## Short Communication

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

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

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

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

Some field-collected RB isolates of RYMV able to induce symptoms in 'Tog 5681' and/or 'Gigante' have been found (Konaté *et al.*, 1997; Traoré *et al.*, 2006). Furthermore, RB isolates can emerge experimentally after serial inoculations (Fargette *et al.*, 2002). In this last case, the progeny of the non-resistance-breaking (nRB) isolate CI4 (GenBank accession no. AJ279905) induced mottling symptoms 30 days post-infection (p.i.) after four passages in 'Gigante' and stunting at the sixth passage (Fargette *et al.*, 2002). The RB variant CI4* was not counterselected in susceptible plants, as it multiplied to a similar level after serial passages, even when mixed with nRB isolate CI4, and it retained its ability to overcome the resistance (Sorho *et al.*, 2005). Nevertheless, the molecular changes from nRB isolate CI4 that led to RB isolate CI4* were not determined. In this study, we identified a candidate mutation in the VPg domain. Nucleotide-specific probes were developed to investigate mutation stability, complementation, reversion and additional mutations during the serial passages. Finally, the involvement of the mutation G1729T in the resistance-breaking phenomenon was validated in an infectious clone.

The full-length sequence of RB isolate CI4*, obtained after six passages in highly resistant plants, was determined by using a method and primers described previously (Fargette *et al.*, 2004). Sequence comparison of the isolates nRB CI4 and RB CI4* showed six differences localized in ORF2 (Table 1; Fig. 1a). Four mutations were synonymous, whilst

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

| Nucleotide no. | ORF | Nucleotide present in: | | Mutation type† | No. sequences‡ the same 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 | − |

†Synonymous (S) or non-synonymous (NS) mutations.

‡Sixteen full-length sequences of nRB isolates [for GenBank accession numbers, see Fargette *et al.* (2004)] were compared at each position and the number of sequences with the same nucleotide as CI4 or CI4* was determined.

§Mutations that were observed only for CI4*.

the other two were non-synonymous, each producing an amino acid change. The polymorphism of these positions was analysed in 16 full-length sequences of nRB isolates described previously, including CI4 (Fargette *et al.*, 2004). Four positions (nt 1544, 1547, 3323 and 3395) of the six mutated sites showed the same nucleotide in CI4* as in several other sequences (7/16, 4/16, 7/16 and 15/16, respectively). The two other positions (nt 1298 and 1729) showed the same nucleotides in all of the isolates as in CI4 (16/16), suggesting that the nucleotide changes observed in CI4* were specific for resistance breaking. The mutation C1298T was synonymous. The mutation G1729T produced a non-synonymous change of Arg374Ile in the polyprotein P2a. The latter mutation was localized close to the conserved motif W(A/G)D (nt 1776–1784) in a D- and E-rich region. This motif is present in analysed and predicted VPg proteins of sobemoviruses (Mäkinen *et al.*, 1995b; Tamm & Truve, 2000). Hence, the mutation G1729T is within the RYMV VPg. This non-synonymous mutation represented a likely candidate for involvement in resistance breaking.

To correlate the presence of the mutation G1729T, detected in the sixth passage, with the RB phenomenon, we developed a three-primer PCR method (Moravec *et al.*, 2003). Four primers were designed to allow the detection of the RYMV polymorphism at position 1729 (Fig. 1b). The flanking primers F1 (nt 1469–1483, 5′-CCCGCTCTACCACAA-3′) and R1 (nt 2131–2117, 5′-AAAGATGACACCCGA-3′) gave a 663 bp fragment, which was used as positive control for PCR in infected plants. Internal primer F2 (nt 1716–1729, 5′-TGGGTGCGTGAGA**G**-3′) was designed to match the sequence of RYMV CI4 to that of isolate CI4*, except for one mismatch (shown in bold) on the 3′ end. This primer, together with R1, gave an additional band of 416 bp when a guanine was present at nt 1729. In contrast, the internal primer R2 (nt 1741–1729, 5′-TGGTACCTCTCT**A**-3′) matched the CI4* sequence, giving an additional band of 273 bp when a thymine was present at nt 1729 when used with primer F1. To perform the single-nucleotide polymorphism detection, first-strand synthesis was performed by using
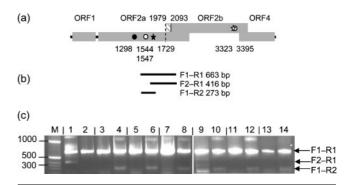


