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ORIGINAL PAPER

Identification of a second major resistance gene to *Rice yellow mottle virus, RYMV2,* in the African cultivated rice species, *O. glaberrima*

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Abstract *Rice yellow mottle virus* (RYMV) is the most damaging rice-infecting virus in Africa. However, few sources of high resistance and only a single major resistance gene, *RYMV1*, are known to date. We screened a large representative collection of African cultivated rice (*Oryza glaberrima*) for RYMV resistance. Whereas high resistance is known to be very rare in Asian cultivated rice (*Oryza sativa*), we identified 29 (8%) highly resistant accessions in *O. glaberrima*. The MIF4G domain of *RYMV1* was sequenced in these accessions. Some accessions possessed the *rymv1-3* or *rymv1-4* recessive resistance alleles previously described in *O. glaberrima* Tog5681 and Tog5672, respectively, and a new allele, *rymv1-5,* was identified, thereby increasing the number of resistance alleles in *O. glaberrima* to three. In contrast, only a single allele has been reported in *O. sativa*. Markers specific to the different alleles of the *RYMV1* gene were developed for marker-assisted selection of resistant genotypes for disease management. In addition, the presence of the dominant susceptibility allele (*Rymv1-1*) in 15 resistant accessions suggests that their resistance is under different genetic control. An allelism test involving one of those accessions revealed a second major resistance gene, i.e., *RYMV2*. The diversity of resistance genes against RYMV in *O. glaberrima* species is discussed in relation to the diversification of the virus in Africa.

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Introduction

Rice (*Oryza sativa*) is a staple food crop in most parts of Africa, but rice production in the continent is far from covering the needs of the growing population. Diseases represent one of the main constraints to rice production worldwide. Among them, the disease caused by the *Rice yellow mottle virus* (RYMV), which was first reported in Kenya in 1966, is one of the most yield-reducing factors in Africa (for review, Kouassi et al. 2005). RYMV, a member of the genus *Sobemovirus*, has only been reported in the African continent. The disease is endemic in most rice-growing areas, especially East and West Africa, where entire fields are often devastated during epidemics. Control measures against RYMV have mainly been geared towards genetic control. Natural sources of resistance have been characterized in the two cultivated rice species, i.e., *O. sativa* (Asian rice) and *O. glaberrima* (African rice). Partial resistance, characterized by a delay in symptom expression and virus accumulation, is widespread in *O. sativa japonica* upland varieties and involves several quantitative trait loci

(QTLs) (Boisnard et al. 2007). On the contrary, high resistance, characterized by a lack of symptoms, undetectable virus content and no yield losses upon field infection, is very rare and only reported in two *O. sativa indica* varieties and a few *O. glaberrima* rice accessions (Ndjiondjop et al. 1999; Rakotomalala et al. 2008; Thottappilly and Rossel 1993). Up to now, a single major resistance gene, i.e., *RYMV1*, has been identified (Ndjiondjop et al. 1999) and mapped on rice chromosome 4 (Albar et al. 2003). This gene encodes the eukaryotic translation initiation factor eIF(iso)4G (Albar et al. 2006). Three recessive alleles responsible for resistance have been described: *rymv1-2* was identified in *O. sativa indica* varieties Gigante and Bekarosaka, whereas *rymv1-3* and *rymv1-4* were found in *O. glaberrima* acccessions Tog5681 and Tog5672, respectively (Albar et al. 2006). However, since RYMV isolates able to break the resistance conferred by different alleles of the *RYMV1* gene have been reported (Traoré et al. 2006), sustainable genetic control of RYMV disease will require the identification and characterization of new sources of resistance.

The African rice species *O. glaberrima* which used to be found in diverse West African agro-ecosystems, e.g., in rainfed environments and deep water floating systems has now been generally abandoned in favor of high-yielding *O. sativa* cultivars (grown worldwide) due to its poor agronomic performance. However, *O. glaberrima* represents a valuable source of genes potentially capable of alleviating many biotic and abiotic stresses (Sarla and Swamy 2005). Concerning RYMV resistance, only limited screenings have been performed in *O. glaberrima* species despite the large number of accessions available in collections. Thottappilly and Rossel (1993) identified a few resistant accessions in *O. glaberrima* and its wild ancestor *Oryza barthii*, suggesting that African rice species could be a valuable source of RYMV resistance. These reports encourage us to initiate intensive screens for RYMV resistance in a large collection of *O. glaberrima* accessions, focusing on *RYMV1* variability. We identified a diverse range of resistance phenotypes, and a second major resistance gene. At the same time, specific markers and convenient procedures were developed to identify distinct *RYMV1* alleles for marker-assisted selection.

Materials and methods

Plant materials

A total of 337 *O. glaberrima* accessions (255 provided by the Africa Rice Center, WARDA, Cotonou, Benin, and 82 provided by the International Rice Research Institute, IRRI, Los Baños, Philippines) were tested in this study. Accessions were selected on the basis of their geographical origins (Fig. 1; Supplemental table) and agroecologies (upland, lowland and floating). The *O. sativa* varieties IR64 (*indica*, highly susceptible to RYMV), Azucena (*japonica*, partially resistant) and the *O. glaberrima* accessions Tog5681 and Tog5672, carrying the *rymv1-3* and *rymv1-4* recessive resistance alleles, respectively, were also included as references in phenotypic evaluations.

F1 hybrids and F2 populations derived from crosses between different *O. glaberrima* accessions were produced to study the genetic control of resistance when necessary.

