The Genetic Basis of High Resistance to Rice Yellow Mottle Virus (RYMV) in Cultivars of Two Cultivated Rice Species

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ABSTRACT

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Three cultivars of *Oryza sativa* (IR64, Azucena, and Gigante) and four cultivars of *O. glaberrima* (Tog5681, Tog5673, CG14, and SG329) were evaluated for their resistance to two isolates of rice yellow mottle virus (RYMV) by enzyme-linked immunosorbent assay (ELISA) and symptomatology. Cultivars Tog5681 and Gigante were highly resistant, and no symptoms were observed when either virus isolate was inoculated at 10 or 20 days postgermination and assayed by ELISA at 7, 14, 22, 35, 50, or 64 days postinoculation. Azucena showed a partial resistance, whereas the other cultivars were susceptible. Symptom appearance was associated with increase in ELISA absorbance in the systemically infected leaves. The best discrimination among the cultivars occurred when the plants were inoculated at 10 days postgermination. Crosses were made between the highly resistant (Gigante and Tog5681) and the susceptible (IR64) cultivars to determine the genetic basis of resistance to RYMV. Evaluation of F1 hybrids and interspecific progenies, as well as the segregation of resistance in F2 and F3 lines of the IR64 × Gigante cross, provided results consistent with the presence of a single recessive resistance gene common to Tog5681 and Gigante.

Additional keywords: Africa, monogenic resistance, sobemovirus

Rice yellow mottle virus (RYMV) is a sobemovirus (10) endemic to the African continent and is one of the most devastating pathogens of cultivated rice throughout West Africa (1), Kenya, Tanzania (1,5,16), and Madagascar (20). RYMV is naturally transmitted mainly by chrysomelid beetles and is experimentally propagated by sap inoculation (4). The disease caused by RYMV is characterized by mottling and yellowing of the leaves, delayed flowering, poor panicle development, and spikelet sterility. Symptom severity and yield losses are variable and depend on genotype. Plant death can occur in highly susceptible cultivars when infected early (3).

Different degrees of resistance to RYMV have been detected in *Oryza sativa* and in *O. glaberrima* (23), and partial resistance was expressed in the upland rice cultivars such as Azucena. The (IR64 × Azucena) doubled haploid (DH) population was analyzed to determine the genetic basis of the quantitative resistance to RYMV (2,9). Alternative resistance sources are

currently being investigated. Recently, the *O. sativa* cultivar Gigante was found to be highly resistant to RYMV during field screening tests of the rice germ plasm at the West African Rice Development Association (WARDA-Bouaké, Côte d'Ivoire). Evaluation of RYMV resistance in *O. glaberrima* also revealed five cultivars, including Tog5681, with high levels of resistance (21,23).

Available techniques of tagging and mapping resistance could help transfer natural resistance found in Tog5681 or Gigante through marker-assisted selection. The main objectives of this study were: (i) to characterize the level of resistance to RYMV in *O. glaberrima* and *O. sativa* cultivars by immunological tests and symptomatology, and (ii) to develop hybrids and progeny between susceptible and resistant cultivars in order to assess the genetic basis of RYMV resistance coming from Tog5681 and Gigante cultivars.

MATERIALS AND METHODS

Experimental plant material and culture conditions. The cultivar Gigante and four cultivars of *O. glaberrima* (Tog5681, Tog5673, CG14, and SG329) were provided by WARDA, while the two *O. sativa* cultivars IR64 and Azucena were the two parents of the DH population currently used for genetic mapping of RYMV resistance (2,9). IR64 is a high-yielding cultivar

developed at the International Rice Research Institute (IRRI, Los Baños, The Philippines), and Azucena is a traditional upland cultivar from the Philippines. Plants were grown in a growth chamber under 12 h of light at 28 ± 1°C (day) and 26 ± 1°C (night). Seeds of each cultivar were sown in two pots. After germination, the plants were thinned to four plants per pot.

