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2 **Emergence of a resistance-breaking isolate of *Rice yellow mottle virus* during serial**
3 **inoculations is due to a single substitution in the genome-linked viral protein VPg**

4
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12
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18 **SUMMARY**

19 The recessive gene *rymv-1* responsible for the high resistance of *Oryza sativa* cultivar Gigante to
20 *Rice yellow mottle virus* (*Sobemovirus*) was overcome by the variant CI4* which emerged after
21 serial inoculations of the non-resistance-breaking (nRB) isolate CI4. By comparison of the full-
22 length sequences of CI4 and CI4*, we identified a non-synonymous mutation at position 1729
23 localised in the putative VPg domain and developed an assay based on this single nucleotide
24 polymorphism. The mutation G1729T was detected as early as the first passage in resistant plants
25 and was found in all subsequent passages. Neither reversion nor additional mutation were
26 observed. The substitution G1729T introduced by mutagenesis in the VPg of an nRB infectious
27 clone is sufficient to induce symptoms in non-inoculated leaves of cultivar Gigante. This is the
28 first evidence that VPg is a virulence factor in plants with recessive resistance against viruses
29 outside the family *Potyviridae*.

30

1 *Rice yellow mottle virus* (RYMV) of the genus *Sobemovirus* (Hull & Fargette, 2005) is present in
2 all rice-growing African countries where it causes heavy yield losses (Abo et al., 1998; Sy et al.,
3 2001). Very few rice varieties are resistant to RYMV. Recently, the available types of resistance
4 were compared (Sorho et al., 2005). The highest level of resistance was provided by one cultivar
5 of *Oryza sativa indica*, i.e. Gigante, and a few cultivars of *O. glaberrima* series Tog (Ndjiondjop
6 et al., 1999; Thottappilly & Rossell, 1993). These varieties show a natural high resistance
7 characterised by a low virus titre and the absence of symptoms (Ndjiondjop et al., 1999). A single
8 recessive gene, *rymv-1*, localised on chromosome 4, is responsible for this phenotype (Albar et
9 al., 2003).

10 Examples of recessive resistance against viruses are numerous (for review (Kang et al., 2005)),
11 but only four other crop genes, all directed against viruses in the *Potyviridae*, have been
12 molecularly identified (Gao et al., 2004; Kanyuka et al., 2005; Nicaise et al., 2003; Ruffel et al.,
13 2002; Stein et al., 2005). These four genes all encode the eukaryotic initiation factor 4E (eIF4E).
14 In addition, artificial mutants in the *Arabidopsis thaliana* gene eIF(iso)4E, the isoform of eIF4E,
15 exhibit resistance to several potyviruses (Duprat et al., 2002; Lellis et al., 2002). Several
16 resistance-breaking (RB) *Potyviridae* variants have been described. Most often, they involve
17 mutations in the VPg (genome-linked viral protein) (Moury et al., 2004), although P3-6K1 and
18 HC-Pro are sometimes involved (Hjulsager et al., 2002; Redondo et al., 2001).

19 Some field-collected RB isolates of RYMV able to induce symptoms in cv. Tog 5681 and/or cv.
20 Gigante have been found {Konaté, 1997 #264; Traoré, 2006 #416}. Furthermore, RB isolates can
21 emerge experimentally after serial inoculations (Fargette et al., 2002). In this last case, the
22 progeny of the non-resistance-breaking (nRB) isolate CI4 (accession number AJ279905) induced
23 mottling symptoms 30 dpi after four passages in cv. Gigante and stunting at the sixth passage
24 (Fargette et al., 2002). The RB variant CI4* was not counter-selected in susceptible plants as it
25 multiplied to a similar level after serial passages, even when mixed with nRB isolate CI4, and it
26 retained its ability to overcome the resistance (Sorho et al., 2005). Nevertheless, the molecular
27 changes from nRB isolate CI4 that led to RB isolate CI4* were not determined. In this study, we
28 identified a candidate mutation in the VPg domain. Nucleotide-specific probes were developed to
29 investigate mutation stability, complementation, reversion and additional mutations during the
30 serial passages. Finally, the involvement of the mutation G1729T in the resistance-breaking
31 phenomenon was validated in an infectious clone.