Virus isolates and propagation

Two RYMV isolates were used: B27 was selected in a collection of isolates from Benin (Séré, unpublished) and BF1 is a highly aggressive isolate from Burkina Faso that was previously used to identify the *RYMV1* gene (Ndjiondjop et al. 1999). B27 and BF1 belong to RYMV strains S1 and S2, respectively, both of which are widespread in West Africa (Traoré et al. 2005). Isolates were multiplied on the standard susceptible rice cv. IR64. Mechanical inoculation was performed with infected leaf samples ground in 100 mM phosphate buffer, pH 7.2 (10 ml/g of leaf sample). Carborundum (600 mesh) was added to the extracts, which were subsequently rubbed onto rice leaves.

Resistance evaluation

An initial phenotypic screening was performed under screenhouse conditions at the WARDA research station

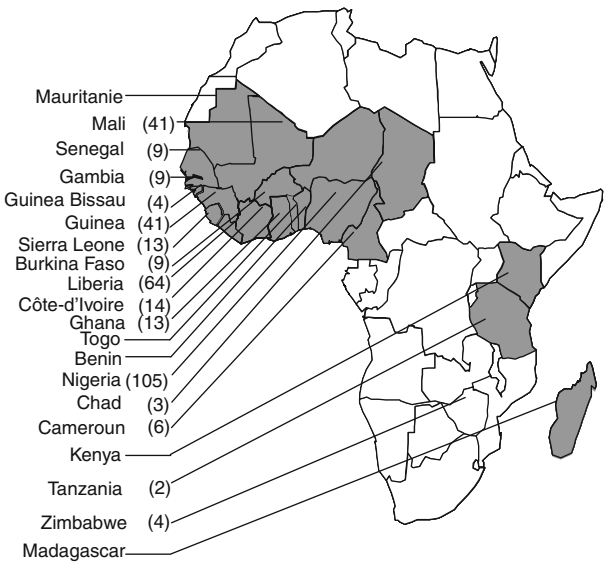

Fig. 1 Countries of origin of the 337 *O. glaberrima* accessions characterized in this study. Countries where the *Rice yellow mottle virus* (RYMV) has been isolated are named and appear in *gray*. *Numbers in parentheses* refer to the number of accessions surveyed per country

(Cotonou, Benin). Two hundred and fifty-five *O. glaberrima* accessions from the WARDA collection were evaluated with the local isolate B27 to avoid introducing another isolate into the experimental area. An augmented randomized incomplete block experimental design with 12 rows was used in the study. The controls (Tog5681, Tog5672, IR64, Azucena) were randomized in each row. Fifteen seedlings of each accession were mechanically inoculated, as previously described, 3 weeks post-germination. Symptom intensity on leaves was monitored every week until heading date using a 1–9 symptom severity scale: score 1 for no symptoms and plants considered as highly resistant (abbreviated as R+), score 3 for sparse dots or streaks, score 5 for general mottling of the leaves, score 7 for yellowing and stunting and score 9 for necrosis and sometimes plant death. The area under the symptom progression curve (AUSPC) was calculated with the formula: AUSPC $= \sum [(S_i + S_{(i+1)} - 2)(t_{(i+1)} - t_i)]/2$, ($S_i$ corresponds to the symptom score at the date t_i, in days). The last fully expanded leaf of each plant was collected for enzyme-linked immunosorbent assay (ELISA), performed as described in N'Guessan et al. (2000). The virus titer was considered significant when the OD values were twofold greater than the mean value of the negative control.

Additional screenings were performed under glasshouse conditions (27–30°C, 60–70% relative humidity) in the IRD laboratory (Montpellier, France). Accessions identified as R+ with isolate B27 in screenhouse conditions and additional ones, from the IRRI collection, were inoculated with the BF1 isolate. A minimum of ten plants per genotype were inoculated 2 weeks post-germination. The presence of symptoms was checked every week for 85 days post-inoculation (dpi). ELISA tests were also performed 85 dpi on systemically infected leaves of each plant. In cases of very late symptom onset (85 dpi), infected leaves were used as inoculum for back-inoculation on the same accession. A subsequent early and generalized infection suggested a resistance-breaking event on a R+ accession.

The pathogenicity of B27 and BF1 isolates was assessed in an experiment involving 14 rice accessions, representative of different resistance levels. Ten seeds of each accession were grown in a randomized complete block design with four replicates. The B27 and BF1 inoculum concentrations were compared by ELISA and adjusted to the same value. Mechanical inoculation was performed 2 weeks post-germination. The symptom intensity was monitored for 30 dpi using the scale described previously. At 30 dpi, systemically infected leaves of the plants of each replicate were pooled and tested by ELISA.

Resistance evaluations of F1 hybrids and F2 populations were performed in glasshouse conditions, after mechanical inoculation of BF1 isolate 2 weeks after germination. The presence of symptoms was checked every week for 12 weeks, and the presence/absence of virus was confirmed by ELISA.

Sequencing of *RYMV1* cDNA and development of *RYMV1* allele-specific markers

Total RNAs were isolated from rice leaves using TRIzol reagent (Invitrogen, Paisley, UK). *RYMV1* cDNAs were obtained by reverse transcription performed with primer 5′-GCCTTGTTCATCTCAGGCTCCA-3′ using ImProm-II™ reverse transcriptase (Promega, Madison, WI, USA) and the protocol recommended by the manufacturer. Sequencing was performed on amplification products by Cogenics Genome Express SA.