Virus source and inoculum preparation. The two viral isolates were collected in Mali (M1) and Burkina Faso (BF1) (West Africa) from field-infected rice plants on the basis of symptom expression. The two isolates belonged to the same group, as determined by monoclonal antibodies and molecular tests (D. Fargette and P. Nguessan, unpublished results), but induced contrasting symptoms: mild for the M1 isolate and severe for BF1. Isolates were recovered by mechanical inoculation of the highly susceptible line BG90-2. Infected frozen leaves were ground in a phosphate buffer (0.1 M KH₂PO₄, 0.1 M Na₂HPO₄, pH 7.2) using 1 g/15 ml ratio. Carborundum was added to the homogenized sap. Mechanical inoculation was carried out by rubbing the extracted sap on the upper and lower leaf surfaces of 2-week-old plants of BG90-2. The leaves exhibiting symptoms were harvested 14 days after infection to prepare a sap inoculum (as described above) that was used for the different experiments.

Protocols. Enzyme-linked immunosorbent assay (ELISA) response of inoculated and systemically infected leaves was measured by direct double antibody sandwich (DAS)-ELISA (6). Polyclonal antisera were prepared against RYMV-purified preparations following the protocol of Fauquet and Thouvenel (8). Antiserum used to determine the virus content of the rice cultivars was directed against the M1 isolate, and that used to test the progeny was against an isolate from Madagascar. Plates (Nunc - Immuno Plate Maxi Sorp) were coated with a 1:2,000 dilution of the polyclonal antiserum in a carbonate buffer (0.015 M Na₂CO₃, 0.034 M NaHCO₃, pH 9.6). After incubation for 2 h at 37°C, wells were saturated with 200 µl of 3% skimmed milk in PBS-T buffer (pH 7.4) for 1 h at 37°C. Plates were washed three times with PBS-T buffer after each step. One gram of inoculated or systemically infected leaves was harvested and ground

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in PBS-T buffer at 1:100 or 1:1,000 dilution depending on the susceptibility of the plants. Samples of 100 μ l were incubated 2 h at 37°C in a microtiter plate, and two replicated wells were used to score optical density (OD) values. One hundred microliters of the secondary antibody conjugated to alkaline phosphatase (dilution 1:1,000) (Roche Molecular Biochemicals, Indianapolis, IN) was then added to the wells. After overnight incubation at 4°C, 100 μ l of a 1 mg/ml solution of *p*-nitrophenyl phosphate in diethanolamine (pH 9.8) (Sigma Chemical Co., St. Louis, MO) was added to each well, and plates were incubated for 3 h at 37°C. The ELISA responses were expressed by scoring the optical densities measured at 405 nm. In all experiments, the uninoculated line BG90-2 was used as a negative control. A positive response was considered if OD values were twofold greater than the mean value of the negative control.

Evaluation of RYMV resistance. A first experiment was conducted to compare the cultivar response for two inoculation dates, 10 and 20 days postgermination (DPG), and for two evaluation times, 7 and 14 days postinoculation (DPI). In a second experiment, virus titer was followed on the same cultivars using 10 DPG for inoculation with the M1 isolate. ELISAs were performed on systemically infected leaves at 7, 14, 22, 35, 50, and 64 DPI, with two replicates of four plants at each date. The cultivar responses to M1 and BF1 infec-

tions were compared under 10 DPG and 7 DPI test conditions, with two replicates of 10 plants for each cultivar in a complete randomized design. In each experiment, symptom appearance, symptom severity, and response to infection assessed in ELISA were scored.

Resistance segregation. To establish the genetic basis of the high resistance to RYMV, different F1 combinations were made between the susceptible IR64 and the resistant accessions Tog5681 and Gigante. As interspecific F1 hybrids between *O. glaberrima* and *O. sativa* are completely male sterile, backcross progenies were derived from the two parents. The standard protocol (inoculation at 10 DPG and 7 DPI for evaluation test) using mechanical inoculation of young seedlings was applied to assess the resistance pattern of an (IR64 $\times$ Gigante) F2 progeny and of individuals coming from rare interspecific backcross-derived fertile plants. F1 hybrids and interspecific backcross-derived sterile plants were duplicated by splitting the tillers of 1-month-old plants. Ten representatives of each F1 combination and a single one for interspecific backcross-derived genotypes were transferred to a growth chamber to be inoculated 7 days later with the BF1 isolate. The response to infection was scored in the systemically infected leaves at 14 DPI. This procedure was previously checked on parents. Plants regenerated by tiller splitting gave the same pattern of virus content as the plants from seeds.

