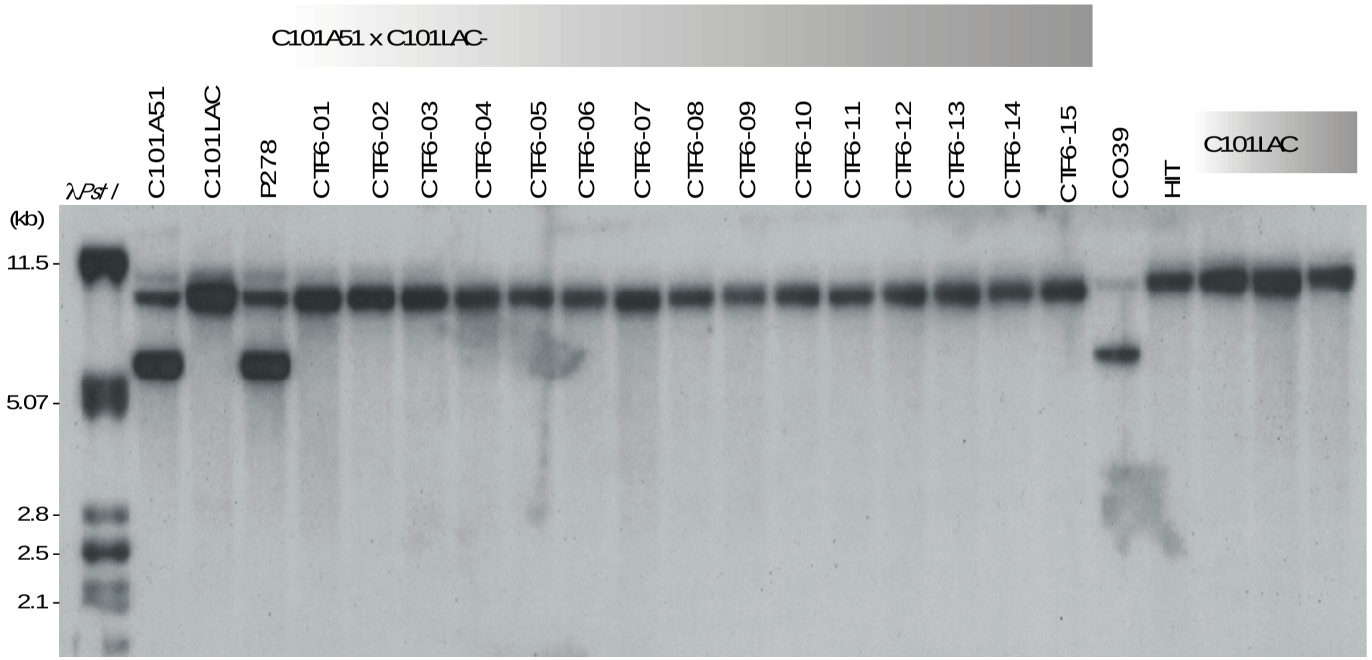

Figure 10. RFLPs with *RZ-536* of *Eco*RI digested DNA from the near-isogenic lines C101LAC *(Pi-1(t)*) and C101A51 (*Pi-2(t)*), the F_6 pyramids derived from their cross and selected at Santa Rosa, and the susceptible check CO39. The homozygosity of the *Pi-1(t)*-linked locus is marked by the 9 kb band in C101LAC, HIT and all the pyramids from CIAT.

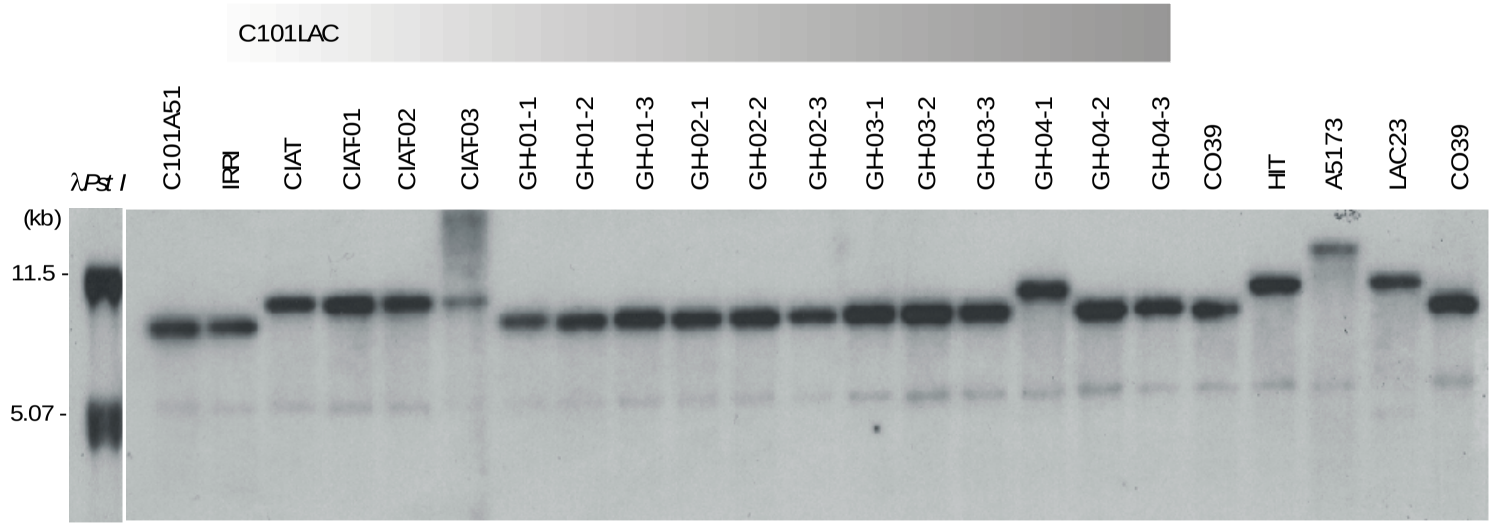

Figure 11. RFLPs for the *Pi-1(t)*-linked probe *RZ-536* on *Dra*I-digested DNA from different accessions of the near-isogenic line C101LAC and the susceptible check CO39. The homozygosity of the *Pi-1(t)*-linked locus is marked by the 11 kb band in LAC23, the C101LAC accesion from CIAT and the pyramid HIT.

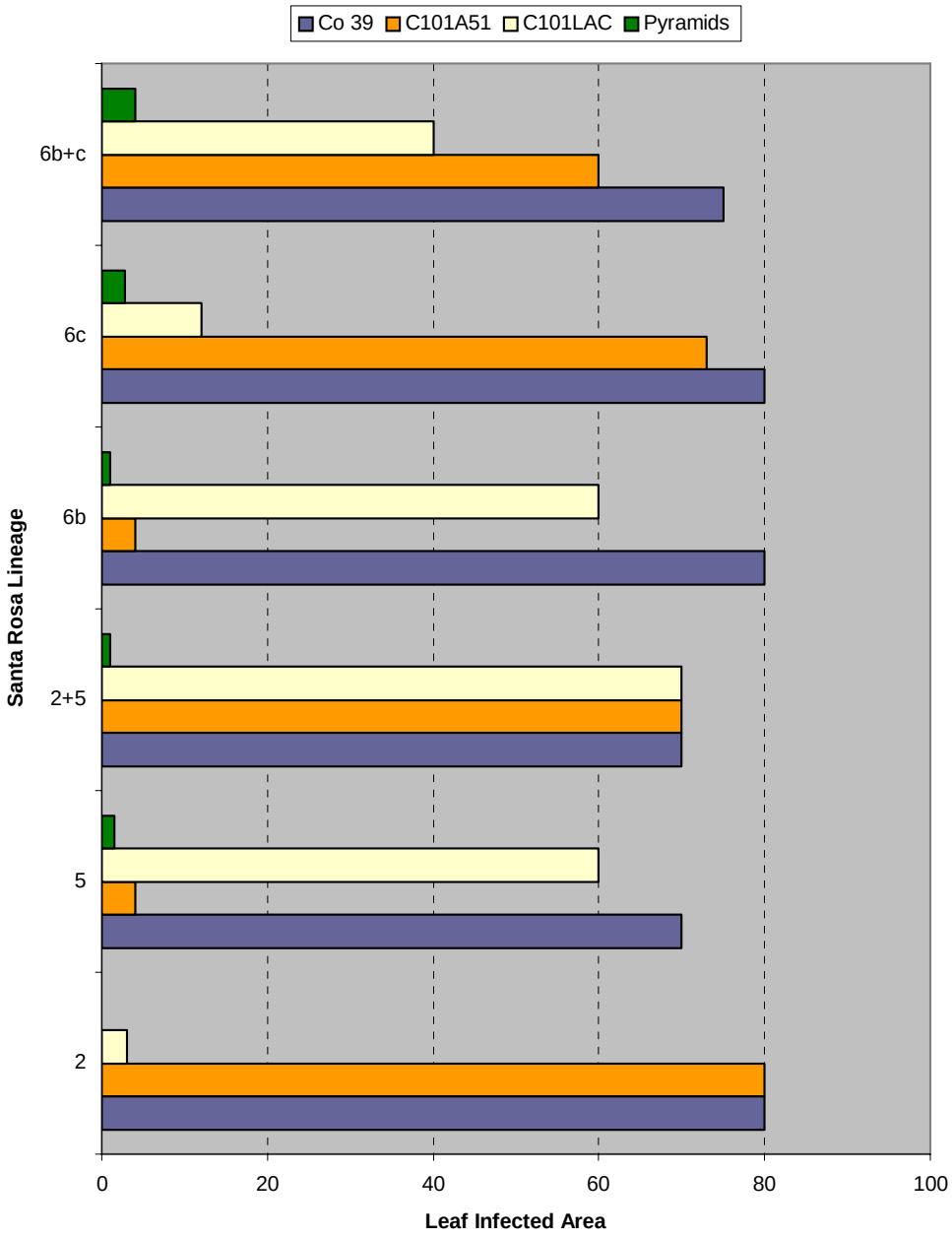

Figure 12. Disease response of the near-isogenic lines C101LAC (*Pi-1(t)*) and C101A51 (*Pi-2(t)*), the pyramids from their cross and the susceptible check CO39 to inoculations with representative isolates of *Pyricularia grisea* from Colombian MGR lineages SRL-2, SRL-5, and SRL-6.

