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Know Your Enemy:

A Novel strategy to develop durable resistance to rice blast fungus through understanding the genetic structure of the pathogen population

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Know your enemy, and know yourself, and in a thousand battles you will win a thousand victories.

- Chinese proverb

THE PROBLEM

Rice is the world's most important food crop, providing the daily staple food for almost 2 billion persons. Almost all live in developing countries. Blast is the most widespread and damaging disease of rice. It is also one of the most variable plant pathogens. New blast strains mutate rapidly, rendering resistant varieties susceptible within 2 or 3 years of release--sometimes, even before the breeding lines reach farmers' fields. The need to continuously produce lines with new sources of blast resistance inflates the cost of rice breeding programs worldwide. More serious, the instability of blast forces farmers to rely on environmentally damaging and costly fungicides for disease control.

THE SOLUTION

CIAT recently bred two resistant rice cultivars suitable for both irrigated and favored upland conditions in a "hot spot" environment for blast. These cultivars have been grown experimentally and commercially for 5 years without breakdown. Resistance remains stable because several resistance sources are combined in the lines, which were selected through a long and complex breeding scheme. An integrated strategy involving classical pathotyping and molecular fingerprinting has provided a novel mechanism to classify more than 50 Colombian pathotypes of blast disease into six distinct genetic lineages or "families." Combined with the use of molecular markers, scientists will now be able to identify and use genes resistant to specific lineages rather than to randomly breed for individual pathotypes.

THE POTENTIAL IMPACT

The proposed integrated breeding strategy, whose living proof is the durability of two cultivars that were independently bred by traditional methodologies, will permit rapid identification of desirable resistance sources and the breeding of stable blast-resistant cultivars for different environments and cropping systems. Lasting resistance will reduce fungicide use and lower plant breeding costs, which will translate into profits for rice farmers and a cleaner environment. Over a 15-year period, including years with both above- and below-average blast epidemics, the net present value of the benefits is estimated at US$1.6 billion from increased crop yield, and in reduced expenditures for rice imports and use of fungicides in Latin America alone. Worldwide, this environment-friendly scientific breakthrough helps resolve one of humanity's most devastating plant diseases.

Rice blast, an intractable worldwide problem

Rice is the world's most important crop, and the major food for a third of humanity.

Rice blast disease is the most widespread and damaging disease of cultivated rice in both tropical and more temperate latitudes. The disease is caused by the ascomycete fungus, *Pyricularia grisea* Sacc. (teleomorph: *Magnaporthe grisea* Barr). The fungus reproduces asexually and is noted for expressing a large number of virulent forms or pathotypes, especially in rainfed or upland areas, where the environment favors epidemics.

Most rice-growing areas in Asia, Africa and Latin America rely upon regular introduction of new blast-resistant rice cultivars as well as fungicide use. Many years of genetic breeding and research have shown that genetic resistance to blast in these regions is short-lived, rarely being effective for more than 2 to 3 years. Several major nationwide resistance breakdowns have been documented in countries as diverse as Egypt, South Korea and the Philippines. One of the most recent breakdown occurred in Texas in 1991. Whether the lack of durable blast resistance reflects significant shifts in the frequency of existing pathotypes or frequent occurrence of new pathotypes by mutation and/or immigration is not fully known. The key to resolving these issues lies in defining the population genetic structure that underlies pathotype variation and in determining how and at what rate pathotypes evolve.

The other control method used by rice farmers, i.e., when resistant varieties are not available, depends heavily on fungicide treatments, usually applied on a calendar basis. Fungicide use may not always be effective or economicaly sound, and is never environmentally desirable. This situation argues in favor of adopting a broader disease management strategy that would allow development of durable resistance and a reduction in fungicide use worldwide.

CIAT's Rice Program has been developing the concept of integrated crop management, which combines breeding for durable disease resistance with rice cultural practices that include disease management concepts. This approach aims to maintain current production levels in regions where yields are already high, and to increase yield where the full varietal genetic potential is far from being realized. Production costs must be lowered if rice production is to remain efficient.

Management of plant disease pathogens, varieties, cultural practices and fungicides, which all too often are treated separately by researchers and extension agents, are considered together in this summary of integrated research. Bringing a range of tools together to guarantee a healthy crop will not only contribute to higher and more stable yields, but should also prolong the lifespan of resistant varieties which are so painstakingly developed. The challenge is then to exploit these integrated management tools so that farmers can maintain high yields at economically attractive production levels with the lowest possible use of external inputs.