RESULTS

Characterization and diversity of RYMV resistance in *O. glaberrima* and *O. sativa* rice cultivars. Initial work in-

vestigated the optimal timing of mechanical inoculation and subsequent ELISAs in order to find the best conditions for discriminating among cultivars. Plants of IR64, Tog5673, SG329, CG14, Azucena, Tog5681, and Gigante were inoculated using the M1 isolate 10 or 20 DPG, and the response to infection assessed in ELISA was scored at 7 and 14 DPI. With the early 10 DPG inoculation, a high response was noticed as soon as 7 DPI in inoculated leaves and confirmed at 14 DPI in systemically infected leaves of the four cultivars IR64, Tog5673, SG329, and CG14 (Fig. 1A and B). With the late 20 DPG inoculation, no positive response was observed at 7 DPI, suggesting that this period was not long enough to discriminate among the cultivars. At 14 DPI, the positive response was observed again for the susceptible cultivars. The intermediate response observed for Azucena at 10 and 20 DPG (Fig. 1) was maintained when the plants were inoculated later (30, 45, or 60 DPG, data not shown), but the discrimination was more precise at the earlier inoculation time (10 DPG) and when leaf tissues were analyzed 7 days after inoculation. Whatever the test conditions, a very low signal, close to the healthy control, was found in leaves of Tog5681 and Gigante.

In a second experiment, each cultivar was infected at 10 DPG and tested by ELISA at 7 DPI (inoculated leaf) and 14, 22, 35, 50, and 64 DPI (systemically infected leaves). For the susceptible cultivar IR64, the ELISA response reached its maximum at 7 DPI and was associated with mottling on the infected leaves (Fig. 2). In Azucena, the response at 7 DPI was significantly lower than that detected in

Fig. 1. Effects of plant age at inoculation (10 or 20 days postgermination) and of days postinoculation (DPI) on enzyme-linked immunosorbent assay (ELISA) response to infection in systemically infected leaves of different rice cultivars. (A) 7 DPI; (B) 14 DPI. Leaves were inoculated with the M1 isolate and tested at 1:100 dilution.

Fig. 2. Enzyme-linked immunosorbent assay (ELISA) response over time to infection of systemically infected leaves of susceptible and resistant rice cultivars. Leaves were inoculated at 10 days postgermination with the M1 isolate and tested at 1:100 dilution. Arrow indicates symptom appearance. The same letter indicates the absence of significant differences in the Newman and Keuls tests between cultivars. a, Tog5681, Gigante, and healthy control; b, Azucena; c, IR64.

IR64 ($F = 822$, $P < 10^{-7}$). It did increase with time and reached a maximum level at 22 DPI corresponding to the beginning of symptom expression. This level remained significantly lower than that detected in IR64 at the later time points ($F = 1133$, $P < 10^{-7}$). Very low absorbances, not significantly different from the healthy control with virus-free leaves, were observed in Tog5681 and Gigante cultivars, even up to 64 DPI ($F = 0.6$, $P = 0.707$). Complete absence of symptoms was also noted even after the flowering stage. On the basis of these observations, an early and heavy symptom development was linked to a high ELISA response in IR64, which is known to be very susceptible to RYMV (2,9).

As the responses of Tog5681 and Gigante cultivars were never significantly different from the noninoculated control, a complementary experiment was carried out to determine if these cultivars could be considered immune to RYMV. Crude extracts of newly emerging leaves from Tog5681 and Gigante plants inoculated at 10 DPG were harvested at 14 DPI. These leaf extracts were used at low dilution (1:10) as sap inoculum to inoculate 10-day-old seedlings of the highly susceptible

check cultivar (BG90-2). Two weeks after inoculation, mottling was observed on the leaves of BG90-2, confirming that leaf sap of resistant cultivars was infectious, and that Tog5681 and Gigante were not immune to RYMV but highly resistant.

To determine whether partial and high resistance shown by some rice cultivars can be overcome by different strains of the virus, the response to infection with the M1 isolate was compared to that with the BF1 isolate, which is known to give earlier and more severe symptoms. The resistant cultivars Tog5681 and Gigante displayed a very low ELISA response with both viral isolates (Table 1). Two-way ANOVA with fixed (isolate, cultivars) effects showed very significant cultivar ($F = 298.2$, $P < 10^{-7}$) and isolate ($F = 24.33$, $P < 10^{-7}$) effects but no significant isolate $\times$ cultivar interaction at the 1% confidence level. Discarding these two extreme accessions for ANOVA, the cultivar effect was still significant ($F = 102.4$, $P < 10^{-7}$). Results confirmed that IR64 and the three *O. glaberrima* cultivars (SG329, CG14, and Tog5673) were the most susceptible, while Azucena showed an intermediate response with either isolate. In a complementary experiment, seven other isolates coming from different African countries were inoculated to Tog5681 and Gigante. Again, the ELISA responses were very low, often not different from the virus-free control (data not shown).