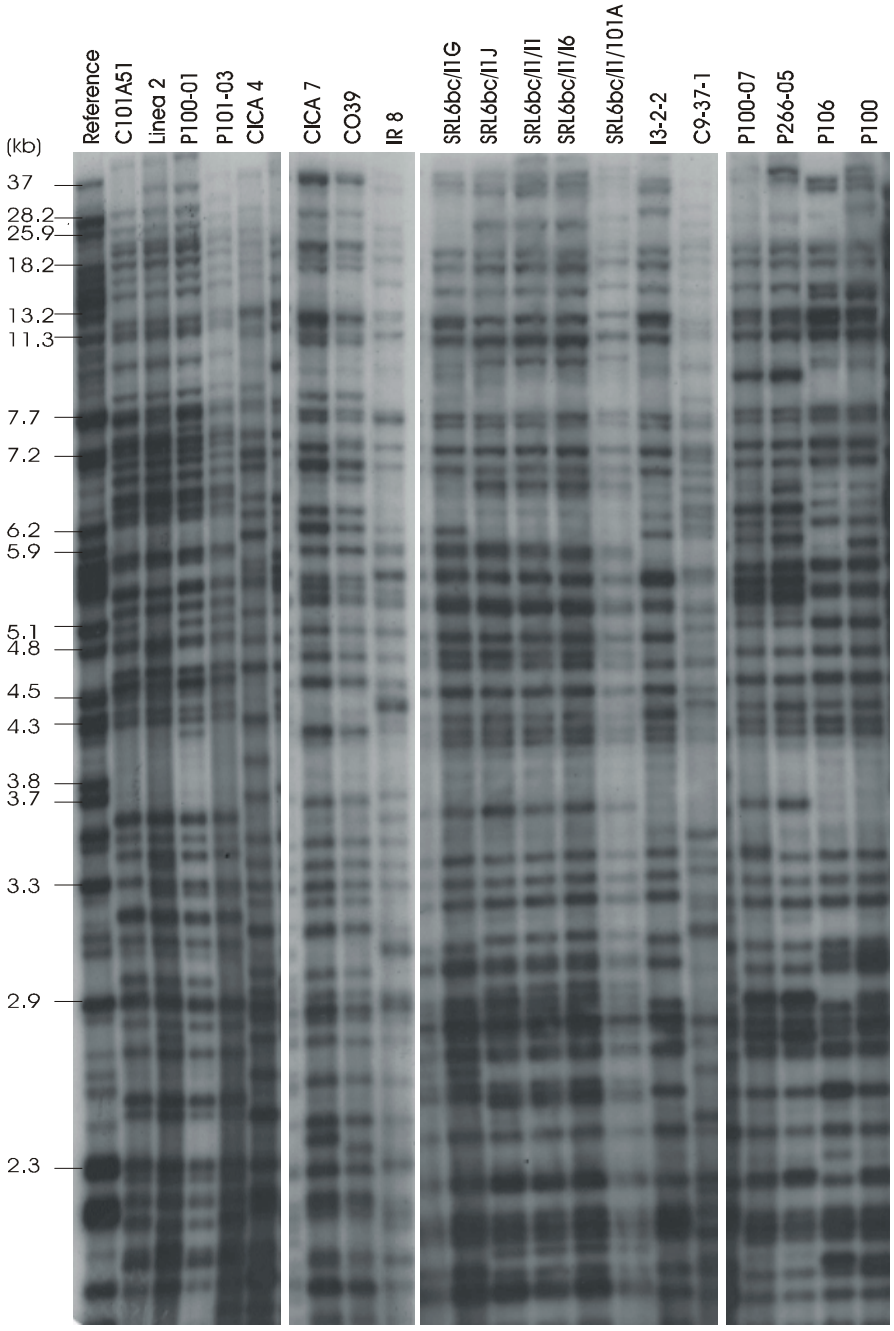

Figure 13. MGR586-DNA fingerprints of isolates of *Pyricularia grisea* from lesions in the C101A51xC101LAC pyramids and other cultivars at field tests, the SRL-6 isolates I3-2-2 and C9-37-1, and from rare and atypical lesions after artificial inoculations with a mixture of representative isolates from the Colombian lineage SRL-6 (labeled SRL6bc/cultivar).

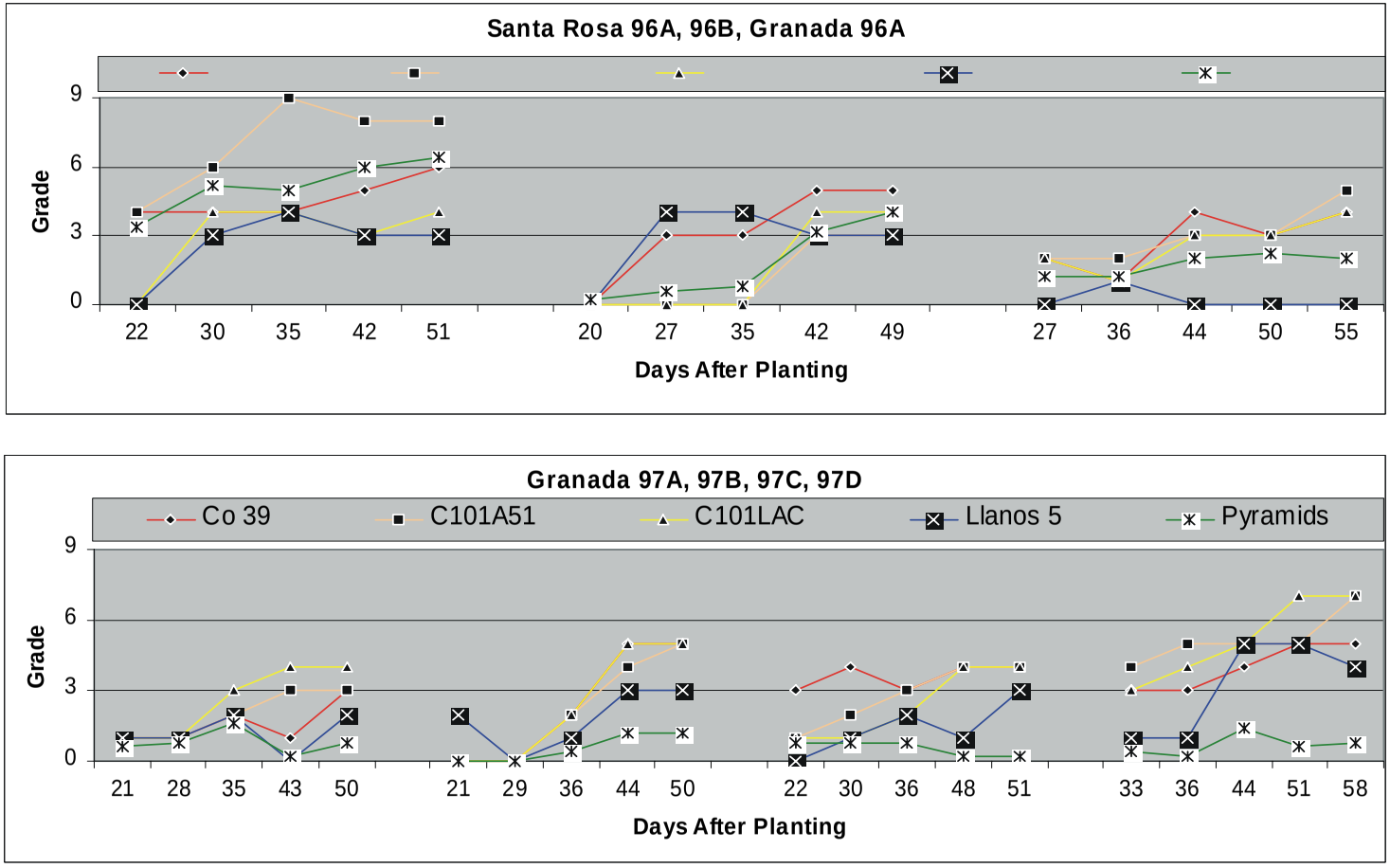

Figure 14. Disease progress on the near-isogenic lines C101A51 (*Pi-1(t)*) and C101LAC (*Pi-2(t)*), the pyramids from their cross, the susceptible check CO39 and the locally resistant cultivar Llanos 5, in field experiments at Santa Rosa, 1996 and Granada, 1996 and 1997. The severity grade was determined according to the 0-9 scale of the Standard Evaluation System for rice blast from IRRI, where 0-4 is resistant to moderatly resistant, 5 is moderatly susceptible and 6 or greater is susceptible. The first date of assessment is indicated above each data plot.