Cultural methods or agronomic practices have been shown to clearly play an important role in developing blast pathogen populations. Using genetic resistance alone in rice may not be an adequate control tactic, particularly in coping with pathogens that exhibit great variation, as in the case of rice blast.

Fertilization, planting and water management practices play an important role in the rapid

breakdown of resistance by encouraging high pathogen population levels. We have accumulated a large set of data at different sites in a tropical blast-prone area in Colombia and can thus assemble and use the available tools properly, keeping in mind farmers' objective of a productive and profitable rice crop.

We have repeatedly demonstrated that higher rice yields are obtained through a combination of lower nitrogen and seeding rates as opposed to the high rates used by rice growers in the study area. Together with optimal water management, total returns may be increased by the equivalent of up to 1 ton/ha of paddy compared with conventional practices.

Rice growers have developed management practices that often incorporate inappropriate land preparation and/or suboptimal chemical control of weeds. Under these circumstances, cultural practices may lose efficiency if resistance levels are not high enough to counteract environmental effects that favor pathogen proliferation and mutation.

CIAT and its collaborators in NARS have generated data that will provide opportunities to weighing management alternatives for rice blast. As an example, one key question is "Does resistance deserve a higher priority within the concept of integrated disease management?" Answers to this question need further analysis using the history of rice varietal development in Colombia since 1960. Incorporating genetic resistance into commercial varieties has clearly been the preferred means for protecting rice from disease. Breakdown of this resistance is common in most rice-growing areas and often occurs shortly after varietal release.

Generating highly resistant and durable varieties for rice-blast-prone areas will have three major economic impacts: (a) eliminate current yield losses because of uncontrolled blast disease in farmers' fields; (b) bring savings from fewer or zero sprayings of fungicides; and (c) minimize environmental hazards from using chemicals on humans, wildlife and the environment in general.

From experimental data, it has been estimated that average yield losses amount to 5-10% in irrigated systems and are more than 20% in rainfed systems in existing production systems using current varieties. According to farm surveys carried out by CIAT and its collaborators, farmers' expenditures on protective fungicides range from 6% to 50% of total crop protection costs. By introducing new, more tolerant and stable rice germplasm to the blast disease, it can be expected that not only will future rice yields stabilize at higher levels but also that unit production costs will decline.

Components of a new integrated strategy

Rice blast disease management, particularly in developing countries, must, for economic reasons alone, rely on effective resistance breeding and gene deployment strategies. This approach is also consistent with overall goals of sustainable agriculture and reduced dependence on chemical-based technologies. Such approaches have been hampered by the diversity and virulence of the fungal pathogen and the inability to combine adequate resistance genes. This summary describes a novel and integrated strategy developed by an interdisciplinary and interinstitutional team from CIAT's Rice Program and Biotechnology Research Unit cooperating with two scientists at Purdue University. The strategy components that follow combine all the tools of classical pathology, breeding, and molecular biology to address this key constraint on global rice production.

Pathotyping

The sole epidemiological tool for studying the rice blast fungus has been a pathogenicity assay, which sorts isolates into pathotypes ("races") by their range of infection on a set of differentially resistant rice cultivars. Assay results can be strongly influenced by host age and condition, inoculum quality and variation in environmental conditions during the assay. Furthermore, isolates may produce a variety of intermediate infection responses (lesion types) on one or more of the differentials, making assay scoring both difficult and subjective. Although an international set of eight differential cultivars has been established to standardize assays, it has become clear that this set cannot decipher the pathotype diversity that occurs in most rice-growing regions.

Many pathogenic races have been identified in *P. grisea*, and this variability has been cited as the cause of resistance breakdown. Controversies over the origins of this diversity are well documented. For example, great pathogenic variation has been reported from single-spore isolates originating from single lesions and monoconidial subcultures, while other studies have shown isolates to be pathogenically stable. In the context of the pathogenic diversity of *P. grisea*, it is recommended that breeding strategies should minimize the probability of escapes. Strategies should also include line evaluation and selection in sites highly favorable for the pathogen, where populations remain high throughout the season and pathogenic diversity is high. These sites are commonly denominated "hot spots" and developing durable resistance is more likely to be successful under such conditions. We have collected evidence over more than eight growing seasons that shows that resistance selected at a "hot spot" site in Colombia is stable over both time and space. A critical evaluation of pathogenic diversity to explain the durability of this resistance has been carried out using both conventional pathotyping and molecular tools such as DNA-fingerprinting.

