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Mutations in the eIF(iso)4G translation initiation factor confer high resistance of rice to *Rice yellow mottle virus*

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Summary

We report here evidence of the role that the isoform of the eukaryotic translation initiation factor 4G (eIF(iso)4G) plays in naturally occurring resistance in plant/virus interactions. A genetic and physical mapping approach was developed to isolate the *Rymv*1 locus controlling the high recessive resistance to *Rice yellow mottle virus* (RYMV) in the rice (*Oryza sativa*) variety Gigante. The locus was mapped to a 160-kb interval containing a gene from the *eIF(iso)4G* family. The stable transformation of a resistant line with the cDNA of this gene, derived from a susceptible variety, resulted in the loss of resistance in transgenic plants. The allelic variability of this gene was analysed in three resistant and 17 susceptible varieties from different cultivated rice species or subspecies. Compared with susceptible varieties, resistant varieties present specific alleles, characterized by either amino acid substitutions or short amino-acid deletions in the middle domain of the protein. The structure of this domain was modelled and showed that the substitutions were clustered on a small surface patch. This suggests that this domain may be involved in an interaction with the virus.

Keywords: rice, *Rice yellow mottle virus*, translation initiation factor, eIF(iso)4G, resistance.

Introduction

Viruses rely heavily on host factors to perform their cycle because of their small genome size and the low number of genes they encode. Specific interactions between host factors and either a virus or viral proteins have been described and shown to be involved in different steps of viral infection, including replication, translation and virus trafficking in the cells (for a review see Ahlquist *et al.*, 2003; Maule *et al.*, 2002). As described by Fraser (1998), either the lack of a given host factor required for the virus cycle or a mutation that impairs the interaction can result in a resistant phenotype. Such resistance is likely to be passive, i.e. it should neither induce a hypersensitive nor a defensive response, and should act under a recessive genetic determinism. Indeed, numerous cases of recessive resistance against viruses have been described (for a review see Diaz-Pendon *et al.*, 2004), whereas they are less frequent against either bacterial or fungal pathogens.

Until recently, however, no gene involved in such recessive resistances had been cloned. The role of the eukaryotic translation initiation factor 4E (eIF4E) in virus resistance was first suggested by its interaction with the potyvirus VPg protein (Leonard *et al.*, 2000; Schaad *et al.*, 2000), and the involvement of VPg in resistance breaking (Keller *et al.*, 1998; Moury *et al.*, 2004; Nicolas *et al.*, 1997). The development of candidate gene approaches for the identification of a resistance gene against the *Potato virus Y* (PVY) in pepper and the screening of *Arabidopsis thaliana* mutants resistant to different potyviruses led, in 2002, to the evidence that either eIF4E or its isoform eIF(iso)4E are required for infection by some potyviruses (Duprat *et al.*, 2002; Lellis *et al.*, 2002; Ruffel *et al.*, 2002). Since then, the role of eIF4E in naturally occurring resistance has been demonstrated in several plant/potyviridae interactions (Gao *et al.*, 2004; Kanyuka *et al.*, 2005; Nicaise *et al.*, 2003; Ruffel *et al.*, 2005; Sato *et al.*, 2005; Stein *et al.*, 2005). In addition, a mutation in the eukaryotic initiation factor 4G (eIF4G) of *A. thaliana* resulted in the impaired multiplication of the *Cucumber mosaic virus* (CMV) and the *Turnip crinkle virus* (TCV) (Yoshii *et al.*, 2004).

Thus, both eIF4E and eIF4G appear to play a significant role in plant/virus interactions.

The *Rice yellow mottle virus* (RYMV), a sobemovirus, is a major pathogen of rice in Africa (for a review see Kouassi *et al.*, 2005). Although partial resistance is widely distributed in *O. sativa* varieties, only some rare varieties from the cultivated rice species *O. sativa* and *Oryza glaberrima* express very high resistance to RYMV (Ndjiondjop *et al.*, 1999; Thottappilly and Rossel, 1993). Highly resistant plants are characterized by the absence of symptoms and a non-significant virus content in the plant based on an ELISA. Impaired cell-to-cell movement has been suggested to be the main mechanism of resistance as virus replication occurred at the same level in protoplasts of resistant and susceptible cultivars (Ndjiondjop *et al.*, 2001). The same locus, called *Resistance to yellow mottle virus*-1 (*Rymv*1), is responsible for the recessive resistance of *O. sativa* cultivar (cv) Gigante and *O. glaberrima* cv Tog5681 (Ndjiondjop *et al.*, 1999). This locus was previously mapped on the short arm of chromosome 4 in a 3.7-cM interval (Albar *et al.*, 2003). Here we show that *Rymv*1 encodes eIF(iso)4G and we analyse its allelic variability.