Inheritance of high RYMV resistance of Tog5681 and Gigante. Plant regeneration by tiller splitting of 1-month-old plants was performed to allow evaluation of the virus titer of F1 hybrids and that of different backcross-derived sterile plants (Table 2). Any F1 combinations between IR64 and the resistant parents, Tog5681 and Gigante, gave high absorbance values when the plants were challenged with the BF1 isolate 15 days after transplanting and analyzed at 14 DPI. Nineteen interspecific backcross individuals (IR64 $\times$ Tog5681) $\times$ IR64 were tested and gave high ELISA

responses similar to that of IR64. These observations indicated the recessive nature of the resistance. The reciprocal backcross (IR64 $\times$ Tog5681) $\times$ Tog5681 gave a clear 1:1 segregation ($\chi^2 = 0.18$), which is compatible with the presence of a single recessive gene in Tog5681 (Table 2). Additionally, a backcross individual BC1F1 was found to be fertile and gave F2 progeny. The test involving this F2 progeny as well as test crosses of the backcross sterile plants with the two parents revealed a very high and homogenous susceptibility of the genetic material through both symptom scoring and ELISA response (Table 2). These results clearly confirmed that a single recessive gene could explain the distribution of interspecific backcross progenies in two resistance classes.

The segregation of resistance was identified in 65 (IR64 $\times$ Gigante) F2 plants inoculated with the BF1 isolate. By 18 DPI, 15 F2 plants could be considered to be completely resistant, while the other 50 F2 individuals displayed various symptoms from the beginning of mottling to leaf necrosis. At that time, newly emerging leaves of each F2 plant were tested by ELISA. The ELISA responses clearly separated the F2 plants into two groups without intermediate values (Table 2). In fact, the plants without symptoms gave the very low ELISA response found in Gigante. The other F2 plants, whatever the symptom severity of the inoculated leaves, gave a high ELISA response similar to that of IR64. This clustering fits well a 3:1 segregation ($\chi^2 = 0.13$) corresponding to the presence of a single recessive resistant gene in this F2 population (Table 3). Altogether, the two sources of resistance (Tog5681 and Gigante) showed the same recessive pattern of heredity. The response to infection of F1 hybrids between Tog5681 and Gigante was very low and close to that of the parents. The resistance observed in Tog5681 $\times$ Gigante F1 hybrids was in favor of an identical resistance locus.

Table 1. Enzyme-linked immunosorbent assay response (A_{405nm}) to rice yellow mottle virus (RYMV) infection of rice cultivars inoculated with a mild (M1) and a severe (BF1) isolate^a

Cultivars	RYMV isolates	
	M1	BF1
IR64	1.50	1.80
Tog5673	1.50	1.75
CG14	1.30	1.60
SG329	1.35	1.60
Azucena	0.20	0.50
Tog5681	0.03	0.06
Gigante	0.02	0.05
Healthy	<0.01	<0.01

^a Crude sap of all cultivars was tested at 1:100 dilution. Plants were inoculated 10 days postgermination and tested 7 days postinoculation.

Table 2. Distribution of enzyme-linked immunosorbent assay (ELISA) responses (A_{405nm}) in the systemically infected leaves of F1 hybrids, backcross, and F2 progenies derived from crosses between the susceptible IR64 and the two resistant rice cultivars Gigante and Tog5681^a

F1 hybrids/progenies	Symptom scoring	No. of genotypes	Distribution of optical density (OD) values			Mean OD values
			0.01-0.05	0.9-1	>1	
Derived from Tog5681						
F1: (IR64 $\times$ Tog5681)	Susceptible	...	...	...	10	1.9
BCS: (IR64 $\times$ Tog5681) $\times$ IR64	Susceptible	19	...	4	15	1.6
BCG: (IR64 $\times$ Tog5681) $\times$ Tog5681	Segregating	22	12	...	10	...
Derived from 1 fertile BCS plant						
BCSF2	Susceptible	11	...	...	11	1.3
BCS $\times$ IR64	Susceptible	1	...	...	1	1.9
BCS $\times$ Tog5681	Susceptible	15	...	...	15	1.9
Derived from Gigante						
F1: (IR64 $\times$ Gigante)	Susceptible	...	...	...	10	1.9
F2: (IR64 $\times$ Gigante)	Segregating	65	15	...	50	...
F1: (Gigante $\times$ Tog5681)	Resistant	...	10	...	...	0.03