Characterizing variability in virulence, a highly necessary step for developing durable resistance, has been conducted in the last four years. It has focused on race composition, compatibility with known resistance genes, frequency of occurrence of virulence factors and variability as reflected by pathogenicity in diverse rice cultivars (Table 1). Studies have also been conducted to determine if the sexual stage of the fungus is playing any role in the origin of this variability. This has not been found to be the case.

Table 1. Frequency of virulent phenotypes in *Pyricularia grisea* isolates collected in Santa Rosa, Colombia.

Cultivar	Known resistance genes	Compatibility frecuency	Isolates tested	Cultivar	Known resistance genes	Compatibility frecuency	Isolates tested
K8		0.03	160	BL-1	Pi-b	0.43	155
Zenith	Pi-z Pi-i Pi-a	0.04	163	Caloro	Pi-k^s	0.46	141
Peta		0.09	67	K 1	Pi-ta	0.47	138
Ceysvoni		0.10	152	Usen	Pi-a	0.57	141
IR 42		0.10	168	Shin 2	Pi-k^s	0.54	136
Fujisaka 5	Pi-i, Pi-k^s	0.14	152	Raminad str 3		0.63	142
Np-125		0.17	150	Dular	Pi-k^a	0.64	154
Fukunishiki	Pi-z	0.22	135	Pi-No. 4	Pi-ta^2	0.64	159
Tsuyuake	Pi-k^m, Pi-m	0.25	118	Kusabue	Pi-k	0.67	141
Tetep	Pi-k^h	0.26	149	Aichi asahi	Pi-a	0.75	145
Kanto 51	Pi-k	0.27	136	K 59	Pi-t	0.78	111
Sha-tiao-tsao	Pi-k^s	0.43	131	Taichung T.C.W.		0.78	145
Chokoto	Pi-k, Pi-a	0.43	124	Fanny		0.86	167

Results from this research indicate that resistance selection in Colombia has been conducted under conditions of exposure to more than 50 international races, representing all 9 international pathotype groups. Pathogen variability has been shown to be far beyond the international designation using differentials because several races could be further differentiated into different pathotypes when local commercial varieties were used as differentials. Compatibility was present in the population for at least 13 known resistance genes and resistance sources tested (Table 1). Virulence frequencies ranged from 0 to 0.86, with no cultivar susceptible to all pathogen isolates, and resistance to all isolates being present only in advanced lines developed at this hot spot site (Table 2). It was very important to find that the lowest virulence frequencies were associated with combinations of resistance genes. Analysis of the frequency of compatibility of isolates recovered from commercial rice cultivars revealed a marked specialization for the cultivar of origin. This is a very important finding given the pathogenic diversity found.

Table 2. Compatibility frequency of virulent phenotypes of *Pyricularia grisea* on commercial Colombian rice cultivars in Santa Rosa.

Commercial Colombian cultivar	Frequency	Number of isolates	Year of release
Oryzica Llanos5	0.00	250	1989
Oryzica Llanos4	0.00	250	1989
Linea 2	0.06	102	1989
Cica 9	0.07	168	1976
Bluebonnet 50	0.08	156	1951 (USA)
Oryzica 2	0.13	168	1984
Cica 8	0.25	168	1978
Cica 6	0.33	168	1974
Cica 7	0.36	167	1976
IR 22	0.42	168	1969
Oryzica 3	0.44	162	1987
Oryzica 1	0.45	168	1982
IR 8	0.50	173	1968
Metica 1	0.54	168	1981
Cica 4	0.66	174	1971

A very important determination was that *P. grisea* isolates may accumulate virulence factors in a common isolate, but no isolate was virulent to all rice cultivars. A combination of virulence factors was detected even for resistance gene combinations never used in the rice breeding lines. Nevertheless, the population already has matching virulence for all resistance genes, indicating that new combinations and/or new resistance genes are needed. Rare compatibilities with particular cultivars suggested that combinations of certain virulence may be associated with poor fitness or with deleterious effects for the pathogen. Further studies on genetic diversity using DNA-fingerprinting indicated that this combination of virulence genes is not present in the pathogen population due to the genetic diversity among the pathogen population that would need the sexual stage for recombination. These results have allowed CIAT to use a precise strategy to develop disease resistance since combinations of resistance genes can now be directed toward resistance to the combination of virulence genes with low chances of occurrence in the pathogen population. Random combinations of resistance genes may be proven to be useless since unnecessary virulence genes are maintained in the pathogen population. This has been observed despite high cultivar specificity.