**Fig. 1.** (a) Genomic organization of RYMV. The dotted line at nt 1979 represents the frameshift signal. The fusion point of the polyprotein P2a+b is unknown and an AUG codon present at the beginning of the ORF2b (nt 2093) is indicated by a vertical line. The location of mutations of the RB isolate CI4* is indicated below. Circles and stars represent synonymous and non-synonymous mutations, respectively. Filled circles and stars represent mutations specific to resistance breaking. (b) DNA fragments PCR-amplified to detect the single-nucleotide polymorphism G1729T. Fragments are represented by horizontal lines. The name of each pair of primers and the size of each PCR-amplified fragment are indicated on the right. F1 and R1 are external primers. F2 and R2 are specific for the presence of the wild-type nt G1729 and the mutated nt T1729, respectively. (c) Detection of mutation G1729T on serially inoculated plants. RT-PCRs were performed with primers F1/R1/F2 (lanes 1, 3, 5, 7, 9, 11 and 13) or F1/R1/R2 (lanes 2, 4, 6, 8, 10, 12 and 14). RNAs were extracted from infected, susceptible plants 'IR64' (lanes 1, 2, 5, 6, 9, 10, 11 and 12) or from resistant plants 'Gigante' (lanes 3, 4, 7, 8, 13 and 14). The isolate nRB CI4 was inoculated onto 'IR64' (lanes 1 and 2), then onto 'Gigante' (lanes 3 and 4) and lastly onto 'IR64' (lanes 5 and 6). After eight passages in 'Gigante' (lanes 7 and 8), the isolate was inoculated once (lanes 9 and 10) or four times (lanes 11 and 12) onto 'IR64' and then onto 'Gigante' (lanes 13 and 14). The name of each pair of primers is indicated on the right. The presence of a band of 416 bp (F2/R1) or 273 bp (F1/R2) was specific for G1729 or T1729, respectively. M, 1 kb molecular mass marker (Invitrogen).

Superscript II reverse transcriptase (Gibco-BRL) with primer R1. The cDNA was used as template for PCR amplification using DyNAzyme EXT DNA polymerase (Finnzymes) with the forward primer F1, the reverse primer R1 and a third primer, either F2 or R2. In susceptible plants inoculated with CI4, only the 416 bp fragment was detected, indicating the presence of G1729 and the absence of T1729 (Fig. 1c, lanes 1 and 2). In contrast, after eight passages in resistant plants, only the 273 bp fragment was detected, indicating the absence of G1729 and the presence of T1729 (Fig. 1c, lanes 7 and 8). Interestingly, T1729 was detected as early as the first passage, whilst G1729 remained undetected (Fig. 1c, lanes 3 and 4). T1729 was retained at each subsequent passage (data not shown). Therefore, only the isolates with a thymine at nt 1729 were detected in resistant plants. The non-mutated nucleotide G1729, whether derived from the initial isolate CI4 or from a reversion, was never detected in resistant plants. After the first passage in resistant plants, the isolate was inoculated back into susceptible plants (Fig. 1c, lanes 5 and 6). Again, only T1729 was detected, indicating that an isolate containing the G1729T mutation multiplied in susceptible plants. The same result was observed with the isolate CI4*, obtained after eight passages in resistant plants and inoculated back into susceptible plants after one or four serial passages (Fig. 1c, lanes 9–12). This latter isolate infected resistant plants and presented T1729 alone (Fig. 1c, lanes 13 and 14). Altogether, these results indicated that an isolate containing the G1729T mutation was not counterselected in susceptible plants and that this isolate was still able to break *rymv-1* resistance. Again, the non-mutated nucleotide, whether derived from the initial isolate CI4 or from a reversion, was not detected in these susceptible plants. Artificial mixed inoculations with CI4 and CI4* performed by Sorho *et al.* (2005) were also assessed. The presence of T1729 was detected in both the susceptible and the resistant plants, whilst G1729 was not detected in resistant plants, suggesting that there was no complementation of nRB CI4 by RB CI4* (data not shown).

The mutation G1729T was detected in the first passage in resistant plants, although no symptoms were apparent. To determine whether the increasing intensity of symptoms through subsequent passages was due to additional mutations around G1729T, each CI4* isolate (obtained in resistant plants) was partially sequenced from nt 1230 to 2090 with primers R9 (nt 1506–1525, 5′-ATAGGTGCTGCGGA-TGGTTA-3′) and R10 (nt 1840–1821, 5′-GCTACGGGATG-CGATGTCTC-3′) after PCR amplification with the primers $B_S$ and $B_{AS}$ as described by Fargette *et al.* (2004). The mutations C1298T, C1547T and G1729T were observed at each passage. The mutation G1544A was not observed in the other CI4* isolates, suggesting that it was not involved in resistance breaking. The only additional mutation detected in the analysed fragment was G1489A at the eighth passage. However, further serial passages in 'Gigante' did not reveal this mutation (data not shown), indicating that it was not stable. Moreover, G1489 is conserved in the 16 isolates that have been sequenced fully. No additional mutation around

G1729T that could explain the appearance of symptoms was observed through serial passages. However, after inoculation with leaf extracts of all previous passages and a longer period of monitoring symptoms, the appearance of mild symptoms from 45 days p.i. was observed (data not shown). As shown by Sorho *et al.* (2005), inoculum concentration is a key factor in overcoming resistance, which suggests that, in the previous experiment, the concentration was below the threshold required to produce symptoms during the first passage.

