Direct Interaction Between the *Rice yellow mottle virus* (RYMV) VPg and the Central Domain of the Rice eIF(iso)4G1 Factor Correlates with Rice Susceptibility and RYMV Virulence

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The adaptation of *Rice yellow mottle virus* (RYMV) to recessive resistance mediated by the *rymv1-2* allele has been reported as a model to study the emergence and evolution of virulent variants. The resistance and virulence factors have been identified as eukaryotic translation initiation factor eIF(iso)4G1 and viral genome-linked protein (VPg), respectively, but the molecular mechanisms involved in their interaction are still unknown. In this study, we demonstrated a direct interaction between RYMV VPg and the central domain of rice eIF(iso)4G1 both in vitro, using recombinant proteins, and in vivo, using a yeast two-hybrid assay. Insertion of the E309K mutation in eIF(iso)4G1, conferring resistance in planta, strongly diminished the interaction with avirulent VPg. The efficiency of the major virulence mutations at restoring the interaction with the resistance protein was assessed. Our results explain the prevalence of virulence mutations fixed during experimental evolution studies and are consistent with the respective viral RNA accumulation levels of avirulent and virulent isolates. Our results also explain the origin of the residual multiplication of wild-type isolates in *rymv1-2*-resistant plants and the role of genetic context in the poor adaptability of the S2/S3 strain. Finally, the strategies of RYMV and members of family *Potyviridae* to overcome recessive resistance were compared.

Plant viruses are important agricultural pathogens worldwide. The use of resistant varieties is the main method of viral disease control. Approximately half of all virus-resistant genes are recessively inherited, whereas resistant genes against other plant pathogens are largely dominant (Kang et al. 2005a). These recessive genes encode plant factors recruited by viral proteins,

and resistance mutations in these factors prevent the viral cycle. To date, all cloned recessive resistance genes in crop species encode translation initiation factors (Truniger and Aranda 2009). Most are resistance genes against family *Potyviridae* and encode eIF4E factors. These small host proteins bind the cap structure of mRNA and form part of a multimeric complex that promotes translation initiation (Browning 2004). The virulence genes have been largely identified as viral genome-linked proteins (VPg). These viral proteins were detected at the 5' end of the viral genomes of several families of viruses (Sadowy et al. 2001). Direct interactions between eIF4E factors and VPg have been previously demonstrated (Charron et al. 2008; Hwang et al. 2009; Kang et al. 2005b; Leonard et al. 2000; Schaad et al. 2000; Wittmann et al. 1997). The resistant phenotypes result from disruptions of the interactions between eIF4Es and VPg (Charron et al. 2008; Kang et al. 2005b; Yeam et al. 2007). However, recessive resistance can sometimes be overcome by the emergence of virulent variants (Ayme et al. 2007; Moury et al. 2004). Some virulence mutations in VPg restore the interactions with proteins encoded by resistance alleles (Charron et al. 2008).

As described above, recessive resistance and virulence factors have been identified, plant and viral mutations have been mapped, and the biochemical roles of these factors have been described in some detail. By contrast, the emergence and evolution of virulent variants in viral populations have been poorly investigated. For most *Potyviridae* studies, data are lacking regarding the mutational pathways that viruses follow, the relationships between these pathways, and the impact of genetic context. However, analyses at both biochemical and population levels should be integrated to better understand virulence acquisition.

Outside the *Potyviridae* family, another biological model has been used to understand the adaptation of viral populations to recessive resistances. *Rice yellow mottle virus* (RYMV) belongs to the unassigned *Sobemovirus* genus. RYMV adaptation to *rymv1-2*-mediated resistance has been previously described. Only two cultivars of the Asiatic rice *Oryza sativa* show a high resistance to RYMV, as characterized by the absence of symptoms and no effect on rice yield (Ndjioudjop et al. 1999; Rakotomalala et al. 2008). The resistance durability of these cultivars has been assessed by experimental evolution, and 15 to 20% of

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the isolates were able to overcome this resistance (Fargette et al. 2002; Hébrard et al. 2006; Pinel-Galzi et al. 2007). The virulence factor of RYMV was identified as a VPg (Hébrard et al. 2006; Pinel-Galzi et al. 2007). Parallel evolution of most RYMV strains results in fixation of the same VPg mutations, predominantly at codon 48 (Pinel-Galzi et al. 2007). The virulent variants have been suggested to emerge from a residual multiplication of wild-type isolates in resistant plants, which was estimated by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to be approximately 10^6 viral RNA copies per milligram of leaves (Poulicard et al. 2010). The origin of this residual multiplication has not yet been identified. A stepwise optimization of virulent variant fitness by a competition or exclusion phenomenon has been observed (Pinel-Galzi et al. 2007). The conserved site at codon 48, corresponding to an arginine (R) in avirulent isolates, was substituted for different amino acids in virulent isolates. The virulence mutations occurred with contrasted frequencies, and several mutational pathways were followed. In the most prevalent mutational pathway, R48 was displaced by glycine (G) in the first step to become fixed at glutamic acid (E) in the second step. By contrast, the virulence mutation R48I (isoleucine) has been identified as an isolate-specific mutational pathway (Hébrard et al. 2006; Pinel-Galzi et al. 2007). The virulent isolates with the 48E mutation restored the optimal multiplication level of 10^{12} viral RNA copies per milligram of leaves in resistant plants, whereas the 48I and 48G mutations induced an intermediate level of accumulation (Poulicard et al. 2010). Increasing affinities between resistance and virulence factors have been proposed to explain the stepwise optimization of virulent variants, but this mechanism remains to be demonstrated (Hébrard et al. 2008).