Strategies to incorporate and accumulate resistance genes that may present barriers to pathogen compatibility are being implemented by characterizing genetic diversity via further pathotyping and DNA-fingerprinting analysis.

MGR-fingerprinting

Recently, 3 DNA probes were developed by Dr. J. Hamer, and they reliably and specifically identify the genetic backgrounds

of the full spectrum of rice blast fungus pathotypes. One of these probes consists of cloned fragments of repeated DNA obtained from the rice blast fungus genome and are called MGR586 (previously referred to as pCB586). The probe hybridizes to approximately 50 EcoRI fragments, ranging in size from 1.5 to 20.0 kb, in the genome of all *P. grisea* isolates that infect rice, but to only one or a few fragments in *P. grisea* isolates limited to other grass hosts. Worldwide conservation of MGR586 sequences in rice blast pathogens indicates that they descend from a common ancestral source, genetically isolated from other host-limited forms of *P. grisea.* Genetic mapping, using MGR, shows that sequences are randomly dispersed on all chromosomes of the pathogens and segregate as genetic loci. Consequently, the MGR586 restriction fragment profiles observed in the natural clones of the pathogen define multilocus haplotypes, which we have referred to as MGR-DNA fingerprints. These can identify both genetic diversity and relatedness among field isolates.

An extensive study of Colombian rice blast diversity was conducted by first analyzing the population structure in a Colombian blast disease "hot spot" where local pathotype diversity is five times greater than in the entire USA. The study site at Santa Rosa Station is a 30-hectare resistance breeding farm located in the savannas of eastern Colombia. An ideal climate and continuous plantings of a wide variety of susceptible cultivars support chronic usage disease levels throughout the 270-day growing season, and the blast population contains more than 50 pathotypes.

A total of 151 field isolates were obtained from 15 different cultivars during 1987 and 1990. The isolates expressed a total of 56 pathotypes which collectively included virulence and avirulence on all international differentials over a broad continuum of combinations. Thirty-one isolates (20% of the sample) had uncharacterized pathogenicity, i.e., pathotype II-1, being avirulent on all differentials. Based on this spectrum of pathotypes and associated inferences of resistance gene frequencies among the differentials, we concluded that few of the known resistance genes or gene combinations would be useful or effective for blast resistance at Santa Rosa.

A total of 115 different MGR-DNA fingerprints (with 42-52 EcoRI fragments per fingerprint) were observed in our sampling. However, all isolate diversity was subsumed in only six distinct MGR-defined lineages (Figure 1). Isolates within each lineage had a 92% or greater average fingerprint similarity, with no significant subclustering among lineage members (not shown). In contrast, the maximum consensus similarity between lineages was 85%, the minimum was 37%, and the average was 49%. All lineages were significantly different (p= 0.001) from each other for genetic distance (Figure 2).

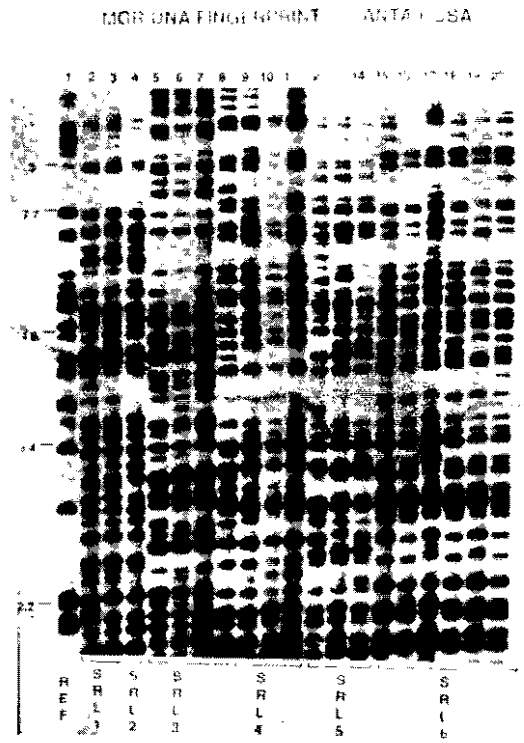

Figure 1. MGR-DNA fingerprints of a representative group of Colombian isolates of *P. grisea* at a "hot spot" site.