32 The full-length sequence of RB isolate CI4* obtained after six passages in highly resistant plants
33 was determined using the method and primers previously described (Fargette et al., 2004).
34 Sequence comparison of the isolates nRB CI4 and RB CI4* showed six differences localised in
35 ORF2 (Table 1, Fig.1A). Four mutations were synonymous (S) whereas the other two each
36 produced an amino acid change (NS). The polymorphism of these positions was analysed in 16
37 full-length sequences of nRB isolates previously described (including CI4) (Fargette et al., 2004).
38 Four positions (1544, 1547, 3323 and 3395) of the six mutated sites showed the same nucleotide
39 in CI4* as in several other sequences (7/16, 4/16, 7/16 and 15/16, respectively). The two other
40 positions (1298 and 1729) showed the same nucleotides in all the isolates than in CI4 (16/16)
41 suggesting the nucleotides observed in CI4* were specific for resistance-breaking. The mutation
42 C1298T (from cytosine at position 1298 to thymine) was synonymous. The mutation G1729T
43 produced a non-synonymous change Arg374Ile (from arginine at position 374 to isoleucine) of
44 the polyprotein P2a. The latter mutation is localised close to the conserved motif W(A,G)D
45 (nucleotides 1776 to 1784) in a D- and E-rich region. This motif is present in analyzed and
46 predicted VPg proteins of sobemoviruses (Mäkinen et al., 1995b; Tamm & Truve, 2000). Hence,

1 the mutation G1729T is within the RYMV VPg. This non-synonymous mutation represented a
2 likely candidate for involvement in resistance-breaking.

3 To correlate the presence of the mutation G1729T, detected in the sixth passage, and the RB
4 phenomenon, we developed a tool using a three-primer PCR method (Moravec et al., 2003). Four
5 primers were designed to allow the detection of the RYMV polymorphism at position 1729
6 (Fig.1B). The flanking primers F1 (nt 1469 to 1483, 5'-CCCCGCTCTACCACAA-3') and R1
7 (nt 2131 to 2117, 5'-AAAGATGACACCCGA-3') give a 663 bp fragment which constitutes a
8 positive marker of PCR in infected plants. Internal primer F2 (nt 1716 to 1729, 5'-
9 TGGGTGCGTGAGAG-3') was designed to perfectly match the sequence of RYMV CI4 except
10 one mismatch (boldface) on the 3' end with the isolate CI4*. This primer, together with R1, gives
11 an additional band of 416 bp when a guanine is present at the position 1729. In contrast, the
12 internal primer R2 (nt 1741 to 1729, 5'-TGGTACCTCTCTA-3') matches the CI4* sequence,
13 giving with F1 an additional band of 273 bp when a thymine is present at the position 1729. To
14 perform the SNP detection, first strand synthesis was performed using Superscript II reverse
15 transcriptase (Gibco-BRL) with primer R1. The cDNA was used as template for PCR-
16 amplification using DyNAzyme EXT DNA polymerase (Finnzymes) with the forward primer F1,
17 the reverse primer R1 and a third primer F2 or R2. In susceptible plants inoculated with CI4, only
18 the fragment of 416 bp was detected, indicating the presence of G1729 and the absence of T1729
19 (Fig.1C, lanes 1-2). Inversely, after eight passages in resistant plants, only the fragment of 273 bp
20 was detected, indicating the absence of G1729 and the presence of T1729 (Fig.1C, lanes 7-8).
21 Interestingly, the nucleotide T1729 was detected as early as the first passage while G1729
22 remained undetected (Fig.1C, lanes 3-4). The nucleotide T1729 was retained at each subsequent
23 passage (data not shown). Therefore, only the isolates with a thymine at position 1729 were
24 detected in resistant plants. The non-mutated nucleotide G1729, whether derived from the initial
25 isolate CI4 or from a reversion, was never detected in resistant plants. After the first passage in
26 resistant plants, the isolate was back-inoculated to susceptible plants (Fig.1C, lanes 5-6). Again,
27 only the nucleotide T1729 was detected, indicating that an isolate containing the G1729T
28 mutation multiplied in susceptible plants. The same result was observed with the isolate CI4*
29 obtained after eight passages in resistant plants and back-inoculated to susceptible plants after
30 one or four serial passages (Fig.1C lanes 9-12). This latter isolate infected resistant plants and
31 presented T1729 alone (Fig.1C, lanes 13-14). Altogether, these results indicated that an isolate
32 containing the G1729T mutation was not counter-selected in susceptible plants and that this
33 isolate was still able to break the *rymv-1* resistance. Again, the non-mutated nucleotide whether
34 derived from the initial isolate CI4 or from a reversion, was not detected in these susceptible
35 plants. Artificial mixed inoculations with CI4 and CI4* performed by Sorho *et al.* (2005) were
36 also assessed. The presence of T1729 was detected both in the susceptible and resistant plants
37 whereas G1729 was not detected in resistant plants, suggesting that there was no
38 complementation of nRB CI4 by RB CI4* (data not shown).