^a ELISA responses were obtained from (i) 10 plants regenerated by tiller splitting for each F1 hybrid combination, (ii) 1 plant regenerated for each interspecific backcross-derived genotype, and (iii) directed tests on young seedlings (inoculation at 10 days postgermination and reading time at 7 days postinoculation) for F2 and fertile interspecific progenies.

To confirm this model of resistance heredity, F3 progeny-tests were carried out from the same (IR64 × Gigante) cross. Eleven F2 plants found to be resistant in the previous ELISAs were transplanted and grown to harvest selfed F3 seeds. A new set of 55 F2 plants was also grown but not inoculated to ensure the F3 seed set whatever the resistance or susceptibility of the genetic material. Each F3 family (10 to 20 plants per family) was inoculated at 15 DPG with the BF1 isolate, and symptom expression was assessed up to 2 months after inoculation for the resistant F3 lines (Table 3). Very susceptible homogenous F3 lines were clearly identified as early as 15 DPI. In pooling data of the segregating lines, the frequency of resistant plants fits with the expected segregation of 3:1 ($\chi^2 = 0.07$). To check the resistance of the plants, symptom expression was assessed until 30 DPI. Resistant F3 families could then be clearly classified, and all F3 lines derived from the 11 resistant F2 plants were found to be resistant. The classification of F3 families fits the 1:2:1 expected distribution with the segregation of a single resistant gene ($\chi^2 = 1.36$). Using the last symptom scoring (45 DPI), the group of resistant plants was split into two subgroups (resistant and highly resistant), assuming the occurrence of few plants with late and discrete mottle apparition. Nevertheless, these observations did not lower the relevance of the monogenic model of resistance inferred for Gigante.

DISCUSSION

An early mechanical inoculation of the plant (10 DPG) was efficient in discriminating among susceptible, partially resistant, and highly resistant cultivars by ELISA, whatever the isolate tested. This indicates that Tog5681 and Gigante are highly resistant and suggests that this resistance is not strain specific. The susceptible cultivars (IR64, Tog5673, CG14, and SG329) were characterized by early symptoms (7 DPI) and high virus accumulation. A very low virus titer and complete absence of symptoms in leaves of Tog5681 and Gigante cultivars were noted. However, they could not be considered immune to the virus, as successful me-

chanical inoculations showed the presence of the virus in systemically infected leaves.

Plant age at inoculation is a key factor of response to infection (13), and virus accumulation varied with plant age at inoculation time for susceptible and partially resistant cultivars. In the case of the susceptible cultivars, virus content increased in the young leaves and decreased when the inoculation was done on the oldest leaves. Our results are similar to those observed with the oldest leaves of sorghum plants infected by maize dwarf mosaic virus (MDMV) (11). As observed by Thottapilly and Rossel (22,23), the determination of virus content in susceptible and resistant lines by ELISA were closely related to visual scoring of symptoms. Comparative analysis between Azucena and IR64 using immunoprinting, ELISA, and tissue fluorescent immunolabeling suggested that partial resistance of Azucena could be attributed to two components (14): (i) a lowered virus movement through the parenchyma cells surrounding the vascular tissue (mestome), specific to the expression of a resistance mechanism, and (ii) differences in the leaf appearance rate between Azucena and IR64 associated with a delay in long-distance movement of the virus.

The high level of RYMV resistance and apparent immunity of some *O. glaberrima* cultivars has already been reported (3,12,23). In our experiments, we confirmed that this resistance is associated with very low virus accumulation throughout plant growth. Such resistant plants, inoculated as early as 10 DPG, could complete a normal growth cycle and produce an abundant seed set. A similar pattern of resistance was found in *O. breviligulata*, which is the direct progenitor of *O. glaberrima* (22,23). Nevertheless, this high level of RYMV resistance is very rare in *O. glaberrima* and found only in five cultivars among several hundred (21,23). This observation could reflect the bottleneck effect usually observed in the domestication process, which drastically reduces genetic diversity in cultivated species.