In order to develop *RYMV1* allele-specific markers, primers targeting the DNA sequence variation which differentiate resistance from susceptibility alleles were designed (Table 1). PCR was performed using genomic DNA extracted from rice leaves as described by Edwards et al. (1991). The 15-µl reaction mix comprised: *circa* 15 ng DNA, 200 µM of each deoxyribonucleotide 5′-triphosphate (dATP, dGTP, dTTP, dCTP), 1.5 mM $MgCl_2$, 0.02 u/µl Go*Taq* DNA polymerase (Promega, Madison, WI, USA), 0.1 µM primers, and 1× buffer. Two primers (F1 and R1) were derived from the *RYMV1* gene, upstream from the sequence corresponding to the MIF4G domain, in order to amplify each of the different alleles in a non-specific manner. The primers F1 and R1 were added to the reaction mix to generate an internal amplification control. The annealing temperature was progressively decreased from 68 to 62°C during the first five cycles and was then maintained at 62°C. Amplification products were analyzed by electrophoresis in a 1.5% or 2.5% agarose gel.

Results

Phenotypic characterization of resistance to RYMV in a collection of African rice

Resistance to RYMV was surveyed in a total of 337 accessions representative of the diversity of the African rice species *O. glaberrima*. In a first step, 255 accessions were evaluated with the B27 isolate at the WARDA research station in screenhouse conditions. A continuous distribution of AUSPC values was observed from high resistance to high susceptibility (Fig. 2). Ninety-one accessions presented clearly visible mottle symptoms associated with virus multiplication, as confirmed by ELISA. The most susceptible accessions developed the first symptoms at 7 dpi, in a similar manner to the susceptible control IR64, and high virus content was detected, accompanied by plant growth arrest and in some cases by

Table 1 Characteristics of primers designed to amplify *RYMV1* allele-specific markers

Name	Sequence (5′ → 3′)	Position (bp)	Allelic specificity			
			Rymv1-1-Og *Rymv1-1-Osi*	*rymv1-3*	*rymv1-4*	*rymv1-5*
F1	CACGTCGGCGGCGCATCCAAG	130 > 150	+	+	+	+
F2	GAGCCCACCTTCTGTCCGATG	3185 > 3205	+	+	+	+
F3	CCTTGGTCAGCTAGAAGAGGCA	2779 > 2800	+	+	+	+
F4	TGGCCCTGACCAAGAGATGA[a]	3364 > 3383	−	−	+	−
F5	CCCTGACCAAGAGATGGAGAAAG	3367 > 3390	−	+	−	−
R1	CGAACACGCTCGCGCACCTCA	862 > 842	+	+	+	+
R2	CAGGGCCAGTCAATTTTGCTATTTC	3371 > 3345	+	+	+	−
R3	CTCTTCACGTCGAGGCACCCA	3820 > 3800	+	+	+	+
R4	CCTCGGTACAACCAAGAGAC	3915 > 3896	+	+	+	+
R5	CAATCCTTTCTTTGTCCCTTCTCTC	3407 > 3383	+	−	−	+
R6	CCATCTCTTGGTCAGGGTTTGC	3383 > 3361	−	−	−	+

Positions refer to the distance of 5′- and 3′-primer ends from *RYMV1* ATG of the Nipponbare DNA sequence. A perfect homology between a primer and the sequence of a given allele is indicated by "+", the absence of a perfect homology is indicated by "−". Bases conferring allele-specificity are underlined on the primer sequences. F1 and R1 primers are non-specific primers used to amplify an internal control

[a] Primer with locked nucleic acid (LNA) base at the 3′ position (Braasch and Corey 2001)

plant death at 30 dpi. Twelve accessions developed the first symptoms later at 14 dpi, similar to the partially resistant control Azucena, whereas the other 42 accessions developed symptoms later again, and for these accessions, the disease had a limited impact on plant growth. The remaining 164 accessions did not show any symptoms. A significant virus titer was observed in 60 of these, while ELISA did not detect any virus in the remaining 104 accessions, which were classified as highly resistant (R+) under these conditions (Fig. 2).

In a second step, accessions identified as R+ at the WARDA station and 82 additional accessions from IRRI collection were evaluated together with the BF1 reference isolate using a simplified susceptible/resistant score in the IRD laboratory, under glasshouse conditions. Among the 104 accessions from WARDA collection, only 17 were confirmed as R+ with the BF1 isolate. Among the additional accessions from IRRI collection, 12 were ranked as R+. Finally, a total of 29 R+ *O. glaberrima* accessions were identified in this study and summarized in Table 2. Therefore, the observed frequency of R+ accessions in *O. glaberrima* was about 8% and contrasted greatly with just two R+ accessions identified to date in *O. sativa* despite intensive screenings. However, it should be noted that, whereas most of the accessions ranked as R+ showed stable high resistance, some of them contained individual plants exhibiting symptoms at 85 dpi due to resistance-breaking events, as confirmed by back-inoculation tests.