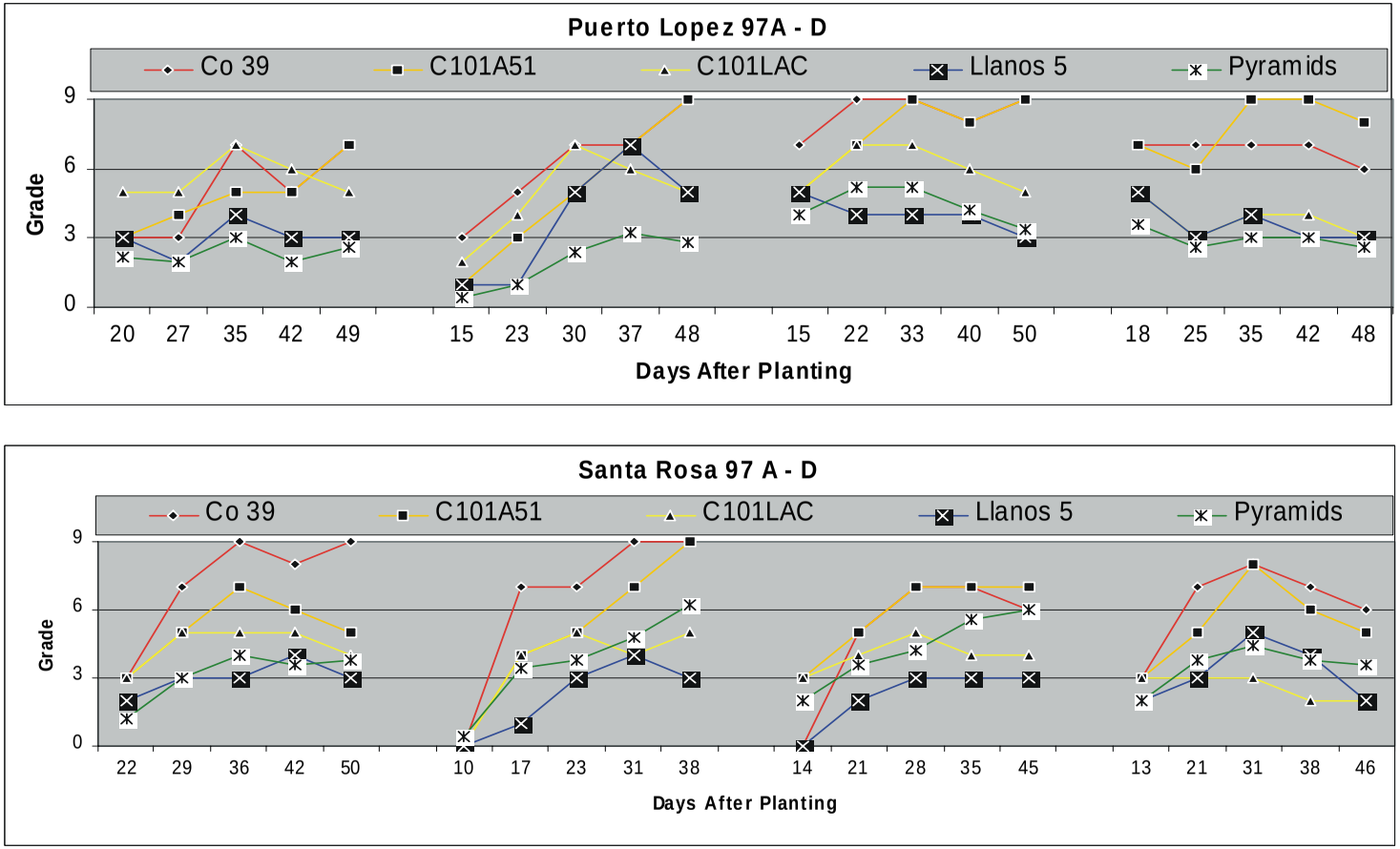

Figure 15. Disease progress on the near-isogenic lines C101A51 (*Pi-1(t)*) and C101LAC (*Pi-2(t)*), the pyramids from their cross, the susceptible check CO39 and the locally resistant cultivar Llanos 5, in field experiments at Puerto Lopez and Santa Rosa, 1997. The severity grade was determined according to the 0-9 scale of the Standard Evaluation System for rice blast from IRRI, where 0-4 is resistant to moderatly resistant, 5 is moderatly susceptible and 6 or greater is susceptible. The first date of assessment is indicated above each data plot.

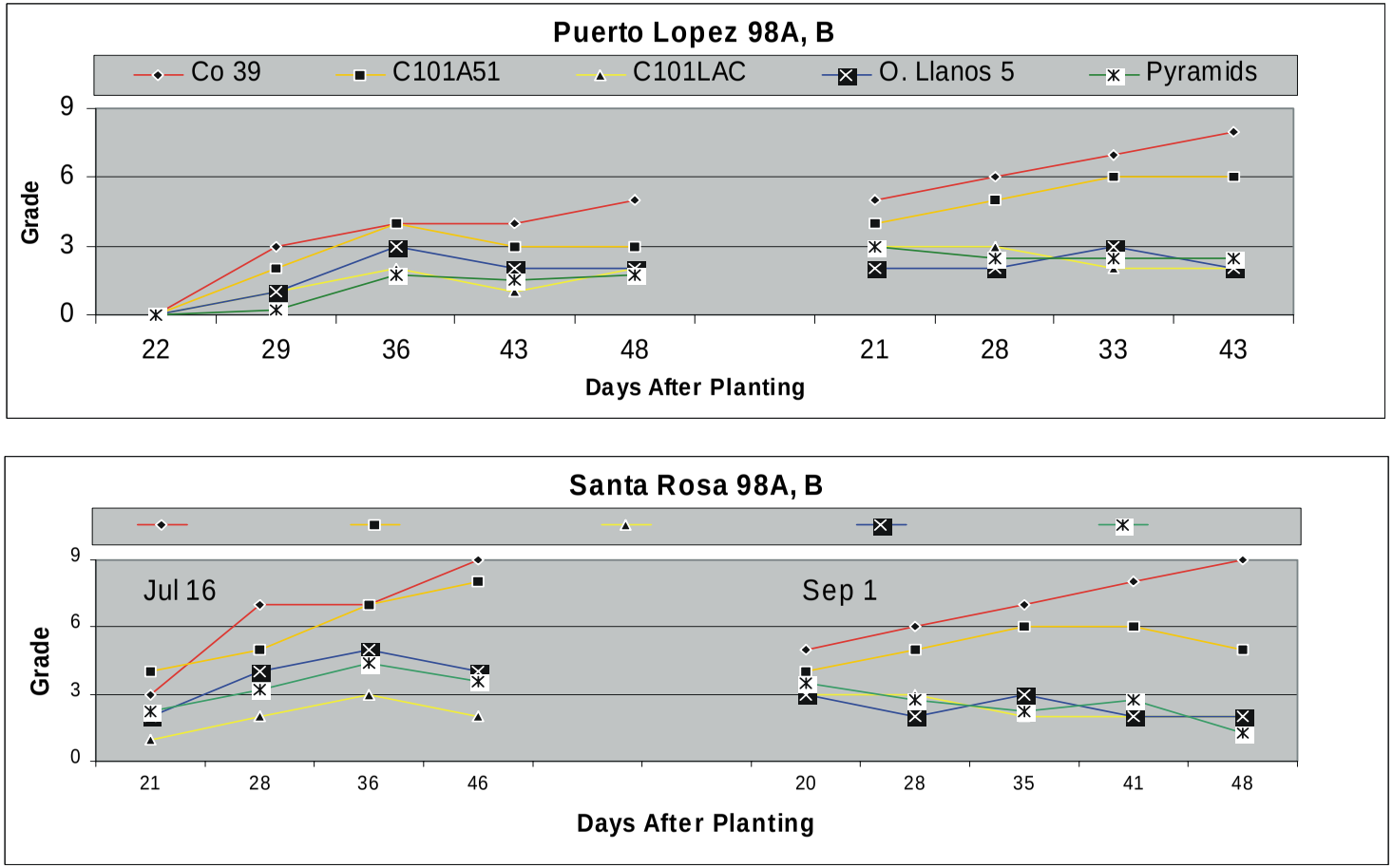

Figure 16. Disease progress on the near-isogenic lines C101A51 (*Pi-1(t)*) and C101LAC (*Pi-2(t)*), the pyramids from their cross, the susceptible check CO39 and the locally resistant cultivar Llanos 5, in field experiments at Puerto Lopez and Santa Rosa, 1998. The severity grade was determined according to the 0-9 scale of the Standard Evaluation System for rice blast from IRRI, where 0-4 is resistant to moderatly resistant, 5 is moderatly susceptible and 6 or greater is susceptible. The first date of assessment is indicated above each data plot.