Results

Fine genetic and physical mapping of the Rymv1 *locus*

We had previously mapped the *Rymv1* locus on chromosome 4 in a 3.7-cM interval flanked by the microsatellite marker RM252 and the amplified fragment length polymorphism (AFLP) marker E-ACG/M-ACA (Albar *et al.*, 2003). The closest marker, i.e. E-ACG/M-ACA, was cloned and sequenced. A BLAST analysis revealed that this sequence also matched the Nipponbare bacterial artificial chromosome (BAC) clone OJ991113_30 (accession number AL662946). Considering the mean 'physical/genetic distance' ratio in rice (about 300 kb/cM), we estimated that *Rymv*1 could be approximately 600 kb downstream from this marker. We identified a contig of eight sequenced BAC clones on the rice physical map (Chen *et al.*, 2002). This contig covers the region about 900 kb below the E-ACG/M-ACA marker and was expected to contain *Rymv*1 (Figure 1a). We generated new markers in the target region based on an *in silico* analysis of the public sequence of rice. Mapping was performed on 906 either F2 or F3 plants from a cross between IR64 (*indica*, susceptible) and Gigante (*indica*, resistant) and 432 F2 plants from a cross between Nipponbare (*japonica*, susceptible) and Gigante. Marker MS604-2, located at the downstream extremity of the candidate contig, confirmed that *Rymv*1 was inside the selected BAC contig. Then additional markers defined a 160-kb interval containing the gene on the overlapping BAC clones OSJNBa0067K08 and OSJNa0029H02 (accession numbers AL606627 and AL606594, respectively) (Figure 1b).

A list of putative genes contained in the 160-kb candidate interval was extracted from the rice genome annotation databases of TIGR (http://www.tigr.org/tdb/e2k1/osa1) and RiceGAAS (http://RiceGAAS.dna.affrc.go.jp/). The TIGR identified 31 putative genes, 25 of which were also identified by the 'Predgeneset' annotation system of RiceGAAS and 18 had a corresponding full-length cDNA in the KOME database (Kikuchi *et al.*, 2003). For seven annotations, no putative function and no homology with known either domains or proteins was reported. No obvious candidate gene was identified except a gene annotation containing the MIF4G and MA3 domains (Pfam accessions number PF02854 and PF02847, respectively), characteristic of eIF4G and its isoform eIF(iso)4G. This gene was considered to be the best candidate for *Rymv*1.

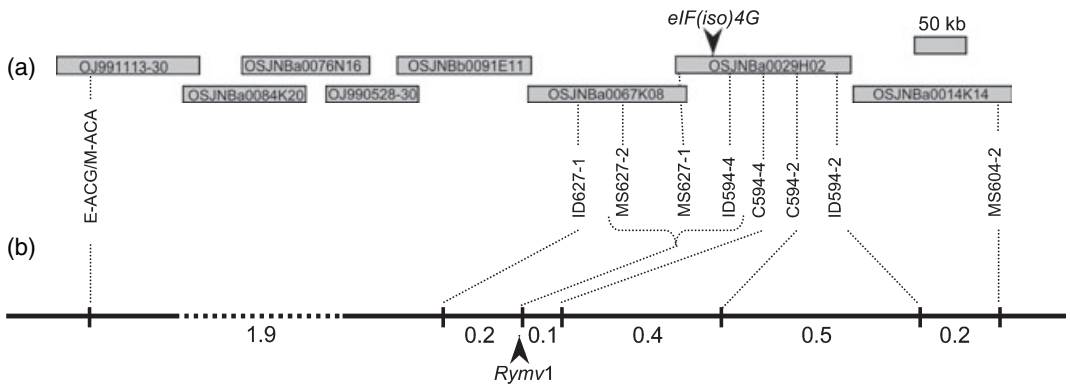

Figure 1. Physical and fine genetic map in the *Rymv*1 region.
(a) A contig of eight sequenced BAC clones covering 900 kb was identified downstream from the AFLP marker E-ACG/M-ACA. The physical position of the markers defined on the BAC clones and of the candidate gene *eIF(iso)4G* are indicated. The marker types are abbreviated as follows: microsatellite (MS), Indel (ID) or CAPS (C). Only the most informative markers are represented here.
(b) Linkage map based on 432 either F2 or F3 plants derived from the Nipponbare/Gigante cross. Distances between the markers are indicated in centimorgans.