Each lineage was associated with a specific subset of cultivars and a specific subset of pathotypes; 90% of all pathotypes were lineage-specific and most were cultivar-specific within a lineage. The hierarchical organization of the Santa Rosa population revealed by MGR polymorphisms indicated that the bulk of the observed pathotype diversity represented selectively neutral variations that were largely irrelevant to field compatibility with the site's cultivars. Generally, isolates of the same lineage were equally compatible with their field host, irrespective of their pathotypes on the differentials under greenhouse conditions. Thus, cultivar range was defined most predictively at the level of the MGR lineage.

CONSENSUS MGR PHENOGRAM FOR SANTA ROSA

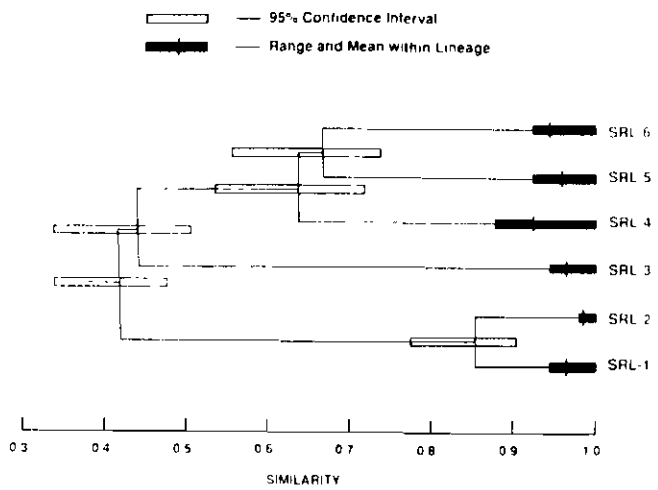

Figure 2. UPGMA phenogram of MGR-DNA fingerprint variation at Santa Rosa. Filled bars denote the range of pairwise isolate similarities within lineages, vertical lines denote the mean similarity within a lineage. Stippled error bars denote 95% confidence interval of between-lineage branch point values from bootstrap analysis, all lineages are significantly different from each other.

Tagging resistance genes with RFLP and RAPD markers

Breeding rice in a blast hot spot was proposed as a way to overcome possible causes of resistance breakdown. Such a strategy has been successful, with the commercial release in 1989 of two cultivars, Oryzica Llanos 4 and Oryzica Llanos 5. These varieties, bred by CIAT in close collaboration with the Colombian national program (ICA) and the Colombian National Association of Rice Growers (Fedearroz), show no signs of susceptibility under either production or experimental conditions of intense disease pressure. So far, Oryzica Llanos 4 and Oryzica Llanos 5 have shown complete resistance when inoculated with some 250 blast isolates prevalent in Colombia (Table 2).

Studies conducted over the past five years on these two cultivars compared with other commercial varieties clearly demonstrated that specific combinations of resistance genes or sets of genes, rather than random combinations of genes, are the most efficient way to develop durable resistance. The breeding effort to incorporate such combinations was slow and laborious due to the inability, at that time, to identify the various genes in a segregating population.

Recent developments described above using molecular tools have provided new horizons for breeders in managing highly variable diseases such as rice blast. Molecular markers offer an efficient way to locate and monitor disease resistance gene transfer in rice breeding programs.

Two types of molecular markers are being used at CIAT to identify, tag and characterize currently used as well as novel resistance genes against Colombian blast isolates. The first type, RFLPs (restriction fragment length polymorphisms), are probes from the molecular map of rice constructed

by Dr. Susan McCouch and Dr. Steve Tanskley and their team at Cornell University. The second type of molecular markers, RAPDs (random amplified polymorphic DNA), are short oligonucleotide primers and were recently demonstrated to be effective in gene tagging in tomato and lettuce. RAPDs offer an inexpensive and efficient way to identify and characterize genomic components. Both markers provide new opportunities to develop or identify breeding lines with specific resistance genes. Using these markers, linkage with genes resistant to isolates belonging to one lineage (SRL-1) has been obtained as a first step in localizing a series of genes resistant to the various lineages found in Colombia.