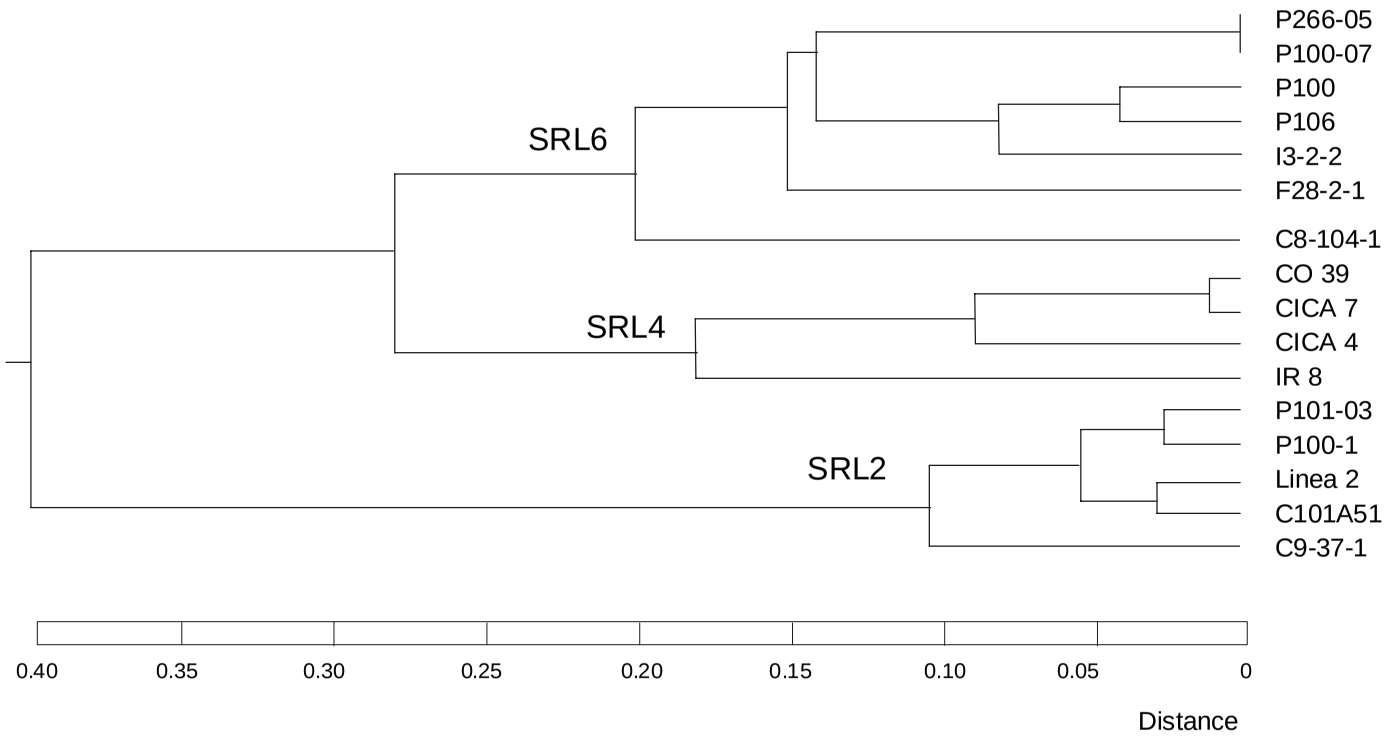

Figure 17. Phenogram drawn after MGR586-DNA fingerprint analysis (UPGMA) of representative isolates from Colombian lineages (SRL-6: I3-2-2, F28-2-1 and C8-104-1; SRL-2: C9-37-1), representative haplotypes from lesions on the pyramids (P#) and commercial cultivars in the field.

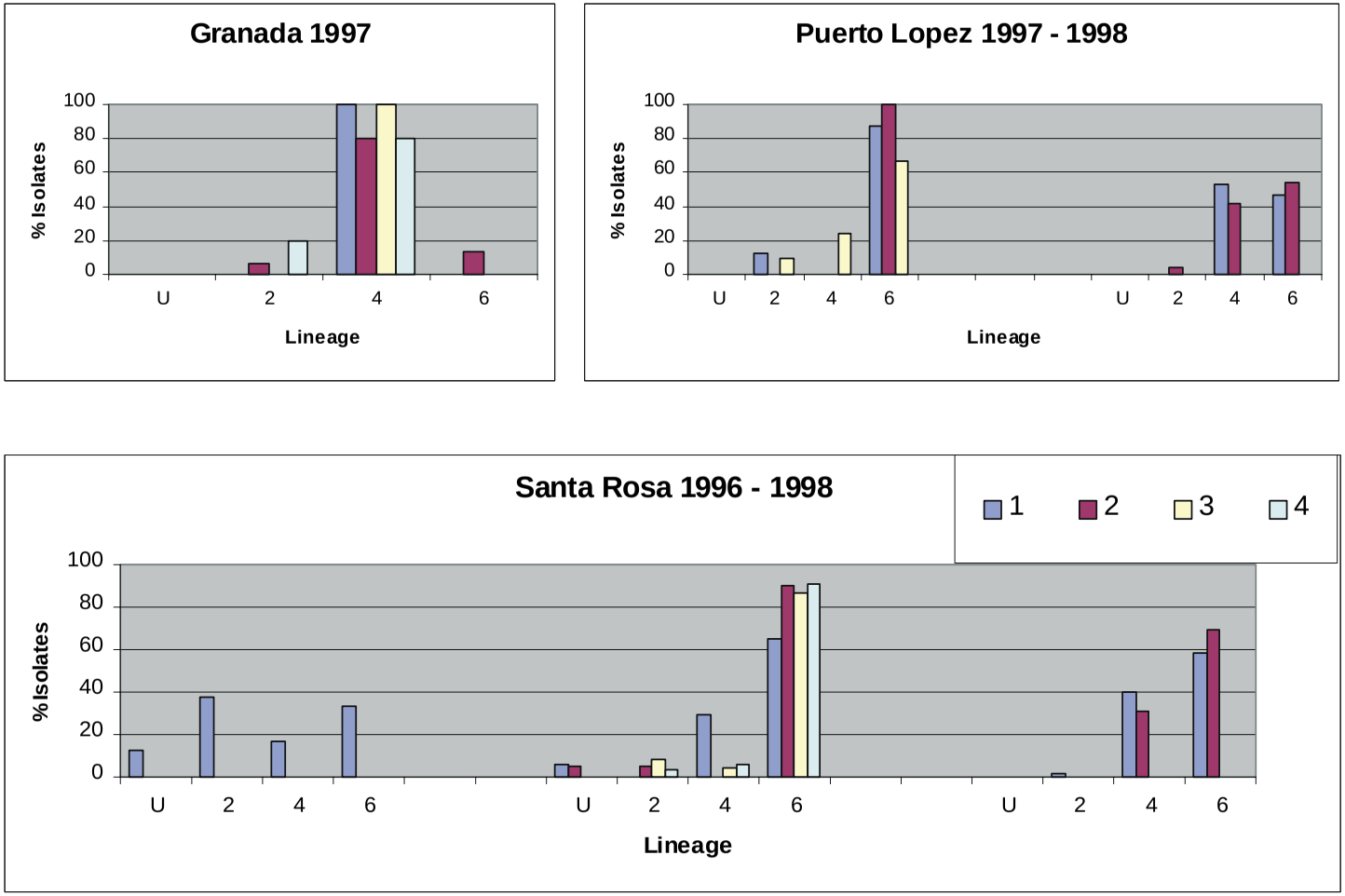

Figure 18. Percentage of isolates by MGR586 lineage (2= SRL-2, 4= SRL-4, 6= SRL-6 and U= unassigned) from the field experiments at Granada, Santa Rosa and Puerto Lopez, Colombia, from 1996-1998.