As the evaluation of plant resistance appeared to be highly dependent on the test conditions (screenhouse conditions with B27 at WARDA and glasshouse conditions

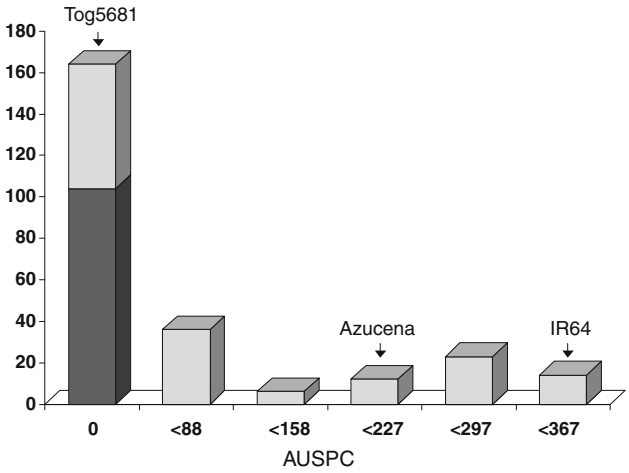

Fig. 2 Distribution of resistance, estimated by AUSPC values, in a collection of *O. glaberrima* accessions inoculated with the B27 isolate. The resistance level of control accessions is indicated. Among symptomless plants, those without any virus detected in ELISA, and considered as R+, are represented in *dark gray*

with BF1 at IRD), the pathogenicity of B27 and BF1 isolates was subsequently compared on 14 accessions of different resistance levels. Irrespective of the isolate, no virus accumulation was detected in the R+ control Tog5681. Contrary to the results obtained at WARDA, both isolates induced symptoms and were detected by ELISA in all of the other accessions (Table 3). A significant accession effect was detected ($F = 70.91$, $df = 12$, $P < 0.001$ for AUSPC) among the accessions with symptoms. Symptom severity was closely correlated with the virus content ($R^2 = 0.97$). Irrespective of the isolate and the character,

Table 2 R+ *O. glaberrima* accessions, their origin and allelic status on RYMV resistance genes

Accessions	IRRI accessions number	Origin	Allelic status on *RYMV1*	Allelic status on *RYMV2*	Resistance-breaking rate
Tog6698	–	Liberia	*Rymv1-1-Og*	–	9/10
Tog5747	112565	Liberia	*Rymv1-1-Og*	–	0/12
Tog12160	–	Guinea	*Rymv1-1-Og*	–	0/13
Tog13943	–	Nigeria	*Rymv1-1-Og*	–	8/10
Tog10434	–	Côte d-Ivoire	*Rymv1-1-Og*	–	12/13
Tog12086	–	Nigeria	*Rymv1-1-Og*	–	0/10
Tog13709	–	Guinea	*Rymv1-1-Og*	–	10/12
Tog14367	–	Guinea	*Rymv1-1-Og*	–	0/12
Tog5882	96809	Liberia	*Rymv1-1-Og*	–	0/12
Tog6356	115598	Liberia	*Rymv1-1-Og*	–	0/10
Tog5307	96726	Nigeria	*Rymv1-1-Og*	–	0/24
Tog6220	112577	Burkina Faso	*Rymv1-1-Og*	–	5/24
Tog7235	103549	Mali	*Rymv1-1-Og*	–	7/24
Tog7291	104589	Burkina Faso	*Rymv1-1-Og*	R allele	0/24
MG12	103544	Mali	*Rymv1-1-Og*	–	0/12
Tog5681	96793	Nigeria	*rymv1-3*	S allele	0/24
Tog5321	–	Nigeria	*rymv1-3*	–	0/10
Tog5418	–	Nigeria	*rymv1-3*	–	0/11
Tog5486	86752	Nigeria	*rymv1-3*	–	0/13
Tog5556	–	Nigeria	*rymv1-3*	–	0/10
Tog8049	–	Nigeria	*rymv1-3*	–	0/10
Tog12249	–	Nigeria	*rymv1-3*	–	0/14
Tog12386	–	Tanzania	*rymv1-3*	–	0/10
Tog12386	–	Tanzania	*rymv1-3*	–	0/11
Tog5672	–	Nigeria	*rymv1-4*	R allele	0/24
Tog5438	–	Nigeria	*rymv1-4*	–	0/11
Tog12188	–	Nigeria	*rymv1-4*	S allele	10/11
Tog5463	–	Nigeria	*rymv1-4*	S allele	8/10
Tog5674	96790	Nigeria	*rymv1-5*	–	0/24

The allelic status on *RYMV1* is based on the complete or partial sequencing of the coding sequence (see Table 4). The allelic status on *RYMV2* (R for resistance allele, *S* for susceptibility allele) is deduced from allelism tests (see Table 5) for five accessions and unknown (–) for the others. The resistance-breaking rate is estimated by the ratio between the number of plants infected 85 dpi on the total number of plants observed at that date; infection was confirmed both by symptoms and ELISA, while symptomless plants were all ELISA negative

the CG14 accession was not significantly different from Azucena, used as a partial resistance control, suggesting that partial resistance is also present in African rice (Table 3). The other *O. glaberrima* accessions showed few symptoms and reduced virus accumulation with B27 but were significantly more susceptible than Azucena and CG14 with BF1 isolate. An isolate effect ($F = 339.5$, $df = 1$; $P < 0.001$) was also detected: higher AUSPC indices were observed with the BF1 isolate which was consistently more severe than B27. The isolate-cultivar interaction ($F = 11.6$, $df = 12$; $P < 0.001$ for AUSPC) was also significant. A main isolate effect and a secondary isolate/cultivar interaction thus explained a major part of the differences. In addition, differences between WARDA and IRD screenings could also be explained by the earlier inoculation date at IRD, as the disease is known to be more severe if inoculation occurs earlier. Finally, the use of a highly aggressive isolate and an early inoculation date proved to be efficient for differentiating susceptible, partially resistant, and R+ *O. glaberrima* accessions. This screening procedure permitted a reduction of the size of the sample selected for fine evaluation of resistance and DNA sequencing efforts.

Allelic diversity at the *RYMV1* gene

Coding sequence variability in the *RYMV1* gene was assessed in the 29 *O. glaberrima* accessions R+ to RYMV.