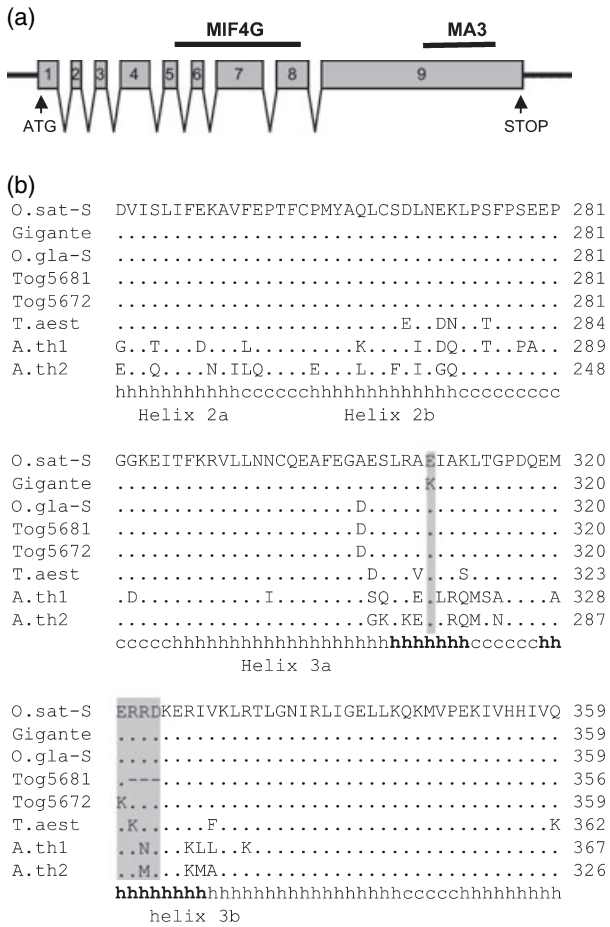

Figure 2. Organization and variability of the candidate gene.
(a) Exons are shown as grey boxes and introns as broken lines. The position of the MIF4G and MA3 domains are indicated.
(b) Alignment of sequences corresponding to exon 7 (part of the MIF4G domain) were performed using Clustal W. 'O. sat-S' represents sequences from 15 susceptible *O. sativa* varieties (including IR64 and Nipponbare), which are identical, and 'O.glab-S' represents sequences from two susceptible *O. glaberrima* varieties, which are also identical. Sequences of the resistant varieties *O. sativa* Gigante and *O. glaberrima* Tog5681 and Tog5672 are indicated by the variety name. 'T.aest', 'A.th1' and 'A.th2' are part of the sequence of *eIF(iso)4G* from wheat and *eIF(iso)4G1* and *eIF(iso)4G2* from *A. thaliana*, respectively. The amino acids that are either mutated or deleted in resistant varieties are shaded. The secondary structure prediction (either helix or coil) and helix names are displayed behind the alignment. Helix parts corresponding to the protrusion compared with human eIF4GII are shown in boldface.

Characterization of the candidate gene

In order to characterize the transcribed sequence of the candidate gene, we sequenced cDNA obtained by reverse transcription (RT) on cv IR64 and cv Gigante RNA. In both varieties, this cDNA included a 2379 nucleotide coding sequence (CDS). The corresponding fragment on the Nipponbare genomic DNA is 5410 nucleotides in length and the comparison with cDNA revealed nine exons. A 70-bp guanine and cytosine-rich region was observed in the first exon, 30-bp downstream from the first ATG codon. CDS encodes a protein of 793 amino acids. The MIF4G and MA3 domains are localized from amino acids 208 to 435 (exons 6–8) and from amino acids 628 to 740 (exon 9), respectively (Figure 2a).

The highest sequence similarity of the putative protein was obtained with the wheat eIF(iso)4G (accession number AAA16209; 85% similarity on the complete sequence and 93% on the MIF4G domain). Comparisons with *A. thaliana* eIF(iso)4G1 and eIF(iso)4G2 (accession numbers AAL07115.1 and AAL32572) revealed 70% and 66% similarity on the complete sequence and 87% and 83% on the MIF4G domain, respectively (Figure 2b). Two other putative *eIF(iso)4G* genes were identified on chromosomes 2 and 6 in the rice sequence; the similarity of the corresponding proteins with wheat eIF(iso)4G was slightly lower (77% and 66%, respectively).

We compared the sequences obtained from IR64 and Gigante varieties and observed only one single nucleotide polymorphism (SNP) (A/G), at exon 7, 925 nucleotides downstream from the ATG, leading to an amino acid substitution (Glu309Lys) in the MIF4G domain (Figure 2b). The Nipponbare sequence is similar to the IR64 sequence for that SNP. 'PCR-confronting two primer pairs' (Hamajima *et al.*, 2002) was used to develop the AG925 marker, which can reveal the allelic status (either A or G) on this SNP (Figure 3a,b). AG925 was analysed in the mapping populations and co-segregated with *Rymv*1.

Functional complementation

In order to validate the candidate gene, a functional complementation approach was carried out using the *Agrobacterium tumefaciens*-mediated transformation method. The complete CDS of the candidate gene, obtained from the susceptible variety IR64, was introduced in the Nip-R line, which is a near-isogenic line of Nipponbare including the resistance allele of Gigante at the *Rymv*1 locus. We analysed the co-segregation on 75 progeny plants (T1) obtained from first generation transgenic plants (T0) that resulted from two independent and monocopy transformation events. Transgene segregation was not different from a 3:1 (presence:absence) ratio ($\chi^2 = 1.55$ and 2.27, 1 degree of freedom), in agreement with a monocopy insertion. Symptom expression showed a clear resistant/susceptible pattern, which was perfectly confirmed by an ELISA: some T1 plants did not show any symptoms, as observed in Nip-R and Gigante, whereas other T1 plants showed clear mottling 10 days after inoculation and generalized yellowing a few weeks later, as observed in IR64 and Nipponbare (Figure 4). Perfect co-segregation was observed between RYMV susceptibility and the presence of the transgene (Figure 4a). Complementation experiments thus demonstrated that the *eIF(iso)4G* allele of the susceptible variety IR64 allowed the restoration of RYMV susceptibility in a rice genotype with a