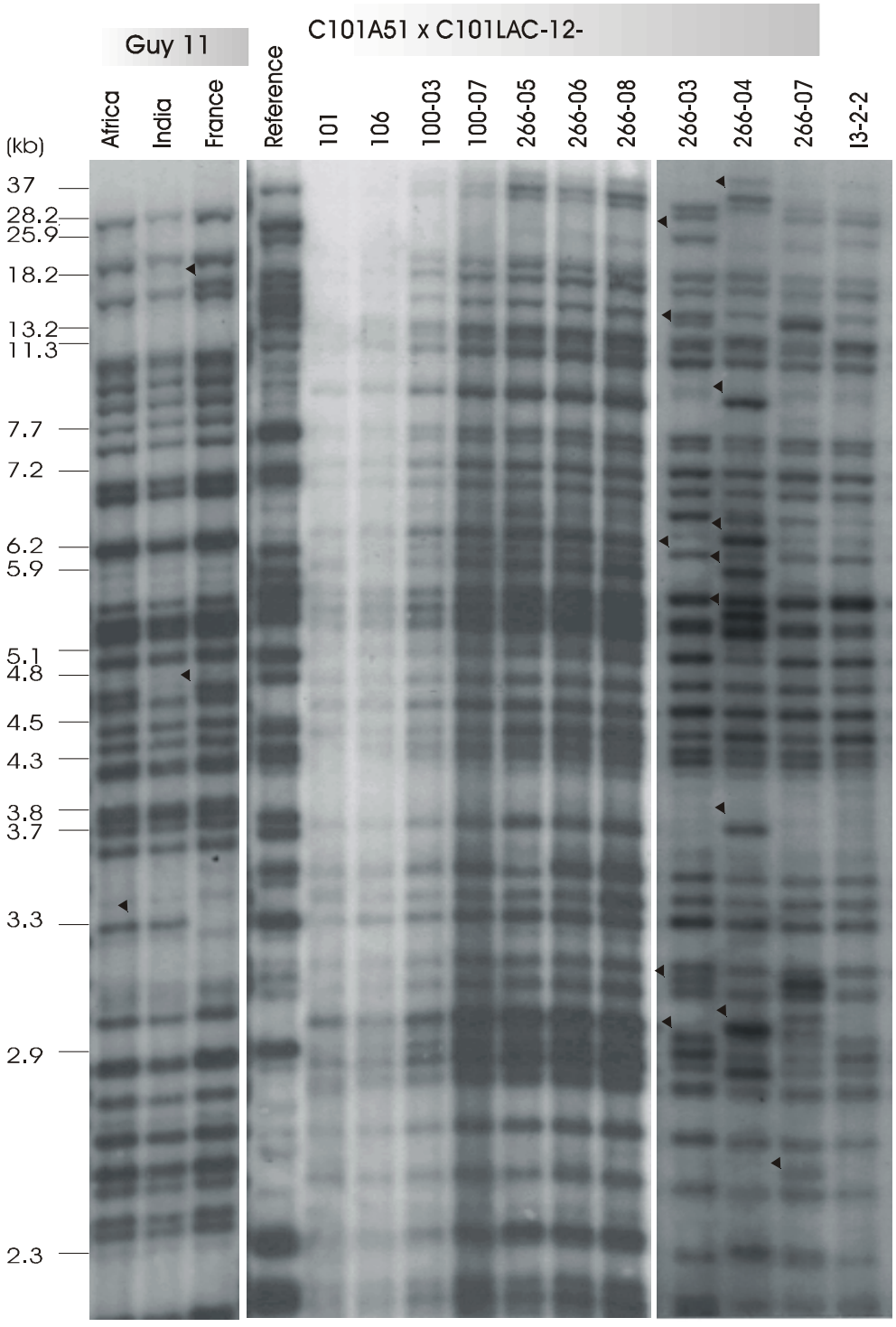

Figure 19. MGR-DNA fingerprints of Guy 11 wild-type strains from different laboratories and isolates of the Colombian MGR lineage SRL-6 from the field infections on the pyramids from the cross C101A51xC101LAC. The arrows indicate differences in RFLPs observed among the haplotypes.

DISCUSSION AND CONCLUSIONS

The research reported here evaluated the design and effectiveness of a lineage-exclusion strategy to confer durable resistance to *Pyricularia grisea* in an environment conducive for rice blast disease in Colombia. The lineage-exclusion strategy was suggested to improve the choice of blast resistance genes for breeding rice (Zeigler et al., 1994). Conventional resistance breeding focuses on combining resistances that exclude all pathotypes (races) observed in the virulence spectrum of the pathogen population. Lineage exclusion adds the consideration of how that virulence spectrum is distributed phylogenetically in the pathogen's population structure. This modification targets component resistances that exclude entire genetic families, i.e., all of the isolates within a lineage, of the pathogen and that complementarily exclude all lineages (Fig. 20). It avoids resistance combinations whose components are susceptible to isolates within a single lineage even if the compatible pathotype has not yet been observed.

Lineage exclusion is based upon outcomes of population studies that helped to explain genetic diversity and pathotype variability in the rice blast pathogen population. Characterized by MGR586 DNA fingerprinting, the rice blast pathogen within a given region typically is composed of a number of discrete lineages, each of which has a limited virulence spectrum (Zeigler et al., 1995; Roumen et al., 1997; Correa-Victoria and Zeigler, 1993; Shen et al., 1998; Sivaraj et al., 2000; Correa-Victoria et al., 2000). The limits are defined by lineage-specific avirulences that, presumably, have evolved under selection and are difficult to overcome (Fig. 20). MGR586 phylogenies thus reflect the inheritance of genetic factors linked to virulence potential. And, given that MGR586 functions as a selectively neutral marker, there are good reasons to believe in this phylogenetic association. Aiding this interpretation, the rice blast pathosystem is

regarded as a classic example of a gene-for-gene system. Single avirulence genes that control the ability of an isolate to infect specific rice cultivars, as well as polygenic factors with minor effects, have been identified in *P. grisea* (Yu et al., 1996; Valent, 1990; Orbach et al., 2000; Chao et al., 1999; Jia et al., 2000). Consequently, when any avirulence is historically conserved within a blast pathogen lineage, a single blast resistance gene can durably deter invasion by that lineage. Given that no individual resistance gene functions to exclude the whole spectrum of virulence, and that genes defeated by a particular lineage can be effective against others, a combination of complementary resistances can result in complete resistance.

The rice blast pathogen population in Colombia is composed of several discrete lineages, most of which have been observed for more than a decade (Correa-Victoria et al., 2000). The *Pi-1(t)* and *Pi-2(t)* resistance genes, although individually defeated by certain isolates, provide complementary resistance to all historically observed pathotypes in Colombia. Furthermore, their combination provides complementary resistance to all Colombian lineages with the exception of lineage SRL-6 (Table 2). However, no archived isolate in lineage SRL-6 could overcome both resistances.

Following conventional breeding approaches, the above two major resistance genes were combined into a pyramid. Crossing two near-isogenic lines, each one of them harboring the already identified gene, double-resistant hybrids were produced. An array of isolates representing the local virulence was used to select true-breeding pyramids in the greenhouse and doubly resistant cultivars were exposed to both commercial and artificially enhanced ("hot spot") conditions for blast disease in the field. The effectiveness and durability of the achieved resistance was inferred by determining the ability of the pathogen to generate new virulences that overcame the pyramids. The lineage specificity of new virulence mutations, the degree of isolate specificity of such mutations, and their overall fitness and stability were also investigated. As the commercial field experiments were located at two different ecological sites within the target region, besides the “hot spot”, the extent of ecological dependence of lineage exclusion-based resistance for rice blast was also investigated.

The pyramids were resistant to all combinations of virulence known to be present in Colombian lineages as tested in the greenhouse. They remained resistant at both of the ecologically differentiated commercial field sites (Granada and Puerto Lopez) during the three years of testing. This happened despite the high disease severities recorded several times on their parental sources of resistance and on susceptible checks at these two sites. However, transient infections at a moderate susceptibility level were recorded on the pyramids at Santa Rosa during three out of the eight tests performed at this "hot spot". While the disease at Santa Rosa generally devastated all susceptible checks and compromised the parental NILs at several times, severe infections on the pyramids were recorded only occasionally during the first two years of testing. No disease was observed on the pyramids at any site during the third year of testing, not even in Santa Rosa, although severe blast infections on susceptible check cultivars were recorded.

Prior to the field testing, no single isolate nor mixture of isolates combining virulence to *Pi-1(t)* and *Pi-2(t)* was able to infect the pyramids at susceptible levels after artificial inoculation in the susceptible three-week seedling stage. Meanwhile, several isolates were capable of infecting the parental sources of resistance, differentially. During a previous three-year sampling period at Santa Rosa only the six lineages initially identified were repeatedly recovered (Levy et al., 1993; Correa-Victoria et al., 2000). Previous sampling in the Altillanura (the local name for the Puerto Lopez area) revealed a more complex population structure, although six of the eighteen lineages identified from this region were composed of a few isolates and found only once (Manry, 1995). Similarly in this study, very few changes were detected in lineage composition in the field. Only three previously detected MGR lineages prevailed through the continuous sampling from 1996-1998 at all three sites, although the sites are located in a prime rice-growing area known for chronically high levels of disease. The nursery trap, although composed of a wide array of resistances to ensure capturing a broad spectrum of virulence available in the eastern plains (Manry, 1995), detected only isolates of lineages SRL-2, SRL-4 and SRL-6. The same lineages continue to be reported as the most

abundant and widespread lineages in Colombian commercial fields currently (Correa-Victoria et al., 2000).

The R-gene Complement *Pi1(t)* + *Pi-2(t)* Provides Lineage Exclusive Resistance

Although isolates belonging to lineages SRL2, SRL4 and SRL6 were identified from lesions on the pyramids occurring in the field, the greenhouse assessment for R-gene compatibility showed that only SRL-6 isolates collected from Santa Rosa were capable of re-infecting their original host pyramids. SRL-6 is the only lineage known to previously express compatibility, although differentially in single isolates, to both pyramided resistance genes. Accordingly, it is concluded that the R-gene complement chosen did provide lineage-exclusive resistance as expected.