Table 3 Comparison of resistance levels of 14 rice accessions inoculated with the B27 isolate in screenhouse conditions and with both B27 and BF1 isolates in glasshouse conditions

Rice accessions	AUSPC[a,b]			ELISA (Glasshouse conditions)[a,c]	
	Screenhouse conditions B27	Glasshouse conditions B27	Glasshouse conditions BF1	B27	BF1
Tog5681	0	0	0	0.05	0.09
Azucena	170	63 ab	69 abc	0.85 a	0.94 a
CG14	170	69 abc	82 c	0.87 a	0.95 a
Tog8537	0	71 abc	109 d	0.88 a	1.10 b
Tog6276	0	62 ab	114 d	0.84 a	1.14 bc
Tog12173	0	58 a	112 d	0.83 a	1.15 bc
Tog12407	87	87 c	116 d	0.96 a	1.15 bc
Tog12430	87	84 c	123 d	0.95 a	1.18 bc
Tog12381	87	84 c	114 d	0.94 a	1.16 bc
Tog7132	18	78 bc	114 d	0.90 a	1.17 bc
Tog6615	154	73 ab	126 d	0.89 a	1.15 bc
Tog5591	154	76 abc	112 d	0.90 a	1.16 bc
Tog5620	337	151 e	151 e	1.23 c	1.26 c
IR64	334	151 e	151 e	1.22 bc	1.24 c

Resistance was estimated by symptom observation (AUSPC) and virus content (ELISA)

[a] Means followed by the same letter do not differ significantly at $P \leq 0.01$. Statistics apply within and across columns for a same trait. Analysis did not include the R+ control, Tog5681

[b] AUSPC are based on symptom observation until heading date for screenhouse conditions and 30 dpi for glasshouse conditions

[c] ELISAs were performed on systemically infected leaves 30 dpi in glasshouse conditions

We first focused on a 227-nucleotide sequence from the MIF4G conserved domain which contains the mutations previously described as being involved in resistance (Albar et al. 2006). Limited intra- and interspecific polymorphism was observed in this conserved domain. Based on the comparison with several *O. sativa* sequences (Albar et al. 2006), a single amino acid substitution at position 303 (Ala303Asp) differentiated *O. sativa* and *O. glaberrima* species, irrespective of resistance level. This substitution differentiated two sequence variants of the dominant susceptibility allele *Rymv1-1*, named afterwards *Rymv1-1-Og* and *Rymv1-1-Osi* and found in *O. glaberrima* Tog5673 and *O. sativa indica* IR64, respectively. Compared with the *Rymv1-1-Og* variant,, the sequence of nine R+ *O. glaberrima* accessions revealed a deletion of nine nucleotides corresponding to three amino acids (Arg Arg Asp 322–324) which characterizes the *rymv1-3* resistance allele previously identified in Tog5681 (Table 4). Four accessions showed a point mutation (A/G) leading to an amino acid substitution (Glu321Lys) which characterizes the *rymv1-4* resistance allele previously identified in Tog5672. Finally, a new sequence variant was observed in the Tog5674 accession. This variant was characterized by an amino acid substitution (Lys312Asn) and a three-amino acid deletion (Leu Thr Gly 313–315). All mutations characteristic of the resistant accessions occurred in a very small interval of 45 nucleotides. The remaining 15 R+ accessions did not show any difference with the *Rymv1-1-Og* variant in the 227-nucleotide domain. The cDNA corresponding to the open reading frame of *RYMV1* was sequenced for six of these accessions (Tog7291, Tog7235, Tog5307, Tog5438, Tog6220, and Tog5674). The *RYMV1* coding sequence of the six accessions was identical to that of the susceptible *O. glaberrima* accessions Tog5673 (Albar et al. 2006), which suggested that genetic control of their high resistance is independent of *RYMV1*.

Identification of a new resistance gene

Genetic complementation analyses were performed to validate the allelic relationships at the *RYMV1* locus and specify the genetic origin of resistance associated with the susceptibility allele at *RYMV1* locus. One of the 15 *Rymv1-1-Og* resistant accessions, namely Tog7291, was selected to develop a 4 × 4 table of resistant × resistant crosses using Tog5681, Tog5672, and Tog5674 representing the *rymv1-3* and *rymv1-4* resistance alleles, and the newly identified variant, respectively (Table 5). The resistance level of F1 hybrids and F2 progenies was based on symptom observation and ELISA tests assessed after BF1 mechanical inoculation. F1 hybrids and F2 populations derived from (Tog5681 × Tog5672), (Tog5681 × Tog5674), and

Table 4 Alignment of the 299-334 region of the *RYMV1* product (part of the conserved MIF4G domain) from susceptible and resistant *O. sativa* and *O. glaberrima* accessions

Allele	Phenotype[a]	Species	Amino acid sequence[b]
Rymv1-1-Osi	S	*O. sativa*	AFEGAESLRAEIAKLTGPDQEMERRDKERIVKLRTLGNIRL
rymv1-2	R	*O. sativa*	----------K----------
Rymv1-1-Og	R/S	*O. glaberrima*	----D----------
rymv1-3	R	*O. glaberrima*	----D----------***----------
rymv1-4	R	*O. glaberrima*	----D----------K----------
rymv1-5	R	*O. glaberrima*	----D--------N***----------

[a] S and R refer to susceptible and resistant phenotypes, respectively; R/S indicates the presence of the variant in both susceptible and resistant accessions

[b] Only amino acids that differentiate a given sequence from the first one are reported. Deletions are represented by "*"