39 The mutation G1729T was detected at the first passage in resistant plants although no symptoms
40 were apparent. To determine whether the increasing intensity of the symptoms through passages
41 was due to additional mutations around G1729T, each isolate CI4* (obtained in resistant plants)
42 was partially sequenced from 1230 to 2090 nt with primers R9 (5'-
43 ATAGGTGCTGCGGATGGTTA-3') and R10 (5'-GCTACGGGATGCGATGTCTC-3') after
44 PCR-amplification with the primers B_S and B_{AS} as described in (Fargette et al., 2004). The
45 mutations C1298T, C1547T and G1729T were observed at each passage. The mutation G1544A
46 was never observed in the other CI4* isolates, suggesting it was not involved in resistance-
47 breaking. No additional mutation was detected in the analysed fragment except G1489A at the

1 eighth passage. However, further serial passages in cv. Gigante did not reveal this mutation (data
2 not shown), indicating that this mutation was not stable. Moreover, the nucleotide G1489 is
3 conserved in the 16 isolates that have been fully sequenced. No additional mutation around
4 G1729T which could explain the appearance of symptoms was observed through serial passages.
5 However, after inoculation with leaf extracts of all previous passages and a longer symptom
6 monitoring, the appearance of mild symptoms from 45 dpi was observed (data not shown). As
7 shown by Sorho *et al.* (2005), inoculum concentration is a key factor in overcoming resistance
8 which suggests that, in the previous experiment, the concentration was below the threshold to
9 produce symptoms at the first passages.