Circumstantially, the field experiments uncovered an apparent shift in aggressiveness in a group of SRL-6 isolates toward the F_3 and F_4 pyramids at the "hot spot" sporadically during the 1996 and 1997 tests. However, these new pathotypes were incompatible to similar advanced generation, F_6 pyramid lines selected at Santa Rosa by CIAT (Correa-Victoria et al., 2000) and to similar F_4 pyramids selected by S. Hittalmani using *Pi-1(t)* and *Pi-2(t)* molecular markers (Kumar et al., 2000). Instead of an aggressiveness shift, there may have been R-gene heterogeneity in some pyramids that was not identified in our greenhouse selection process. This possibility was further suggested by the complete absence of infection recorded on the pyramids during the 1998 Santa Rosa field tests. Because the initial pyramid stocks were exhausted, the seed for the 1998 field tests was obtained from uninfected plants in the previous planting. It is surmised that during the 1997 field tests at Santa Rosa unrecognized segregants may have been eliminated because of SRL-6 infection and probably only truly double-resistant, high *Pi-1(t*) penetrance genotypes were chosen for the 1998 experiments. The recent discovery of a second unlinked resistance gene, *Pi-11(t),* in the genetic makeup of the *Pi-1(t)* donor, C101LAC, by French researchers at CIRAD (F. Correa-Victoria, pers. comm.) complicates the matter. Although the potential lineage-excluding resistance

provided by *Pi-1(t)* remains supported by Hittalmani's marker-aided selection, the contribution of *Pi-11(t)* to pyramid behavior remains unresolved.

Whether or not the capacity of SRL-6 isolates for defeating the R-gene pyramids involved unexpected segregation or low penetrance of the *Pi-1(t)* gene in the 1996-97 pyramid lines, it was clear that recurrent pyramid infection was limited to this lineage. Further, pyramid vulnerability to SRL-6 was expressed only in the Santa Rosa trials where chronically severe infection levels, and the high inoculum levels they imply, were present. The small size of the testing plots and the artificial infective environment at this "hot spot" still cannot be compared to the levels of host selection enforced in massive commercial monocultures. However, as predicted by the lineage-exclusion design, isolates within lineage SRL-6 are the most likely source of new pathotypes that will overcome the selected two-gene pyramid in the field. Overall, the tests conducted during the present study confirmed the capacity of *Pi-1(t)* + *Pi-2(t)* pyramids to exclude all of the current virulence spectra from lineages in Colombian commercial fields located in the eastern plains.

Durability of the Resistance Provided by the *Pi-1(t)* + *Pi-2(t)* Combination

Durable resistance can be recognized only after a cultivar has been extensively grown for years, in regions where environments are favorable for the disease. The term by itself is only descriptive and the association of durability to any breeding strategy has been extrapolated mostly on partial results (Agrios, 1997). Durable resistance has been associated with horizontal resistance because of the effects of the latter in slowing the progress rate of epidemics. Techniques for distinguishing horizontal resistance in breeding lines have been difficult to apply to rice (Villareal et al., 1981). Some success has been achieved by screening rice breeding lines with differential *P. grisea* strains and testing consecutive progenies in the field against parameters like infection rate (Yu et al., 1987; Yeh and Bonman, 1986). However, rice cultivars that exhibited resistance to blast in certain regions were susceptible when planted in different environments (Bonman et al., 1991). Whether pathotype variation or environmental factors were responsible for

this shift has not been conclusively determined. The fact remains that durable blast resistance has been observed (Bonman and Mackill, 1988), but mostly in cultivars with complex genetic backgrounds, e.g., Moroberekan and Oryzica Llanos 5, that also have limited yields or narrow ecological requirements.

Due to its ease of implementation and other advantages, the most popular breeding strategy to obtain resistance to blast disease has been the incorporation of complementary major (or qualitative) R-genes into a cultivar or pyramid (Nelson, 1978; Ou, 1979). Although no pyramid is actually built, the term has been chosen to indicate the additional strength introduced by a broader composition of the new cultivar's genetic base. Resistance gene combinations may contribute to the durability of resistance by several potential mechanisms. In general, the probability that a pathogen can simultaneously mutate to virulence at multiple loci corresponding to combined resistance genes is lower than mutations at a single gene (Mundt, 1991). Factors other than gene number may be closely associated with durability as well (Zeigler et al., 1994). Specific combinations of virulence in the pathogen also may have fitness disadvantages, making it more difficult to evolve to those multiple virulence genotypes. Thus, enhanced durability is expected to result less from the inability of the pathogen to mutate than from the fitness constraints required to maintain the necessary gene combinations. Clearly, no single breeding strategy is likely to confer durable resistance to every pathogen. However, achievements attained thus far with the rice blast pathosystem are strongly in favor of accumulation of resistance genes (Nelson, 1978; Ou, 1979; Bonman et al., 1992).

The major constraint until recently has been choosing the appropriate genes. Knowledge of the effectiveness of the chosen R-genes in regard to the virulence characteristics of the pathogen population in the target region must be thoroughly understood to succeed. Population studies based on molecular markers have made this knowledge available and have prompted the lineage-exclusion strategy (Zeigler et al., 1994).

Deployment of locally defeated genes, arranged in novel combinations of overlapping resistances that exclude the observed virulence spectra of all lineages of the

pathogen population, may confer more durable resistance if lineage composition is stable, the pathogen does not freely recombine and/or the mutation rates for virulence are low. The durability of lineage-exclusion resistance combinations thus depends on the reduced potential of new and high fitness virulences to emerge in the field from resident lineages.

Stability of Regional Lineage Composition

Currently, the frequencies of MGR586 lineages in the eastern plains region of Colombia have been narrowed from their typical expression because of shifts in farmers' preferences for specific cultivars and their associated lineage-specific susceptibilities. Lineages SRL-1 and SRL-3 are now rare and the frequency of SRL-5 has been reduced drastically (Correa-Victoria et al., 2000) with the elimination of cultivars Cica 9, Fanny and Cica 8, respectively. The long predominant lineage SRL-6 also has decreased as plantings of Oryzica 1 have been reduced. Increased planting of Linea 2 is associated with increases of lineage SRL-2 while cultivars Oryzica Caribe 8 and Oryzica Yacu 9 have promoted the emergence of lineage SRL-4 as the predominant lineage in the region. The latter increase has also been accompanied by a shift in virulence spectrum. Stimulated by host selection, some lineage SRL-4 isolates have become virulent to *Pi-2(t)*, a feature that had not been recorded in the past. Nonetheless, all previously identified lineages are still resident in the area as revealed by nursery traps in Santa Rosa (Correa-Victoria et al., 2000). The recent introduction of new cultivars has not diminished the occurrence of rice blast disease as most cultivars apparently harbor similar resistance genes. This is a common criticism to the rice blast resistance-breeding program in Colombia which it seems has not yet been amended. The durable (1989-present) resistance of Oryzica Llanos 5, whose breeding sources exhibit complementary lineage-exclusive resistances, remains a notable exception.

The field experiments conducted in this research confirmed previous findings about the resident structure of *P. grisea* populations. While the three lineages SRL-2, SRL-4 and SRL-6 were consistently registered in nursery traps at all three experimental sites, the conditions in Granada favored the almost exclusive expression of lineage SRL-4

in contrast to the predominance of lineage SRL-6 in Santa Rosa and Puerto Lopez. The extensive cultivation of mostly upland, SRL-4-susceptible cultivars like Oryzica Caribe 8 in the Granada area (Correa-Victoria et al., 2000) seemed to have shifted the blast pathogen population in favor of this lineage at this site. A more diverse rice cultivar composition on the largely irrigated lands area near Santa Rosa and Puerto Lopez allowed other lineages to be part of the test inoculum. The overwhelming majority of Latin American rice cultivars are susceptible to lineage SRL-6 virulence (81%) compared to 17% and 26% to lineages SRL-4 and SRL-2, respectively (Correa-Victoria et al., 2000).

Despite shifts in lineage frequency, no new lineages (nor obvious interlineage recombinants) were detected during the three years of the field experiments. Consequently, the emergence of new pyramid-breaking virulences must have been limited to the virulence potential of the previously identified resident lineages in the region. Such lineage stability thus favored the effectiveness of the R-gene combination used for lineage-exclusion resistance.

Lineage Specific Virulence Potential

The association between MGR586 lineages and virulence spectrum as defined by rice cultivar compatibility has been well established for several rice blast pathogen populations. In the United States, one or a few pathotypes predominate in each lineage (Levy et al., 1991). Similar associations but with greater pathotype diversity were found in Colombia (Correa-Victoria et al., 1994; Manry, 1995). In the European rice blast pathogen population, lineage-virulence associations were discrete enough that lineage was easily inferred by the pathotype (Roumen et al., 1997). Most importantly, MGR586 lineages have also been shown to have their virulence spectrum limited by lineage-specific avirulences to single resistance genes. This characteristic was originally described in populations from the Philippines (Zeigler et al., 1995) and has been verified for a diverse array of rice blast populations in China (Shen et al., 1998), southern India (Sivaraj et al., 2000), Thailand (Mekwatanarkarn et al., 2000), and Colombia (Correa-

Victoria et al., 2000). The durability of lineage-exclusion pyramids depends on the conservation of lineage-specific avirulences.