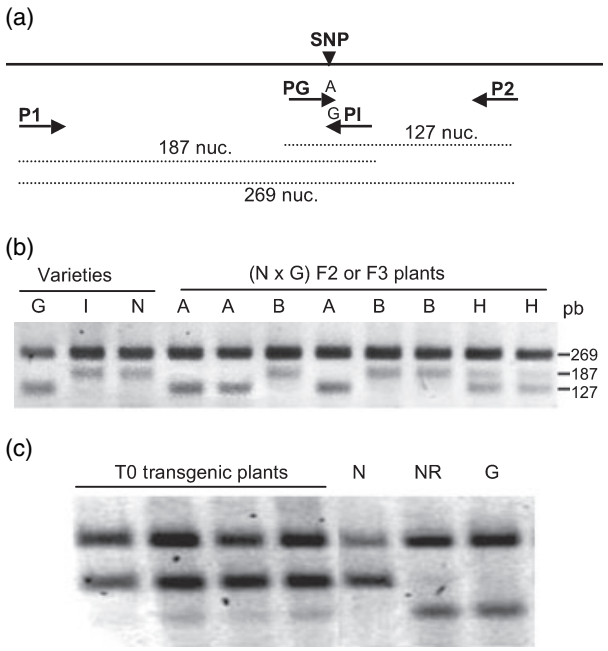

Figure 3. Amplification profile of the AG925 marker that is able to differentiate IR64 and Gigante alleles on the candidate gene.
(a) Allele-specific PG and PI primers possess G and A nucleotides, respectively, on their 3′-extremity. PCR with the four primers generated a fragment of 269 nucleotides and fragments of either 127 or 187 nucleotides, depending on the allele amplified.
(b) Amplification profiles of AG925 on DNA. Fragment sizes were determined by reference to a molecular weight marker. Gigante variety (G) amplified the 127-bp fragment, whereas IR64 (I) and Nipponbare (N) varieties amplified the 187-bp fragment. Amplification profiles were confirmed on F2 and F3 plants derived from the Nipponbare/Gigante cross, as shown here for eight plants. Genotypes with markers flanking the candidate gene are indicated with A (homozygous for Gigante allele), B (homozygous for Nipponbare allele) or H (heterozygous).
(c) Amplification profile of AG925 on cDNA obtained from four T0 transgenic plants expressing a transgenic copy of the gene obtained from IR64, Nipponbare (N), Gigante (G) and Nip-R line (NR). In transgenic plants, the transgenic copy, under 35S control, is overexpressed as compared with the endogeneous copy, which enhances the detection of the 187-bp fragment.

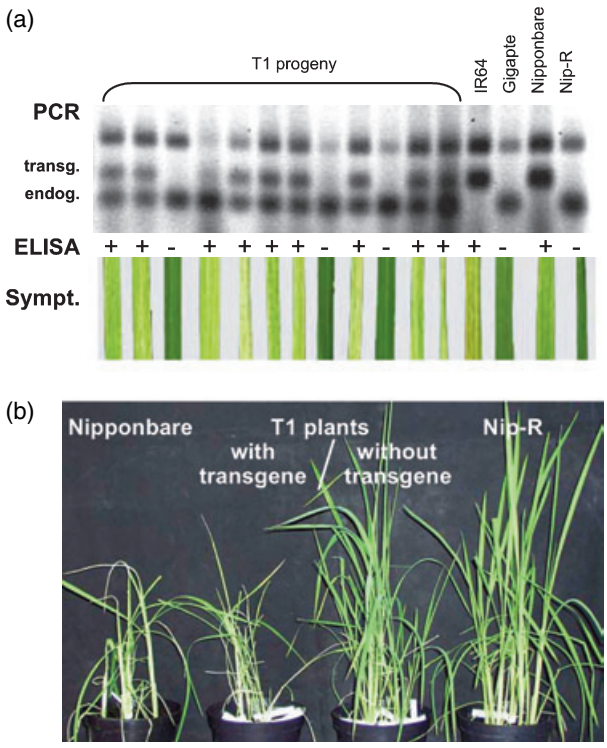

Figure 4. Transgene presence and RYMV resistance in T1 progenies.
(a) Analysis of 12 T1 progenies obtained from a T0 plant with a single copy of the transgene. The presence of the transgene was analysed using the AG925 marker. Resistance versus susceptibility to RYMV was assessed using virus detection with either ELISA or symptom observation after mechanical inoculation.
(b) Effect of virus inoculation on plant development on T1 plants, with or without the transgene, compared with the Nipponbare and Nip-R controls. Plants were inoculated 2 weeks after sowing and observed 4 weeks after inoculation.

resistance allele at the *Rymv*1 locus. This confirmed that the candidate gene corresponds to *Rymv*1.

Allelic diversity of Rymv1

In order to analyse the sequence variability of *Rymv*1, we sequenced the complete CDS on two resistant accessions (Tog5681 and Tog5672) and one susceptible accession (Tog5673) of the cultivated African rice species *O. glaberrima*. Two mutations were observed in the MIF4G domain, on exon 7: compared with Tog5673, the Tog5681 sequence had a deletion of three amino acids (Arg Arg Asp 322–324) and a point mutation (A/G) in the Tog5672 sequence leads to an amino acid substitution (Glu321Lys) (Figure 2b). These mutations and the SNP that differentiates Gigante from IR64 occur in a 48-nucleotide interval. An additional SNP (G/A) on exon 9, which leads to an amino acid substitution (Ser573-Asn), distinguished Tog5681 from Tog5673 and Tog5672.