10 The mutation G1729T was introduced in a nRB infectious cDNA clone to validate its role in
11 resistance-breaking. As the clone of CI4 was not available, the infectious cDNA clone of the nRB
12 isolate CIa was used (Brugidou *et al.*, 1995). Two PCR experiments were performed
13 independently with a flanking primer and a primer containing the mutation, FL5R5bis (nt 892 to
14 911, 5'-CCCGGGGTACGTGGTTGAC-3') and RmIIR374I (nt 1739 to 1717, 5'-
15 TACTTCGTTATCTCACGCACCC-3'), R14bis (nt 2424 to 2401, 5'-
16 ACTTCGCCGGTTTCGCAGAGGATT-3') and FmIIR374I (nt 1714 to 1738, 5'-
17 CCTGGGTGCGTGAGATAACGAAGTA-3'), respectively. The products of the two PCR
18 applications were mixed and used as template for another PCR experiment with the two flanking
19 primers. PCR fragment was cloned in the infectious cDNA clone CIa after double-digestion by
20 *Afl*II and *Avr*II (Biolabs). The codon 1728-1730 AGG was mutated to ATA as in RB CI4* to
21 produce a clone called CIa*. The mutation was confirmed by sequencing the fragment. However,
22 two other mutations A1414G and G2095A were randomly introduced during the mutagenesis
23 process. The latter is localised at the ATG of ORF2b (Fig.1A). These mutations were not
24 observed in the 16 nRB isolates that have been sequenced (Fargette *et al.*, 2004). The infectivity
25 of the mutated clone CIa* was tested in susceptible plants after *in vitro* transcription with T7
26 RNA polymerase (Promega) performed as described in (Brugidou *et al.*, 1995). Despite the three
27 non-synonymous mutations, *in vitro* transcripts from the clone CIa* were infectious. This result
28 demonstrated that, as predicted by homology with *Cocksfoot mottle virus* (Mäkinen *et al.*, 1995a),
29 the ATG codon of ORF2b was not a start codon, and that the ORF2b was translated in fusion
30 with ORF2a from the frameshifting signal at 1979. Moreover, yellow mottling symptoms
31 appeared on inoculated and non-inoculated leaves in susceptible plants. High virus content was
32 detected at 21 dpi by DAS-ELISA performed as described in (N'Guessan *et al.*, 2000). No
33 significant difference in virus content was observed in susceptible plants infected with the
34 progeny of the isolates CIa*, CIa, CI4 and CI4*. Moreover, the isolate CIa* overcame the high
35 resistance of cv. Gigante. Symptom intensity and virus content at 45 dpi in non-inoculated leaves
36 were similar to those of CI4*. This experiment was repeated twice on 20 plants each with
37 different virus preparations. Hence, the mutation G1729T is sufficient to induce symptoms of
38 resistance-breaking. After multiplication in resistant plants, isolate CIa* was again sequenced
39 full-length and the presence of the mutations introduced by mutagenesis was confirmed. Another
40 mutation C3541T, which was absent in the original mutated clone CIa* but present in the 16 full-
41 length sequences, was detected.

42 It is notable that isolates CI4* and CIa* belong to two different RYMV strains (S1 and S3,
43 respectively) (Pinel *et al.*, 2000) which are geographically, ecologically and genetically distant
44 (Traoré *et al.*, 2005). Moreover, the surrounding context of the mutation G1729T (*i.e.* Arg374Ile)
45 is different. In particular the two downstream codons encoded for amino acids which possess
46 different physico-chemical properties in isolates CI4* and CIa* (*i.e.* Glu375Thr and Arg376Lys).

1 We conclude that the mutation G1729T is sufficient to induce resistance-breaking in non-
2 inoculated leaves in different strains and genetic contexts.

3 Taken together, the data presented here provide the first evidence for a virus outside the family
4 *Potyviridae* that VPg is the virulence factor toward recessive resistance. The RYMV/rice
5 pathosystem presents several specificities. First, no amino acid sequence similarity has been
6 detected between the VPgs of RYMV and *Potyviridae*. Second, the genome organisation of
7 sobemoviruses differs from that of *Potyviridae* (Tamm & Truve, 2000): the 3'-end of RNA is not
8 polyadenylated and the VPg is localised downstream of the protease. Finally, the resistance gene
9 *rymv-1* appears to be different from the well-studied eIF4E. We recently identified *rymv-1* as a
10 truncated isoform of the eukaryotic translation initiation factor 4G (eIF(iso)4G) (Albar L,
11 Bangratz-Reiser M, Hébrard E, Njiondjop MN, Jones M, Ghesquière A, unpublished results).
12 The factors 4E and 4G and their isoforms provide different functions in the translation initiation
13 complex. While eIF4E binds directly to the cap of mRNA, the high molecular weight eIF4G acts
14 as a scaffold for other components of the complex such as eIF4E. Recently, eIF4G was reported
15 to be involved in recessive resistance against viruses of families *Bromoviridae* and
16 *Tombusviridae* (Yoshii et al., 2004). Whereas direct interactions between eIF4Es and VPgs of
17 *Potyviridae* were demonstrated (Leonard et al., 2000, Leonard et al., 2004, Schaad et al., 2000,
18 Wittmann et al., 1997), the interactions between eIF4G and these other viruses, which do not
19 possess a VPg, are not known. Further investigations on the molecular properties that enable
20 RYMV to overcome the resistance of *Oryza sativa* cv. Gigante would contribute to the
21 understanding of recessive resistances mediated by eIF4G genes.