During the three years of pyramid field tests, the virulence of the resident rice blast lineages toward the pyramided resistances remained generally the same as in previous characterizations over the last 10 years (Levy et al., 1993; Manry, 1995). Lineages SRL-2 and SRL-4 remained completely avirulent for *Pi-1(t)* and, as before, only a small minority of SRL-6 isolates expressed a low level of aggressiveness toward this resistance gene. Although *Pi-2(t)* compatibility arose in a sector of SRL-4 isolates, as this lineage became increasingly frequent throughout the study area, no SRL-4 isolate was recovered from a non-segregating pyramid. The only isolates infecting the pyramids did so transiently at the Santa Rosa hot spot and were solely from a unique sector of lineage SRL-6 isolates.

Only four SRL-6 haplotypes were identified by MGR586 fingerprints from lesions on the pyramids and only two of these prevailed throughout the entire sampling period and were re-infective to the pyramids. Moreover, these haplotypes clustered close to an archived SRL-6c isolate, rather than to the SRL-6b haplotypes, used for the greenhouse testing (Fig. 19). SRL-6c isolates are efficient *Pi-2(t)* breakers in Colombian rice fields but only comprise a minority of isolates in this widespread lineage. SRL-6b isolates show low-level virulence towards *Pi-1(t)* and are much less frequent than SRL-6c isolates (Correa-Victoria et al., 2000). The issue of low penetrance of the *Pi-1(t)* gene in the pyramids again complicates interpretation of potential virulence shifts. However, the circumstantial evidence suggests that the rare pyramid-infecting haplotypes originated from a pre-existing SRL-6c genotype. In any case, the new virulence arose solely from the only lineage known to express compatibility with both resistance genes.

Evidence for the conservation of lineage-specific avirulence in Colombia is also available from characterization of *AVR-Pita,* formerly *AVR2-YAMO* for avirulence on the rice cultivar Yashiro-mochi (Valent and Chumley, 1994), the first *M. grisea* avirulence gene to be fully characterized (Orbach et al., 2000). In multiyear sampling, lineages SRL-1, SRL-2, SRL-3 and ALL-7 (an Altillanura lineage) were each

characterized as monomorphic for *Eco*RI digests of *AVR-Pita* and associated pathogenicities (Montenegro-Chamorro, 1997). All isolates in lineages SRL-1 and SRL-2 contain a 3.2 kb RFLP and are avirulent on Yashiro-mochi (and other cultivars bearing the resistance gene *Pi-ta*; (Levy, unpub. data.) Isolates in lineage SRL-3 express a 7.5 kb RFLP and those in ALL-7 express 1.9 kb and 7.5 kb RFLPs, each derived from a separate locus. Both lineages are virulent on Yashiro-mochi. Sequencing of PCR-DNA amplified fragments from representative isolates indicated that all of the lineage-specific alleles showed strong conservation of the coding sequence originally identified in the avirulent isolate O-137 from China (Valent and Chumley, 1994). The inferred protein structure for the avirulent alleles from SRL-1 and SRL-2 were nearly identical to that of O-137 (Montenegro-Chamorro, 1997). The inferred proteins of the virulent alleles differed from the avirulent alleles by seven shared amino acid substitutions, only one of which appears to be responsible for the shift to virulence (Bryan et al., 2000). Clearly, all of the alleles appear to be functional.

However, the conservation of *AVR-Pita* is not universal among Colombian blast lineages. The gene is absent in lineages SRL-4 and SRL-5, with each expressing a mixture of avirulent and virulent isolates. Lineage SRL-6 is polymorphic for the presence/absence of a 1.85 kb RFLP but with uniform virulence among isolates. Sequencing of the SRL-6 allele revealed a frameshift mutation encoding an inferred truncated non-functional polypeptide (Montenegro-Chamorro, 1997). The key for durable lineage exclusion is, thus, targeting only strictly conserved avirulences within lineages.

Fitness and Stability of New Virulences

In the field experiments only two pyramid-breaking SRL-6 haplotypes were detected and these were only sporadically present at the Santa Rosa disease nursery where pathogen diversity was highest and conditions favored chronic and severe disease levels. It was not possible to reproduce such novel pathotypes in the lab either by mass selection of isolates compatible with one of the resistant genes or by serial re-inoculation

with cultures from unusual lesions occurring on sheath tissues of artificially inoculated pyramids. While the number of isolates tested in the lab was small, both results suggest that mutations shifting to relevant new virulence combinations are not frequent in Colombian blast lineages. This coincides with findings by Latterel (1971), and contrary to Ou (1979), that pathotypes typically are conserved and not randomly variable in most blast isolates.

Rapid shifts to virulence have been reported due to spontaneous mutations (mostly deletions) at the avirulence gene, *AVR2-Yamo* (now called *AVR-Pita*), from the Chinese rice field isolate O-137 (Valent and Chumley, 1994). The close proximity of the locus to a telomere in this isolate is probably responsible for the high frequency of mutations to virulence observed. Genes in the pathogen coding for recognition by products of resistance genes may play a negative role in adaptation and are believed to be prone to be lost (Chasan, 1994). The *AVR-Pita* locus is absent or non-functional in Colombian lineages SRL-4, SRL-5 and SRL-6. Thus, it can be speculated that rapid virulence shifts can occur whenever *AVR* gene loss or eliminated expression has little fitness cost.

The function of the vast majority of *AVR* genes already cloned is unknown but several *AVR* genes are also associated with general fitness (Dangl, 1994). In such cases there is a trade-off, i. e., losing such a gene to gain a novel host would jeopardize the pathogen´s general fitness and adaptability (Zeigler et al., 1994). Reciprocally, to maintain high fitness the pathogen would be selected for a limited host range. The strong host specificity apparent in Colombian rice blast lineages suggests that such a trade-off is operating. Nonetheless, virulence shifts do occur in the field, as evidenced by the ability of some lineage SRL-4 isolates to acquire *Pi-2(t)* virulence. This may mean that a broad variety of mutational events other than the loss of avirulence genes may result in virulence gains in the field.

For example, single base-pair changes in the *AVR4* gene of *Cladosporium fulvum* make tomato genotypes containing the complementary resistance gene *Cf4* unable to prevent infection from previously avirulent strains (Johal et al., 1995). Similarly,

single amino acid changes in the *Avr-Rrs1* gene of avirulent *Rhyncosporium secalis* isolates make them virulent to previously resistant barley cultivars (Rohe et al., 1995). Mutations causing deletion of the carboxy-terminal end of the protein encoded by the avirulence gene *Avr-Pto* make tomato cultivars carrying the *Pto* gene susceptible (Tang et al., 1996).

Mutations in avirulence genes also do not always reduce pathogenicity. Most relevantly, the sequences of alleles for *AVR-Pita* in Colombian rice blast lineages that condition virulence in SRL-3 and ALL-7 isolates appear to encode a functional polypeptide. However, these alleles differ from avirulent *AVR-Pita* alleles by seven specific amino acid substitutions, all corresponding to the same base substitutions (point mutations) in the coding sequence. Clearly, these point mutations accumulated non-randomly over time and only one of them has been shown to cause a shift to virulence (Bryan et al., 2000). These features suggest that avirulence gene function has been conserved in these lineages as well as in those where the avirulence phenotype has been maintained.

The evolution of novel virulence in Colombian blast pathogens appears to be constrained by the history of host selection interacting with a genetic architecture that results from predominant asexuality. Not all virulence changes are possible from any resident lineage background, at least in the short term. Fitness effects of specific avirulence genes may not allow for random changes. However, the number, allelic diversity, and phylogeographic distribution of *AVR* genes (as well as R-genes) in the rice blast pathosystem remains unknown. Consequently, exactly what high-fitness novel types are possible depends on local pathosystem structure. Interestingly, the *Pi-2(t)* R-gene is broadly resistant to blast lineages world wide (Zeigler et al., 1995; Shen et al., 1998; Sivaraj et al., 2000) but highly susceptible to all three of the prevalent lineages in the eastern plains of Colombia. It may be reasonable to expect that R-gene pyramids containing *Pi-2(t)* might be especially vulnerable to rapid and multilineage breakdown in Colombia. This has not yet been observed. Perhaps the avirulence alleles that condition incompatibility with *Pi-2(t)* and *Pi-1(t)* have mutually exclusive functions. This would make modification of both alleles extremely difficult because of low fitness.

Recombination and Lineage Exclusion

A limited potential for genetic recombination via parasexual exchanges among rice blast isolates has been demonstrated repeatedly in the laboratory (reviewed in Zeigler, 1997). If realized in the field, such recombination between lineages could rapidly assemble novel virulence genotypes that could defeat any lineage-exclusion resistance design. No such interlineage recombinant was detected in the course of the field experiments; the consensus MGR586 fingerprints of the prevalent Colombian lineages are so distinct that partial recombination should have been detectable. Similarly, the joint culture of isolates from lineages SRL-2 and SRL-5, that were pathogenically complementary to resistance genes *Pi-2(t)* and *Pi-1(t)*, never produced an isolate with combined virulence. Isolates obtained from unusual sheath lesions on the pyramids expressed the same MGR586 profiles as their parental sources and were never re-infective. The reasons why these, as well as mixtures of SRL6-b and SRL6-c isolates, were unable to provide isolates with complementary virulences will require further tests.