Table 5 Segregation of resistance in F2 populations derived from crosses between the four resistant accessions Tog5681, Tog5672, Tog5674, and Tog7291

Crosses	*RYMV1* genotype	F1 phenotype	F2 segregation[a] R/S/χ^2
(Tog5681 × Tog5672)	*rymv1-3* × *rymv1-4*	Resistant	54/0/–
(Tog5681 × Tog5674)	*rymv1-3* × *rymv1-5*	Resistant	58/0/–
(Tog5681 × Tog7291)	*rymv1-3* × *Rymv1-1-Og*	Susceptible	75/81/1.18 (ns)
(Tog5672 × Tog5674)	*rymv1-4* × *rymv1-5*	Resistant	50/0/–
(Tog5674 × Tog7291)	*rymv1-5* × *Rymv1-1-Og*	Susceptible	75/100/0.06 (ns)
(Tog5672 × Tog7291)	*rymv1-4* × *Rymv1-1-Og*	Resistant	88/12/79.57**

[a] R/S/χ2 refer to the number of resistant plants (*R*), the number of susceptible plants (*S*) and the χ^2 test statistics calculated for the segregation of two recessive resistance genes (7R:9S). Significativity of level of χ^2 is indicated by "*ns*" (non-significant) or "**" (Significant $P \leq 0.05$)

(Tog5672 × Tog5674) crosses were resistant, confirming the allelic relationships at the *RYMV1* locus to explain the resistance. The variant observed in Tog5674 was thus confirmed as a new resistance allele, and named *rymv1-5*. F2 populations (Tog5681 × Tog7291 and (Tog5674 × Tog7291) showed clear 7R:9S segregation, in agreement with the segregation of two recessive resistance genes and suggesting that an additional gene was controlling the resistance in Tog7291. This gene was named *RESISTANCE TO YELLOW MOTTLE VIRUS 2* (*RYMV2*), as recommended by McCouch and CGSNL (2008). Interestingly, segregation in F2 (Tog5672 × Tog7291) was not in accordance with the expected 7R:9S segregation, and there was only a very small proportion of susceptible plants. Back-inoculation on Tog5672 and Tog7291 parental lines using susceptible F2 plants as inoculum source revealed that those susceptible plants corresponded to resistance-breaking events. Therefore, Tog5672, known to possess the *rymv1-4* resistance allele on the first gene, was interpreted as possessing a resistance allele on the second locus *RYMV2*.

In conclusion, at least two resistance genes against RYMV were found to be present in *O. glaberrima*. Three different recessive resistance alleles of *RYMV1* were confirmed by allelism tests. All the corresponding accessions were from Nigeria, except for two accessions reported to be from Tanzania, whose origin was considered suspicious since *O. glaberrima* originated from West Africa. No resistance breaking of *rymv1-3* and *rymv1-5* was observed with the BF1 isolate (Table 2). Conversely, the second high-resistance gene, *RYMV2*, seems to be more widespread in West African countries and can

occasionally give rise to some resistance-breaking events by BF1 (Table 2).

RYMV1 allele-specific markers

Primers targeting mutations/deletions characterizing the different alleles of *RYMV1* were designed in order to improve marker-assisted selection for the introduction of resistance alleles in *O. sativa*- or *O. glaberrima*-susceptible accessions (Table 1). For practical purposes, primers were designed to visualize the markers directly on agarose gel, and an internal amplification control was included. The markers are dominant and two PCR reactions using additional complementary markers are thus necessary to determine heterozygous genotypes in segregating progenies. For example, F5/R4 primer pairs amplified a 540-bp band on the *rymv1-3* allele, but not on *Rymv1-1-Og* or *Rymv1-1-Osi* (Fig. 3b), and conversely, F2/R5 primer pairs amplified a 223-bp band on *Rymv1-1-Og or Rymv1-1-Osi,* but not on *rymv1-3*. Similar markers have been developed to differentiate *rymv1-4* and *rymv1-5* from *Rymv1-1-Og* and *Rymv1-1-Osi* (Fig. 3c, d).

Discussion

Here, we report the results of a comprehensive screening of a large representative collection of African rice (*O. glaberrima*) for resistance to RYMV. We identified 29 resistant accessions which likely correspond to different cultivars and we excluded duplication of samples based on a description of the plant type, growth duration together with panicle, and grain characteristics. Therefore, high resistance to RYMV may occur more frequently in *O. glaberrima* than in *O. sativa*, where only two resistant cultivars, sharing the same *rymv1-2* resistance allele, have been detected to date, despite intensive RYMV resistance screening by national and international institutions (WARDA, IITA). Different alleles were observed at the *RYMV1* locus, but high resistance was conditioned by a new recessive resistance identified in this study. A single resistant accession possessing the susceptibility allele at the *RYMV1* locus, Tog7291, was used in allelism tests. The results revealed other resistant *O. glaberrima* accessions exhibiting the susceptibility allele at *RYMV1* locus. These accessions may harbor resistance alleles in *RYMV2* or additional resistance genes.