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38
39

1 Table 1: Mutations of the nRB isolate CI4 after six serial inoculations on *O. sativa* cv. Gigante
 2

Nucleotide number	ORF	Nucleotide in		Mutation type†	Number of sequences‡ as		Specific mutation§
		CI4	CI4*		CI4	CI4*	
1298	2a	C	T	S	16/16	0/16	+
1544	2a	G	A	S	9/16	7/16	-
1547	2a	C	T	S	12/16	4/16	-
1729	2a	G	T	NS	16/16	0/16	+
3323	2b	T	C	S	9/16	7/16	-
3395	2b	C	T	NS	1/16	15/16	-

3
 4 † Synonymous (S) or non-synonymous (NS) mutations.
 5 ‡ Sixteen full-length sequences of nRB isolates (see accession numbers in (Fargette et al., 2004))
 6 were compared at each position and the number of sequences with the same nucleotide as CI4 or
 7 CI4* were determined.
 8 § Mutations which were only observed for CI4*.
 9

1 Figure 1. (A) Top: Genomic organisation of RYMV. The dotted line at nucleotide 1979
 2 represents the frameshifting signal. The fusion point of the polyprotein P2a+b is unknown, and an
 3 AUG codon present at the beginning of the ORF2b (nucleotide 2093) is indicated by the vertical
 4 line. Bottom: Mutation localisation of the RB isolate CI4*. The mutation positions are indicated
 5 in bold. Circles and stars represent synonymous and non-synonymous mutations, respectively.
 6 Solid black circles and stars represent mutations specific to resistance-breaking. (B) DNA
 7 fragments PCR-amplified to detect SNP G1729T. Fragments are represented by horizontal lines.
 8 The name of each pair of primers and the size of each PCR-amplified fragments are indicated on
 9 the right. F1 and R1 are external primers. F2 and R2 are specific for the presence of the wild-type
 10 nucleotide G1729 and the mutated nucleotide T1729, respectively. (C) Detection of the mutation
 11 G1729T on serially inoculated plants. RT-PCRs were performed with primers F1/R1/F2 (lanes 1,
 12 3, 5, 7, 9, 11, 13) or F1/R1/R2 (lanes 2, 4, 6, 8, 10, 12, 14). RNAs were extracted from infected
 13 susceptible plants cv. IR64 (lanes 1, 2, 5, 6, 9, 10, 11, 12) or from resistant plants cv. Gigante
 14 (lanes 3, 4, 7, 8, 13, 14). The isolate nRB CI4 was inoculated to cv. IR64 (lanes 1-2), then to cv.
 15 Gigante (lanes 3-4), then to cv. IR64 (lanes 5-6). After eight passages in cv. Gigante (lanes 7-8),
 16 the isolate was inoculated one (lanes 9-10) or four times to cv. IR64 (lanes 11-12), then to cv.
 17 Gigante (lanes 13-14). The name of each pair of primers is indicated on the right. The presence of
 18 a band at 416 bp (F2-R1) or 273 bp (F1-R2) is specific for G1729 or T1729, respectively. M,
 19 molecular weight marker (1kb Invitrogen).