Vegetative incompatibility barriers between MGR lineages may explain the absence of intralineage recombinants between Colombian lineages performed during this research. Correll et al. (2000) recently reported a complete correspondence between vegetative compatibility groups, MGR586 lineages and mating type for the four prevalent lineages in the USA based on extensive samples of field isolates collected between 1991-1997. This is consistent with a strictly asexual mode of reproduction. However, they also reported a small number of archival isolates (collected in the 1970s and 1980s) having some incongruence regarding strict asexuality. Two isolates grouped in different lineages and with opposite mating type, were vegetatively compatible in lab culture. The authors suggested that such compatibility could be the remains of sexual or parasexual recombination occurring sometime in the past. However, the MGR profiles of the two USA lineages involved in this highly unexpected compatibility have been grossly distinctive for over 20 years (Levy et al., 1991). This indicates that the interlineage compatibility observed in the lab has not been taking place in the field. Otherwise, the lineages would have either not differentiated or would not be becoming more similar with

time. By the same reasoning, there is no evidence of interlineage recombination in Colombia over ten years of sampling (Levy et al., 1993; Correa-Victoria et al., 2000).

However, the Correll et al. (2000) study highlights the possibility that isolates within the same MGR-defined lineage will be members of the same vegetative compatibility group and would likely have more potential for genetic exchange. If such recombination does occur within lineages it will tend to preserve lineage-specific virulence differences while preventing lineage decay due to accumulated mutations. An additional consequence is that it will enforce the lineage-specific virulence potentials assumed in a lineage-exclusion resistance breeding design.

Breeding Strategies: Options

The lineage-exclusion design described in this research used combinations of already defeated R-genes. It may be argued that, where lineage composition is stable, arrays of such R-genes leave little room for new virulences to be generated. A possible pitfall in such a scenario is migration of low-frequency isolates with undetected combined virulence. Breeders who are reluctant to give the pathogen a chance for uncovering new virulences would be more sympathetic to the use of already defeated genes rather than introducing foreign resistance genes, whose effects or impact may not have been thoroughly tested. Instead of gaining a broader spectrum of resistance in the field the introduction of new resistance genes can seriously disturb the local pathogen structure. The blast pathogen population in Colombia has proven to be genetically constrained to overcome combination of R-genes *Pi-1(t)* and *Pi-2(t)*. These two locally defeated R-genes have been present long enough for different components of the local pathogen population to evolve specificities for each R-gene.

Since the discovery that resistance to rust in wheat was heritable (Farrer, 1898 cited by de Wit, 1992) and that resistance to yellow rust obeys Mendel's laws (Biffen, 1905 cited by de Wit, 1992), great expectations grew among plant pathologists and plant breeders for obtaining genetic resistance to pathogens. These expectations were aided by the discovery of the gene-for-gene concept by Flor working with *Melampsora lini* on flax

(Flor, 1945) and by Oort working with *Ustilago tritici* on wheat (Oort, 1944 cited by de Wit, 1992). The initial expectations for durability of genetic resistance, based on the assumption of slow evolution in the pathogen, were rapidly swept away by adaptation of new pathogenic variants. Not very long after the initial success, only a few of the resistance genes introduced in commercial crops were able to confer resistance that remained effective in the field. Because of this failure, several strategies for enhancing the durability of resistance have been suggested. However, the types of interactions between hosts and pathogens are so diverse that probably no single model will account for the establishment of a unique strategy to obtain resistance that remains durable in the field.

Breeding cultivars with single major R-genes is a strategy of choice because major genes are easily recognized by their specificity and thus can be rather easily manipulated by the breeders (Agrios, 1997). The attained resistance is effective until a new strain of the pathogen to which the incorporated genes do not confer resistance becomes established. Crill et al. (1981) suggested timely rotations of rice varieties with major R-genes to increase the longevity of resistance to blast in the field. By following local changes in specific virulences, new R-genes would be deployed to replace the old ones likely to be defeated by the virulence shifts. The use of multilines, or mixture of lines carrying one or a few major R-genes, also has been suggested as a means to reduce the epidemic rates of the rice blast disease (MacKenzie, 1979). Lines must be carefully chosen as their resistance effectiveness will be related to the predominant virulence composition of the pathogen population in the field. Both conventional R-gene pyramids and multiline strategies failed to provide resistance that remained durable in Colombia. Resistant cultivars to replace the defeated ones were not available on time and the effort to develop multilines was succumbed by the increasing susceptibility of the lines.

A recent large-scale experiment (requiring a huge cooperative effort) performed in the Yunnan Province of China claimed crop heterogeneity as a solution to the vulnerability of monoculture crops to rice blast disease (Youyong et al., 2000). Susceptible highly valued rice cultivars were planted in mixtures of 1 row per 6 rows of resistant hybrids. The area of the study was expanded to a total of 3,342 ha in 1999 and

showed a dramatic reduction of disease incidence such that no foliar fungicide application was made (less than 1% of total panicle infections). The region is well known for a weather regime that favors blast and averaged panicle severities were 20% on the susceptible (monocultured) cultivars. Increased distance between plant genotypes was considered as the most important factor for the disease reduction in cultivar mixtures. Although impressive because of the area involved, the limited extent of susceptible genotypes within the mixture and the reduced level of susceptibility make the real impact of genotype diversity accounting for important disease severity reductions still unresolved. Blast panicle severities in Colombia, for instance, can easily exceed 80% in susceptible cultivars. Moreover, the strikingly different rice cultivars in the Yunnan study had to be hand harvested. This is an untenable condition for most rice growing areas in the Americas.

Plants may exhibit some level of non-specific (partial) resistance to pathogens (Agrios, 1997; van der Plank, 1963). This is a type of resistance that is often controlled by the action of multiple genes in an additive fashion. Each of the genes alone may not confer a high level of resistance but once combined they would provide a degree of resistance that may be effective for certain environments. Although it does not prevent infection, it does play a role in slowing down the development of an epidemic in the field. Partial resistance has been frequently suggested as a means to increase durability of resistance to rice blast (Villareal et al., 1981; Ou, 1979; Kiyosawa, 1972; Bonman et al., 1992). However, the phenotypic expression seems greatly influenced by the environment. More recently, significant progress has been made in mapping quantitative trait loci (QTL) for resistance towards diseases like rice blast, potato late blight and gray leaf spot in maize, among others (Young, 1996). The addition of QTL linked to partial resistance against rice blast to lineage-exclusion devised pyramids could provide an extra buffer against the development of combined virulences in the field.

Although at the experimental level yet, the choosing of R-genes based on a lineage-exclusion strategy has proven effective for conferring durable resistance to rice blast disease in Colombia. Similar studies conducted by CIAT in Colombia, S. Hittalmani and R. Sivaraj in India have also proved so. However, the extent of

ecologically diverse environments, crop cultural conditions and infection levels attained during the tests conducted make the research reported here the most documented attempt for testing lineage-exclusion in the rice blast pathosystem up to date.

Following a gene-for-gene interaction	
R_j vs A_j	incompatible
R_j vs A_i	compatible
R_j vs a_i	compatible
$R_1R_2R_3R_4$ *vs* $a_1a_2a_3a_4$	compatible

Lineages have historically defined incompatibilities

Lineage 1	Lineage 2
$A_{1..}A_2a_3a_4$	$a_1A_2a_{3..}A_4$
$A_{1..}a_2A_3a_4$	$A_1a_2A_{3..}A_4$
$A_{1..}A_2a_3A_4$	$a_1A_2a_{3..}A_4$
$A_{1..}a_2A_3A_4$	$A_1a_2A_{3..}A_4$
$A_{1..}$	$_{..}A_4$

Figure 20. Schematic diagram of the gene-for-gene basis of the lineage exclusion strategy. MGR586 lineages have evolved with lineage-specific avirulences (represented as *A* or *a*). Each lineage has historically defined incompatibilities (A_1 in lineage 1 and A_4 in lineage 2) that are difficult to overcome.

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VITA

Anibal Leonidas Tapiero-Ortiz was born on June 3, 1951 in Pitalito, Huila, Colombia. He attended high school at the Instituto del Carmen in Bogotá, Colombia, where He graduated in 1969. Afterwards, He attended the Faculty of Agronomy Engineering of the National University of Colombia in Bogotá and graduated in 1978. By 1979 He joined a collaboration project between the United Nations Children's Found (UNICEF) and the Presidency of the Republic of Colombia in the Amazon forest during three years. His role in the project was to conduct research and be an extension agent on native horticulture. Afterwards, He enrolled the Colombian Institute of Agriculture Investigations (ICA) where He worked on cocoa, plantain and rice diseases, in the eastern plains of Colombia known as "Los Llanos". While at ICA He traveled to Ascot, Berkshire, UK to pursue his Master of Philosophy (M.Phil.) degree from the University of London. He graduated as a Plant Pathologist at the Department of Biology of the Imperial College of Science and Technology in 1991. His submitted thesis is entitled "Resistance of rice to blast disease caused by strains of *Pyricularia oryzae* Cav. from Colombia". Back in Colombia, He joined the recently created Colombian Corporation of Agricultural Research (CORPOICA) to work in rice disease management and plant breeding at La Libertad research station in Los Llanos. He came to Purdue University in 1994 granted by a Rockefeller Foundation scholarship and after completing his Ph.D. studies in the Department of Botany and Plant Pathology by 2000, returned to his country. Currently, He is working in soybean and citrus diseases as an associated scientist in CORPOICA La Libertad, Villavicencio, Colombia.