The finding of an exceptional cultivar of *O. sativa* with resistance similar to that of an *O. glaberrima* resistant cultivar is novel

for the following reasons: (i) it is the first time that such high resistance has been reported in *O. sativa* collections and nurseries despite intensive RYMV resistance screening by national and international institutions (WARDA, IITA); (ii) Gigante showed the typical morphological traits and lowland adaptation of a traditional *indica* cultivar, whereas all the other *indica* cultivars were found to be highly susceptible to RYMV. Moreover, the classification of Gigante in a representative rice collection using 9 microsatellite loci among the set mapped by Chen et al., (7) on IR64 × Azucena DH population has confirmed that this cultivar is clustered unambiguously within the *indica* group (C. Patry and A. Ghesquière, unpublished results); (iii) only partial and quantitative resistance has been observed in upland rice cultivars in *O. sativa*. Thus, the genetic basis of the high resistance found in this *indica* cultivar is probably completely different from Quantitative Trait Loci (QTL) associated with the partial resistance of upland rice cultivars such as Azucena. In this instance, genetic mapping of RYMV resistance in the IR64 × Azucena DH population through the evaluation of virus content and field resistance assessment revealed a complex pattern of resistance heredity involving several QTLs and interactions with morphology (2,19).

Little is known about the genetic basis of the high resistance to RYMV. However, diallel analysis involving susceptible and resistant *O. glaberrima* cultivars revealed the recessive nature of this resistance and predominantly additive effects with little room for other Mendelian loci (18). In our study, interspecific hybrids and backcross progenies confirmed the recessive pattern of the resistance coming from *O. glaberrima* and the ratios obtained in F2 and F3 progeny tests of IR64 × Gigante crosses could be explained by the presence of a single recessive resistant gene. Our results are also compatible with the hypothesis that the high resistance to RYMV is controlled by the same resistance locus in the two Gigante and Tog5681 cultivars. As slight variations between F3 lines and delay in symptom appearance were observed, resistance QTLs and interactions with plant morphology probably also act, but the quantitative resistance of the *O. sativa* genetic background did not mask the effect of the major resistance gene provided by Gigante.

These results indicate that this high resistance mechanism is completely different from the polygenic partial resistance system found in upland cultivars of *O. sativa*. Virus replication, cell-to-cell movement, and long-distance movement in the vascular system are the different steps involved in the RYMV spread throughout the plant (17). Consequently, this major resistance gene could act through a specific plant-virus interaction during one of these criti-

Table 3. Segregation of rice yellow mottle virus (RYMV) resistance in (IR64 × Gigante) F3 progenies^a

Resistance classes	No. of F3 progenies	No. of plants			Frequency of resistant plants
		Total	Susceptible	Resistant	
Susceptible	15	191	191	0	0
Segregating	30	343	262	81	0.24
					$\chi^2 = 0.07$ (3:1)
Resistant	4	45	14	31	0.69
Very resistant	6	87	0	87	1
Resistant* ^b	7	73	23	50	0.68
Very resistant*	4	56	0	56	1

^a Inoculation was made from 10 to 17 days after germination with BF1 isolate, and symptom scoring was followed for 45 days after inoculation.

^b * F3 progenies derived from resistant F2 plants analyzed by enzyme-linked immunosorbent assay.

cal steps. The high resistance to RYMV provides a new and interesting plant virology model to dissect the different components involved in virus spreading within the plant and to elucidate a key factor of the plant-RYMV interaction through the assessment of coat protein, P1 movement protein, and RNA accumulation in inoculated and systemically infected leaves. The identification and mapping of resistance markers is also of great interest in plant breeding applications for transferring this resistance into the susceptible irrigated and lowland rice cultivars through marker-assisted selection. While the appropriate interspecific genetic material is currently developed to carry out allelism tests between the two resistance sources, the two groups of highly susceptible or very resistant F2 plants and F3 lines derived from IR64 × Gigante crosses will serve to identify resistance markers through bulked segregant analysis, as described by Michelmore et al. (15), using amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA markers. Positive markers will then be mapped on the reference (IR64 × Azucena) genetic linkage map for further fine mapping.

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