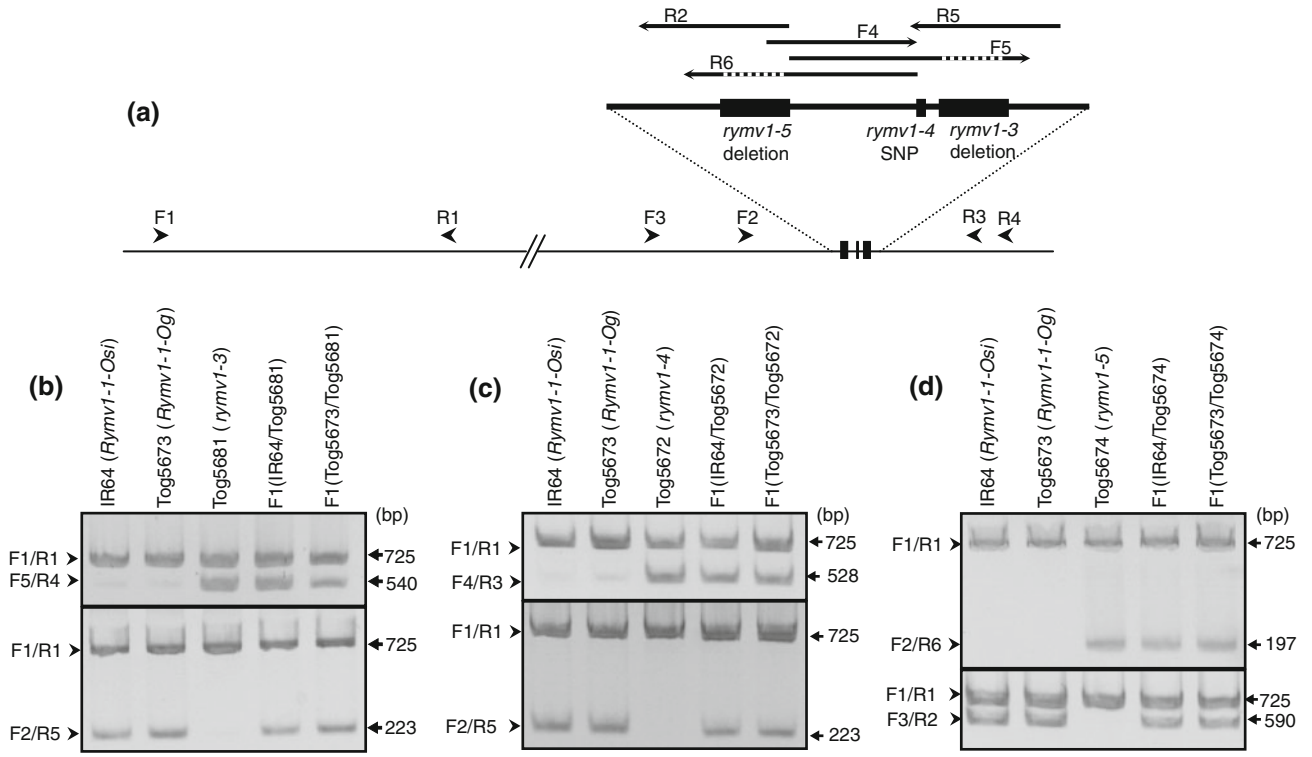

Fig. 3 *RYMV1* allele-specific markers and amplification profiles. **a** Relative positions of primers. **b** Differentiation of *Rymv1-1* (*-Og and –Os variants*) and *rymv1-3* using F2/R5 and F5/R4 pair of primers. **c** Differentiation of *Rymv1-1* and *rymv1-4* using F2/R5 and F4/R3 pair of primers. **d** Differentiation of *Rymv1-1* and *rymv1-5* using F3/R2 and F2/R6 pair of primers IR64 and Tog5673 represent *Rymv1-1-Osi and Rymv1-1-Og* variants of the susceptibility allele. Tog5681, Tog5672 and Tog5674 *O. glaberrima* accessions represent *rymv1-3*, *rymv1-4* and *rymv1-5* resistance alleles, respectively. F1 hybrids between X and Y genotypes were indicated as $F1_{x/y}$. Marker F1/R1 was used as an amplification control

CG14 accession was characterized by a significant but reduced virus accumulation, delayed symptom onset, as well as lower symptom intensity, which is very similar to the pattern observed in the partially resistant Azucena cultivar. Nevertheless, it has been proposed that a component of the partial RYMV resistance in Azucena is a consequence of the slower plant development and morpho-physiological characteristics of upland rice varieties (Albar et al. 1998). The evidence of partial RYMV resistance in some *O. glaberrima* accessions with early growing habit, such as CG14, is of great interest and suggests that genes and interaction mechanisms differing from those in *O. sativa* are involved. Some NERICA rice varieties, which have been developed by WARDA from interspecific crosses between *O. sativa* and *O. glaberrima*, have CG14 in their parentage and show partial resistance to the virus.

The two cultivated rice species exhibit a different pattern of resistance to RYMV. While *O. sativa* shows frequent partial resistance associated with upland *japonica* varieties and very rare high resistance, *O. glaberrima* harbors more frequent and diverse high resistance and also partial resistance, independent of genetic differentiation. The origin of RYMV resistance in *O. glaberrima* may be inferred since it is expected that a majority of resistance genes in *O. glaberrima* originate in its wild progenitor *O. barthii*. Preliminary assessment of a sample of *O. barthii* accessions identified some R + plants, and sequencing of the MIF4G domain in one of these accessions did not reveal any differences with the *Rymv1-1-Og* variant of the susceptibility allele, supporting the hypothesis that the resistance of Tog7291 was derived from its wild progenitor (data not shown). However, evidence now suggests that some *O. glaberrima* accessions are derived from introgressions with *O. sativa* (Semon et al. 2005), and that the resistance diversity pattern of *O. glaberrima* needs to be reconsidered from this perspective. Some, but not all, of these admixtures can show attributes of the weedy form of *O. barthii* with high photoperiod susceptibility, coarse and awny spikelets and high shedding ability. Some of our R+ *O. glaberrima* accessions possessing a susceptibility allele on *RYMV1* showed this plant type (Tog5672, Tog7235). In addition, the R+ accession Tog5307 was confirmed to have originated by a natural interspecific hybridization as evidenced by a genome-wide analysis of diversity (data not shown). Therefore, the unexpected diversity of resistance genes and phenotypes found in *O. glaberrima* could be the result of (1) the *O. barthii* gene pool (*RYMV1* and *RYMV2* genes), and (2) introgressions with *O. sativa* leading to different gene or allele interactions, each contributing to a greater range of resistance phenotypes.