As these first sequencing results pinpointed interesting mutations on exon 7, we enlarged the sequencing of this region to include 14 additional susceptible varieties (seven *O. sativa indica*, six *O. sativa japonica* and one *O. glaberrima*) in order to obtain an overview of *Rymv*1 diversity in 17 susceptible and three resistant rice varieties. The sequence obtained on these additional *O. sativa indica*, *O. sativa japonica* and *O. glaberrima* varieties did not differ from the sequence obtained on IR64, Nipponbare and Tog5673, respectively, i.e. none of them had either the point mutations or the deletion identified in Gigante, Tog5681 and Tog5672. Three different haplotypes were thus identified in the 17 susceptible varieties, in agreement with the genetic structure of cultivated rice: two SNPs characterized the *O. sativa japonica* varieties, one SNP characterized the *O. sativa indica* varieties and another SNP characterized the *O. glaberrima* varieties.

To assess the frequency of the SNP that distinguishes *Rymv*1 alleles from Gigante and IR64, the AG925 marker was used to characterize a collection of 96 susceptible varieties, representative of the genetic diversity in *O. sativa*. None of these additional varieties had the profile of Gigante, which is consistent with the rarity of the high RYMV resistance in rice varieties.

Predicted three dimensional (3D) structure of the MIF4G domain

A structure modelling study was undertaken to localize the position of amino acid substitutions present in highly resistant varieties. The sequence of the predicted protein encoded by *Rymv*1 was submitted to a fold-recognition metaserver @TOME (Douguet and Labesse, 2001). Significant structure-compatibility scores and high identity sequence scores were observed for the central domain, i.e. MIF4G (see supplementary information at http://www.info-biosud.cnrs.fr/bioserver/eIFiso4G/suppl.html). The best two templates corresponded to the human eIF4GII middle and C-terminal domains (Protein Data Bank code 1HU3 and 1UG3, respectively). These templates were used for further structure modelling. The two crystal structures exhibited a crescent-like shape with five anti-parallel α-helical hairpins (HEAT-repeats) arranged in a helical stack (Marcotrigiano *et al.*, 2001). However, three regions of the template structures presented poorly resolved electron density, in particular the loop between helix 3a and 3b containing the studied substitutions. The secondary structure prediction showed that this region in the rice protein is composed of two longer α-helices than those found in human eIF4GII (Figure 2b). Structure modelling was performed using this secondary structure prediction as an additional restraint. The deduced model of the rice protein was evaluated using the PROSA program and Verify3D and had good quality scores, equivalent to those of the 1HU3 crystal structure. The two α-helices would form a protrusion, perpendicular to the convex face of the crescent-like shape (Figure 5). The model suggested that the two substitutions present in the highly resistant varieties (Glu309Lys and Glu321Lys) are localized in the same small region of the long α-helical hairpin. Moreover, these two positions appeared on the surface, on the same side and in the same direction, suggesting that the interaction domain with the viral components would be localized in this region. The three deleted amino acids at position 322–324 did not enable reliable modelling in the case of Tog5681.

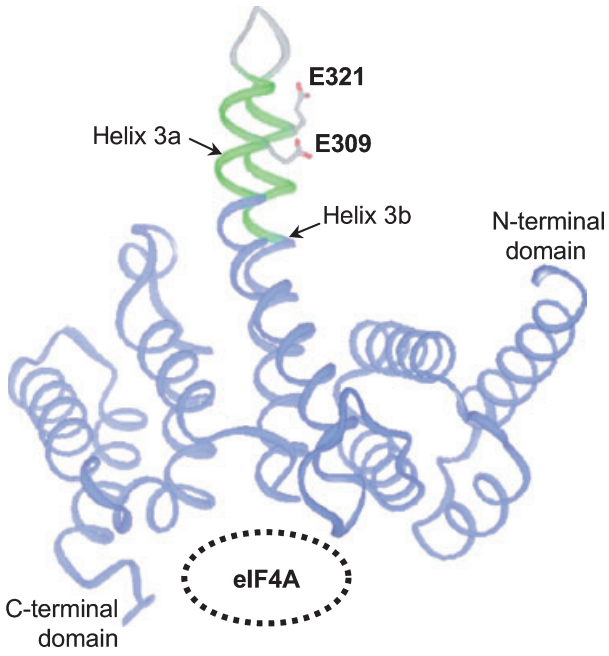

Figure 5. Three-dimensional model of the MIF4G domain (208–435) of *Rymv*1.
The common core and protrusion, compared with 1HU3, are respectively shown as blue and green ribbons. The position of eIF4A is shown as an oval. The two positions involved in high resistance of *O. sativa* Gigante and *O. glaberrima* Tog 5672 are shown as wireframes (Glu309 and Glu321, respectively).

Discussion

Here we report the isolation of a recessive resistance gene of rice against RYMV. This gene is of outstanding interest as it is the only one controlling the high resistance of rice against RYMV that has been described so far. In addition this result is of general interest in furthering the understanding of plant resistance to viruses. Recently published results emphasize the role of eukaryotic initiation factors in plant/virus interactions, and in particular the role of eIF4E in plant/potyviridae interactions. Our result underlines that viruses from various genera and families, here a sobemovirus, require translation initiation factors to perform their cycle, and that different initiation factors – not only eIF4E but also eIF(iso)4G – can be involved in naturally occurring resistance.