A new breeding strategy

These exciting results allow a fundamental redirection of rice breeding schemes. They imply that we should focus on genes resistant to lineages, rather than on genes resistant to specific isolates as inferred from pathotype analysis (Table 3). As an example, Oryzica Llanos 5 presents combinations of resistance genes to the six genetic lineages found in the pathogen. Not even the most virulent isolates of the blast pathogen studied exhibited virulence to the susceptible cultivar Cica 9, one of the parents of Oryzica Llanos 5. Compatible isolates with Cica 9 belong to the genetic lineage SRL-1 or SRL-2, and do not present virulence genes for resistance genes associated with the genetic lineages SRL-3, SRL-4, SRL-5 and SRL-6. Achieving such levels of resistance requires an intensive crossing and selection scheme. The new strategy combining pathotyping, MGR-fingerprinting and molecular marker-assisted gene tagging provides a way to define the genetic organization and distribution of rice blast pathogen diversity and to incorporate appropriate resistance genes. Such a strategy to attack a formerly intractable problem will provide the most efficient means available for improved blast disease management and could serve as a model system to tackle similar fungal diseases such as potato blight, wheat rust or downey mildew in sorghum and millet.

Table 3. Reaction of commercial rice varieties to six genetic lineages of blast pathogen in Colombia.

CULTIVARS	LINEAGE					
	1	2	3	4	5	6
IR8	S	S	R	S	S	S
ORYZICA 1	R	S	R	S	R	S
ORYZICA 3	R	R	R	S	S	S
METICA 1	R	R	S	S	R	S
CICA 4	S	S	R	S	S	S
CICA 8	R	R	R	S	S	S
CICA 9	S	S	R	R	R	R
Oryzica Llanos 4	R	R	R	R	R	R
Oryzica Llanos 5	R	R	R	R	R	R

Potential impact

The potential impact of developing durable resistance for rice in Latin America has been estimated at more than US$400 million per year in years of high disease pressure. This is equivalent to 7.4% of the total value of rice production in the region. The benefits amount to approximately US$200 million and US$80 million in years of normal and low disease pressure, respectively. For a 15-year production series, the net present value of these benefits is estimated at US$1.6 billion, assuming a probability a priori of 33% for years of low, normal and high disease pressure, respectively. This also means that, on average, the region would save about US$210 million through reduced imports of rice and lower cost of fungicides, with very positive implications for its food balance and sustainability of rice production.

We hesitate to magnify these estimates to a global scale and will leave this to others. We who make up this multidisciplinary team are

proud to have worked together to solve this difficult problem. We are confident that the results will lead to major benefits for mankind in rice and that the methodologies, once applied to other crops and variable pathogens, will provide even further proof that good science, wisely managed, is an excellent investment.

Interdisciplinary team

The interdisciplinary and interinstitutional team is composed of the following scientists, listed in alphabetical order:

Fernando Correa-Victoria, Plant Pathologist from CIAT, who carried out cultural management research, pathotyping and stability studies on resistance to the rice blast pathogen.

Morris Levy, Evolutionary Biologist from Purdue University, who implemented the MGR-fingerprinting on the Colombian isolates and conducted the statistical analysis of genetic distance.

John Hamer, Molecular Biologist from Purdue University, who identified the MGR probe and characterized its structure and participated in MGR-fingerprinting.

César Martínez, Plant Breeder from CIAT, who participated in the selection of rice-blast-resistant cultivars and the establishment of a routine anther culture scheme to provide for double-haploid production for gene tagging.

William Roca, Cell Physiologist and Head of the Biotechnology Research Unit at CIAT, who established anther culture research procedures and supervised gene tagging.

Joe M. Tohme, Plant Geneticist from CIAT, who implemented the RFLP and RAPD gene tagging and participated in developing the breeding strategy.

Robert Zeigler, Plant Pathologist and former Leader of the CIAT Rice Program, who led the interdisciplinary team and participated in stability and pathotyping studies and in developing the breeding strategy.

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