Each of the four resistance alleles identified to date in *RYMV1* has a specific signature characterized by a deletion or a substitution in the same conserved domain. Breaking down of *rymv1-2* resistance has been well characterized and involves mutations observed mainly at codon 48 of the viral genome-linked protein (VPg) (Hébrard et al. 2006). The 3D topology and biochemical properties of virulence mutations suggest site-to-site binding between position 48 of VPg and position 309 of eIF(iso)4G, in a compatible interaction, that would be disrupted in an incompatible interaction due to the *rymv1-2* resistance allele (Hébrard et al. 2008). Resistance breaking is interpreted as involving restoration of this interaction by appropriate mutations at codon 48 of VPg. Resistance breaking of the *O. glaberrima rymv1-3* allele has also been reported (Traoré et al. 2006). The ability to overcome the *rymv1-2* or *rymv1-3* alleles appeared to be associated with polymorphism in the VPg sequence at position 49, a site that is under very strong positive selection (Pinel-Galzi et al. 2007; Traoré and Pinel-Galzi, unpublished results). Here, a threonine residue confers a strong ability to break *rymv1-3* resistance, whereas strains possessing glutamic acid at this position are more adapted to *rymv1-2* resistance breaking. The BF1 isolate did not show any ability to break *rymv1-3* or *rymv1-5* resistance, despite the presence of T at position 49. We found evidence for resistance-breaking of the *rymv1-4* allele in some accessions presumed to possess only the *RYMV1* resistance gene. Furthermore, *RYMV1* and *RYMV2* resistance breaking appeared to be independent, since preliminary sequencing data revealed that BF1 variants resulting from *RYMV2* resistance breaking are not mutated in the VPg sequence, suggesting a distinct mechanism of interaction.

The greater diversity found in the MIF4G domain of eIF(iso)4G in *O glaberrima* despite the lower general genetic diversity attributed to the cultivated African rice (Second 1982) may have an evolutionary significance in terms of host-pathogen relationships. The *eIF4E* genes of *Capsicum annuum* are involved in various recessive resistances against potyviruses, and amino-acid variation conferring resistance has shown significant positive selection (Cavatorta et al. 2008; Charron et al. 2008). In rice/RYMV interactions, competition experiments between T49 and E49 S1 strains of the virus suggest that T49 strains are more adapted to *O. glaberrima* (Poulicard, unpublished results). Nevertheless, both the life history traits of the virus together with domestication and extension of rice cultivation in Africa are not in favor of a long direct co-evolution between *O. glaberrima* and RYMV leading to the diversification of initiation factors related to RYMV virus resistance. East Africa is considered as the center of diversification of the virus with a maximum divergence time of 200 years before the present (Fargette et al. 2008), whereas *O. glaberrima* was domesticated much earlier in West Africa (Portères 1950). Epidemics of RYMV in East and West Africa are recent (Bakker 1974; Fauquet and Thouvenel 1977) and took place at a time when the

importance of *O. glaberrima* in rice cultivation was already significantly reduced. The advent of RYMV epidemics coincided with the introduction of high-yielding *O. sativa* varieties susceptible to RYMV. Closely related viruses may rely on the same host factors to complete their cycle, for example, the *eIF4E* gene is involved in resistance against different potyviruses, i.e., *Potato virus Y* and *Tobacco etch virus* in *Capsicum* spp. and *Lycopersicon* spp., *Lettuce mosaic virus* in *Lactuca* spp., and *Pea seed-borne mosaic virus* in *Pisum sativum* (Robaglia and Caranta 2006). The greater *RYMV1* gene diversification in Nigeria may thus have involved an ancient selection pressure on the *O. barthii–O. glaberrima* populations by a RYMV-related sobemovirus, which may now be extinct or may have diverged in different hosts. The observation that the *Rottboellia yellow mottle virus* and *Panicum mosaic virus* were isolated on wild Gramineae in Nigeria (Thottappilly et al. 1992) and the *Imperata yellow mottle virus*, closely related to RYMV, was isolated from *Imperata* spp. and experimentally transmitted to maize (Sérémé et al. 2008) support this scenario.

Sequencing of the MIF4G domain of *RYMV1* and its paralogs in representative *O glaberrima* and *O. sativa* accessions is in progress to determine whether amino-acids associated with RYMV resistance are under positive selection. Mapping of *RYMV2* in a progeny derived from Tog7291 to aid its positional cloning is also under way. Defining the *O. sativa* gene content in natural admixtures with respect to RYMV resistance together with a more in-depth evaluation of resistance and genetic diversity of *O. barthii* will help elucidate the origin of RYMV resistance in African rice. Although *RYMV2*, when alone, seems to be relatively easy to overcome, the natural accession Tog5672 has resistance alleles on both *RYMV1* and *RYMV2* and is reported to be much more difficult to breakdown. This should provide new opportunities to construct genotypes with more diversified and sustainable resistance.

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