In plants, eIF4E and eIF4G form the eIF4F complex, and eIF(iso)4E and eIF(iso)4G form the eIF(iso)4F complex. These complexes are involved in the fixation of the mRNA cap and ribosome recruitment in the initial steps of translation. Two models are generally accepted for the translation of cellular mRNA. The first model is cap-dependent translation initiation, which requires several initiation factors and the closed-loop of the mRNA via cap-eIF4E-eIF4G-PABP-poly(A) interactions (Wells *et al.*, 1998). Most cellular mRNAs use this model. The second model involves an internal ribosomal entry site (IRES), defined by the sequence and structure of a particular mRNA region, but little is known about the role of initiation factors in this cap-independent model (Hellen and Sarnow, 2001). Viruses have developed different strategies for translation (for a review see Thivierge *et al.*, 2005)

and they often require translation initiation factors from their host. On the one hand, RNA viruses with 5′-cap and 3′-poly(A) as potexviruses, are likely to follow the cap-dependent model, whereas some viruses have adapted this model to their own characteristics with either viral protein or RNA structure replacing either one component or more of the model; for instance, Goodfellow *et al.* (2005) suggest that feline calcivirus VPg may act as a 5′-cap substitute in order to initiate translation. On the other hand, cap-independent translation, via IRES, has been described for several animal viruses and requires either none or most of the initiation factors (Bergamini *et al.*, 2000; Pestova *et al.*, 1996, 1998). Considering the diversity of translation models used by viruses, it is not surprising to find different initiation factors involved in resistance. In the rice/RYMV interaction, a mutation in the eIF(iso)4G factor is responsible for resistance, which suggests an interaction between a viral component and eIF(iso)4G. The localization of substitutions characteristic of high resistance on the modelled structure of eIF(iso)4G suggests that resistance involves subtle changes in the surface interaction rather than more pronounced structural/functional changes. The next steps of our work will focus on gaining further insight into the interaction with a viral component. As the breakdown of resistance is associated with a mutation in the VPg protein of RYMV (Hebrard *et al.*, 2006), we will first test a possible VPg/eIF(iso)4G interaction. We will also analyse the putative consequences of such an interaction on the binding of other components of the complex, in particular eIF4A. Indeed, the eIF4A-binding domain of the human eIF4GII middle domain was localized and the surface contact was modelled (Oberer *et al.*, 2005): it maps to a region opposite the loop containing the resistance-associated substitutions in *Rymv*1 (Figure 5).

A mutation in a translation initiation factor required by the virus and a defective interaction between cellular and viral components may impair the virus cycle at different levels. The mutation can directly affect the main steps of the virus cycle. First, the most evident consequence could be an either reduced or totally blocked translation. This hypothesis is supported by the reduced translation of CMV RNA in *cum1* and *cum2 A. thaliana* mutants, mutated in eIF4E and eIF4G, respectively (Yoshii *et al.*, 2004). Effects on another virus cycle step cannot be excluded. It has been proposed that eIF4E may stabilize viral RNA (Lellis et al., 2002), which is supported by observations in yeast highlighting the protection of RNA against degradation factors when interacting with eIF4E (Vilela *et al.*, 2000). However, this hypothesis is less relevant for eIF(iso)4G. In addition, in the rice/RYMV interaction, the same virus RNA content has been detected in protoplasts from susceptible and resistant varieties (Ndjiondjop *et al.*, 2001), suggesting that neither replication nor RNA stability is affected. Consequently either eIF(iso)4E- or eIF(iso)4G-mediated resistance may also be linked to virus trafficking in the cells through eIF4(iso)E/eIF4(iso)G and eIF(iso)4G/microtubule interactions (Bokros *et al.*, 1995). This latter hypothesis is in agreement with the role of eIF4E in the cell-to-cell movement of the *Pea seed-borne mosaic virus* (PSbMV) in pea (Gao *et al.*, 2004). Lastly, instead of a deleterious effect on a specific step of the virus cycle, such as RNA stability, translation or movement, a resistance-associated mutation could decrease the capacity of the virus to compete with host cell mRNAs for access to translation initiation factors. Indeed, some viruses reroute cellular translation machinery from cellular mRNAs to viral RNA. For instance, either viral RNA or viral proteins may have a much higher affinity for initiation factors than cellular mRNA have, as described for either the interaction between the *Turnip mosaic virus* (TuMV) VPg and eIF(iso)4E (Miyoshi *et al.*, 2006) or the interaction between picornavirus RNA and the eIF4G factor truncated by viral protease (Ali *et al.*, 2001): this higher affinity results in a competitive advantage for virus translation. All of these putative mechanisms underline the probable complexity of interactions between translation initiation factors and viruses. In the rice/RYMV interaction, the step of virus cycle affected in eIF(iso)4G-mediated resistance remains to be investigated.