The mutation G1729T was introduced in an nRB infectious cDNA clone to validate its role in resistance breaking. As the clone of CI4 was not available, the infectious cDNA clone of the nRB isolate CIa was used (Brugidou *et al.*, 1995). Two PCR experiments were performed independently with a flanking primer and a primer containing the mutation, FL5R5bis (nt 892–911, 5′-CCCGGGGTTACGTGGTTGAC-3′) and RmIIR374I (nt 1738–1717, 5′-TACTTCGT**TA**-TCTCACGCACCC-3′), and R14bis (nt 2424–2401, 5′-ACTTCGCCGGTTTCGCAGAGGATT-3′) and FmIIR374I (nt 1714–1738, 5′-CCTGGGTGCGTGAGA**TA**ACGAAGTA-3′), respectively. The products of the two PCR applications were mixed and used as template for another PCR experiment with the two flanking primers. The PCR fragment was cloned in the infectious cDNA clone CIa after double digestion with *Afl*II and *Avr*II (Biolabs). The AGG codon at nt 1728–1730 was mutated to ATA, as in RB CI4*, to produce a clone called CIa*. The mutation was confirmed by sequencing the fragment. However, two other mutations, A1414G and G2095A, were introduced randomly during the mutagenesis process. The latter was at the ATG of ORF2b (Fig. 1a). These mutations were not observed in the 16 nRB isolates that have been sequenced (Fargette *et al.*, 2004). The infectivity of the mutated clone CIa* was tested in susceptible plants after *in vitro* transcription with T7 RNA polymerase (Promega) performed as described previously (Brugidou *et al.*, 1995). Despite the three non-synonymous mutations, *in vitro* transcripts from the clone CIa* were infectious. This result demonstrated that, as predicted by homology with *Cocksfoot mottle virus* (Mäkinen *et al.*, 1995a), the ATG codon of ORF2b was not a start codon and ORF2b was translated in fusion with ORF2a from the frameshift signal at nt 1979. Moreover, yellow mottling symptoms appeared on inoculated and uninoculated leaves in susceptible plants. A high content of virus was detected at 21 days p.i. by double antibody-sandwich ELISA, performed as described previously (N'Guessan *et al.*, 2000). No significant difference in the content of virus was observed in susceptible plants infected with progeny of the isolates CIa*, CIa, CI4 and CI4*. Moreover, the isolate CIa* overcame the high resistance of 'Gigante'. Symptom intensity and virus content at 45 days p.i. in uninoculated leaves were similar to those of CI4*. This experiment was repeated twice on 20 plants, each with different virus preparations. Hence, the mutation G1729T was sufficient to induce symptoms of resistance breaking. After multiplication in resistant plants, isolate CIa* was again fully sequenced and the presence of the mutations introduced by mutagenesis was confirmed.

Another mutation, C3541T, which was absent in the original mutated clone CIa*, but present in the 16 full-length sequences, was detected.

It is notable that isolates CI4* and CIa* belong to two different RYMV strains (S1 and S3, respectively) (Pinel *et al.*, 2000) that are geographically, ecologically and genetically distant (Traoré *et al.*, 2006). Moreover, the surrounding context of the mutation G1729T (i.e. Arg374Ile) is different. In particular, the two downstream codons encode amino acids that possess different physicochemical properties (Glu375 Arg376 in CI4* and Thr375 Lys376 in Cla*). We concluded that the mutation G1729T was sufficient to induce resistance breaking in uninoculated leaves in different strains and genetic contexts.

Taken together, the data presented here provide the first evidence that VPg is the virulence factor for recessive resistance in a virus outside the family *Potyviridae*. The RYMV–rice pathosystem has several specificities. First, no amino acid sequence similarity has been detected between the VPgs of RYMV and members of the family *Potyviridae*. Secondly, the genome organization of sobemoviruses differs from that of members of the family *Potyviridae* (Tamm & Truve, 2000): the 3′ end of the RNA is not polyadenylated and the VPg is localized downstream of the protease. Finally, the resistance gene *rymv-1* appears to be different from the well-studied eIF4E. Recently, we identified *rymv*-1 as a truncated isoform of the eukaryotic translation initiation factor 4G [eIF(iso)4G] (L. Albar, M. Bangratz-Reiser, E. Hébrard, M.-N. Ndjiondjop, M. Jones & A. Ghesquière, unpublished results). The factors 4E and 4G and their isoforms provide different functions in the translation-initiation complex. Whilst eIF4E binds directly to the cap of mRNA, the high-molecular-mass eIF4G acts as a scaffold for other components of the complex, such as eIF4E. Recently, eIF4G was reported to be involved in recessive resistance against viruses in the families *Bromoviridae* and *Tombusviridae* (Yoshii *et al.*, 2004). Whilst direct interactions between eIF4Es and VPgs of members of the family *Potyviridae* were demonstrated (Léonard *et al.*, 2000, 2004; Schaad *et al.*, 2000; Wittmann *et al.*, 1997), the interactions between eIF4G and these other viruses, which do not possess a VPg, are not known. Further investigations on the molecular properties that enable RYMV to overcome the resistance of *O. sativa* 'Gigante' would contribute to the understanding of recessive resistance mediated by eIF4G genes.

## Acknowledgements

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