A first analysis of *Rymv*1 diversity was undertaken. In susceptible varieties, a clear-cut differentiation was observed between *O. sativa indica*, *O. sativa japonica* and *O. glaberrima*, in agreement with the known genetic diversity of rice (Ishii *et al.*, 1993; Second, 1991). Except for some specific additional mutations, the three resistant varieties presented the typical profile of their species or subspecies. This dismisses a possible introgressive origin of the resistance gene via interspecific hybridization, and supports the hypothesis of independent point mutations in the different genetic backgrounds, as previously suggested by the neutral diversity around the gene (Albar *et al.*, 2003). All three resistant varieties analysed here present specific mutations in the same 16-amino-acid interval of the MIF4G domain. The presence of different alleles in Gigante, Tog5681 and Tog5672 is in agreement with the observation that the resistance-breaking ability of some virus isolates is variety specific (Fargette *et al.*, 2002; Konate *et al.*, 1997). We propose to name these alleles *Rymv*1-2, *Rymv*1-3 and *Rymv*1-4, respectively. Considering the lower genetic diversity observed in African rice (Second, 1982), it was quite surprising to observe two distinct resistance alleles in *O. glaberrima*, whereas only one was found in *O. sativa* despite the fact that there have been many more extensive surveys conducted to screen for varietal resistance in this species. We now plan a wider analysis of *Rymv*1 diversity in rice varieties and their wild relatives, particularly by verifying other *O. glaberrima* varieties that Thottappilly and Rossel (1993) described as being resistant. A greater understanding of the molecular basis of resistance and resistance-breaking mechanisms, as well as experimental control of allele durability, are essential to ensure the efficient deploy-

ment of sustainable natural RYMV resistance genes for use in breeding programs.

Experimental procedures

Mapping populations and resistance evaluation

Mapping populations were derived from crosses between the susceptible varieties, either IR64 (*O. sativa indica*) or Nipponbare (*O. sativa japonica*), and the resistant variety Gigante (*O. sativa indica*). F2 populations and F3 populations derived from heterozygous F2 plants were developed and analysed with two different strategies. First, the genotype at markers that flanked the *Rymv*1 locus was scored for all plants and progenies of recombinants were evaluated for resistance. Second, plants were screened for virus resistance and only resistant plants were retained for genotype scoring at flanking markers. RYMV resistance was assessed on the basis of symptom intensity. Two weeks after sowing, plants were inoculated with an RYMV isolate from Burkina Faso, as described in Ndjiondjop *et al.* (1999). Susceptible plants developed clear yellow mottles 10–15 days after inoculation, whereas resistant plants had no symptoms at all. Progeny evaluations focused on about 20 plants per progeny.

Development of molecular markers

Different types of markers have been used for *Rymv*1 mapping. The AFLP marker E-ACG/M-ACA was amplified, cloned and sequenced as described in Albar *et al.* (2003). Other markers are PCR-based markers designed on the public genomic rice sequence. On the one hand, simple sequence repeats were identified on the Nipponbare sequence and used to design microsatellite markers, as described by McCouch *et al.* (2002). On the other hand, putative *indica/japonica* polymorphisms, potentially useful in our Nipponbare (*japonica*) × Gigante (*indica*) mapping population, were identified through a comparison between publicly available sequences of Nipponbare (*japonica*) and 93-11 (*indica*), as described by Shen *et al.* (2004). We thus designed Indel markers corresponding to 10–100 bp insertion/deletion and cleaved amplified polymorphic sequence (CAPS) markers corresponding to SNPs.

Primers were designed using the Primer3 software package (Rozen and Skaletsky, 2000). DNA was extracted as described by Edwards *et al.* (1991). The PCR was performed with 200 μM of each deoxyribonucleotide 5′-triphosphate (dATP, dGTP, dTTP, dCTP), 2 mM $MgCl_2$, 0.02 U μl^{-1} Taq DNA polymerase (Promega, Madison, WI, USA), 0.2 μM primers, and about 1 ng μl^{-1} DNA. The annealing temperature decreased from 60 to 55°C during the first five cycles and was then maintained at 55°C. In some cases, however, $MgCl_2$ concentrations and annealing temperatures were adapted according to the primers. Large-size polymorphisms were detected on agarose gels, whereas small-size polymorphisms were detected on 6.5% acrylamide gels using a LI-COR Genotyper (LI-COR Inc., Lincoln, NE, USA). In this case, one primer of each pair was tailed during synthesis with the M13 sequence (CACGACGTTGTAAAACGAC) on the 5′-extremity; an IRDye™-labelled (LI-COR Inc., Lincoln, NE, USA) M13 primer was included in the reaction (0.2 μM) and annealed to the PCR fragment synthesized using the tailed primer, thus labelling the PCR product.

We generated a total of 32 primer pairs. Twenty-four pairs (nine microsatellites, eight indels and seven CAPS markers) gave an amplification product (Table S1). Fifteen markers (four microsatellites, five indels and six CAPS markers) revealed polymorphism between Gigante (*indica*) and Nipponbare (*japonica*), whereas only the microsatellite marker MS604-2 was polymorphic between the *indica* varieties IR64 and Gigante.

Sequence analysis of the candidate gene

The cDNA of the candidate gene was sequenced in the *O. sativa* varieties IR64 and Gigante and in *O. glaberrima* varieties Tog5681 (resistant), Tog5672 (resistant) and Tog5673 (susceptible). Total RNA was isolated from either leaf tissue or cell suspensions with TRIzol reagent (Invitrogen, Paisley, UK). Retrotranscription was performed with an oligo(dT) primer for IR64 and Gigante and a *Rymv*1 specific primer (5′-GCCTTGTTCATCTCAGGCTCCA-3′) for *O. glaberrima* varieties, using Superscript II retrotranscriptase (Invitrogen) and the protocol recommended by the manufacturer. Sequencing reactions were carried out with Applied Biosystems BigDye terminator and analysed on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA). The sequences are registered in the EMBL data library under accession numbers AM156957 (IR64), AM156958 (Gigante), AM156959 (Tog5681), AM156960 (Tog5673) and AM156961 (Tog5672).

Marker AG925, which can reveal the allelic status on SNP differentiating IR64 and Gigante alleles, was developed according to Hamajima *et al.* (2002) (Figure 3a). SNP non-specific primers (P1, 5′-GAGCCCACCTTCTGTCCGATG-3′; P2, 5′-AGTAGCTCACCAATTAGACGGA-3′) are localized at 163 bp upstream and 107 bp downstream from the SNP. SNP-specific-primers PG (5′-GTGCTGAGAGCCTAAGGGCTA-3′) and PI (5′-CAGGGCCAGTCAATTTTGCTATTTC-3′) end on the SNP and were specific to Gigante and IR64 sequences, respectively. Amplification was as described above for molecular markers, except that the four primers were included in the PCR reaction and that the annealing temperature was 61°C. The amplification profile was revealed on a 2% agarose gel.

In order to analyse the allelic variability of the gene, a PCR fragment including exon 7 was amplified from genomic DNA, and sequenced for the susceptible varieties BG90-2, Bouaké 189, Jaya, MTU9, ASD1, Carreon, IR5 (*O. sativa indica*), Hawmom, Canaroxa, Carolina, Pate Blanc, Azucena, Moroberekan (*O. sativa japonica*) and CG14 (*O. glaberrima*). Ninety-six additional susceptible varieties representing *indica* (35 varieties), *japonica* (46) or iso-enzymatic groups II and V (15), according to both Glaszmann (1987) and Second and Ghesquière (1995), were analysed for marker AG925.

Functional complementation

The complete CDS of the candidate gene obtained from IR64 was cloned between the CaMV-35S promoter and the nopaline synthase (nos) 3′-terminator. The resulting cassette was introduced in the multiple cloning site of the binary plasmid pC5310, derived from the plasmid pCambia 1300 (CAMBIA, Canberra, Australia) by inserting a catalase intron in the hygromycin resistance gene (*hpt*) and the green fluorescent protein gene (*sGFP*). This plasmid was then transferred into *A. tumefaciens* strain LBA4404 by electroporation.

As *A. tumefaciens*-mediated stable transformation is more convenient for *japonica* genotypes, we developed a near-isogenic line of Nipponbare with the Gigante allele at the *Rymv*1 locus. Introgression was monitored by phenotypic and marker-assisted selection. The resistant BC3F4 obtained and used for transformation is referred to as Nip-R.

Preparation of *Agrobacterium* cells, induction of embryogenic tissues and transformation procedures were as described in Sallaud *et al.* (2003). First generation transgenic plants (T0) were selected based on their resistance to hygromycine and confirmed by PCR

amplification of transgene-specific DNA fragments. In order to determine the transgene copy number, we performed Southern blot analysis as described in Sallaud *et al.* (2003) using the 3′-part of the candidate gene as a probe. Transgene expression was tested using the AG925 marker, which is able to distinguish the transgenic copy from the endogenous copy (Figure 3c).

T1 progenies were checked for the presence of the transgene with marker AG925. For phenotypic screening, T1 plants were mechanically inoculated with RYMV. We detected symptoms and performed ELISA as described in Ndjiondjop *et al.* (1999).

Structure modelling

The fold compatibility between the target and Protein Data Bank entries was analysed using the meta-server @TOME (Douguet and Labesse, 2001). The query sequence is sent automatically to six distinct fold recognition or protein structure prediction servers: 3D-PSSM (Kelley *et al.*, 2000), PDB-BLAST (http://bioserv.cbs.cnrs.fr), FUGUE (Shi *et al.*, 2001), GenTHREADER (McGuffin *et al.*, 2000), SAM-T99 (Karplus *et al.*, 1998) and J-PRED2 (Cuff *et al.*, 1998), with default parameters except for PDB-BLAST (10 iterations). Each structural alignment was further evaluated using TITO (Labesse and Mornon, 1998). A consensus ranking was deduced for each template by taking into account its score and ranking (both computed by the original server), the TITO score and the sequence identity level. For all targets, three models were built directly using MODELLER (Sali and Blundell, 1993) for the top-ranking structural alignments. Models were evaluated using PROSA (Sippl, 1993), Verify3D (Eisenberg *et al.*, 1997) and Errat (Colovos and Yeates, 1993). Side-chain modelling in the common core (as defined by target-template alignment) was also performed using SCWRL 2.8 (Dunbrack and Karplus, 1993), and similarly evaluated but not further refined. These supplementary results are available online (http://www.infobiosud.cnrs.fr/bioserver/eIFiso4G/suppl.html).

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Molecular markers used for *Rymv*1 mapping. Marker type (microsatellite, Indel or CAPS), primer sequences, amplification conditions and polymorphism obtained between the IR64, Gigante and Nipponbare varieties are indicated. Supplementary information about the 3D modelling of the MIF4G domain is presented at http://www.infobiosud.cnrs.fr/bioserver/eIFiso4G/suppl.html

This material is available as part of the online article from http://www.blackwell-synergy.com

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