The viral genetic context influences RYMV adaptability to resistant plants. In contrast to virulent variants, the widely spread West African S2/S3 strain was poorly efficient at overcoming *rymv1-2*-mediated resistance, as more than 98% of S2/S3 isolates failed to evolve toward virulent variants (Pinel-Galzi et al. 2007). S2/S3 virulent variants did not emerge from the main mutational pathway. However, directed mutagenesis to 48I or 48E in a S2/S3 infectious clone could induce virulence. The 48E artificially mutated S2/S3 isolate showed an accumulation rate similar to the S1 strain (Poulicard et al. 2010). Interestingly, artificial mutation to 48G is lethal in the S2/S3 genetic context. In this strain, the main mutational pathway appeared to be blocked at its first step. The codon 49, which is under diversifying selection, has been previously sug-

gested to be involved in antagonistic epistasis with the codon 48 (Pinel-Galzi et al. 2007).

Although RYMV is a good model for understanding the emergence and evolution of virulent variants in resistant plants, detailed knowledge of the molecular interactions mediating these processes is lacking. Resistance gene *RYMV1* of rice was the only example of a recessive resistance gene from a crop species that does not encode eIF4E (Truniger and Aranda 2009). *RYMV1* encodes the eukaryotic translation initiation factor eIF(iso)4G1, a scaffold protein that interacts with eIF4E, eIF4A, and the poly-A binding protein (PABP) (Albar et al. 2006; Browning 2004). The *rymv1-2* allele is characterized by an amino acid substitution of glutamic acid (E) to lysine (K) at position 309 of eIF(iso)4G1. However, a direct or indirect interaction between eIF(iso)4G1 and the RYMV VPg has not been demonstrated. In this paper, we investigated i) the nature of this interaction, ii) the effects of resistance and virulence mutations on this interaction, and iii) the role of genetic context in the poor adaptability of the S2/S3 strain to *rymv1-2*-mediated resistance. Our results provide a molecular explanation for the frequencies of virulence mutations observed during experimental viral evolution and are consistent with the respective RNA accumulation levels of avirulent and virulent isolates. Furthermore, a residual interaction was detected that could be the source of residual multiplication of wild-type isolates in *rymv1-2*-resistant plants. Negative epistasis in the VPg sequence, which could have explained the genetic constraints observed in the S2/S3 strain, was not observed. Finally, RYMV and potyvirus models were compared.

RESULTS

Structural characterization of the central domain of rice eIF(iso)4G1.

The rice resistance gene *RYMV1* has been identified as the eukaryotic translation initiation factor eIF(iso)4G1. Compared with the well-known human eIF4Gs (Lamphear et al. 1995; Marintchev and Wagner 2005), rice eIF(iso)4G1 is shortened at its two extremities, similar to other plant eIF(iso)4G (Fig. 1A). The N-terminus of rice eIF(iso)4G1 comprises a conserved eIF4E-interacting domain, a Tyr-X₄-Leu-Leu motif (Mader et al. 1995) and a PABP-interacting domain that has not been precisely mapped. The amino-acid sequence of the central region of rice eIF(iso)4G1 showed an identity score of 37% with the central domain (named the MIF4G domain) of human eIF4GII,

Fig. 1. Organization of the MIF4G central domain of rice eIF(iso)4G1. **A**, Human eIF4GII is a modular protein containing three regions separated by flexible hinges. The N-terminal region contains the binding site for the poly-A binding protein (PABP) and the eIF4E-binding motif Y-X₄-L-L. The MIF4G central region of eIF4GII binds to eIF4A and eIF3. The C-terminal domain comprises the MA3 and W2 domains. The MA3 domain carries a second eIF4A-binding site. The W2 domain, also called an AA (acidic/aromatic) box-containing repeat, binds to mitogen-activated protein kinase-interacting kinases. Shorter than human eIF4GII, rice eIF(iso)4G1 has a length of 806 aa and lacks the PABP-containing N-terminal and W2-containing C-terminal extensions. Conserved domains are shown in black. They include the conserved eIF4E-interacting domain and an amino-acid sequence sharing 37 and 23% identity with the human MIF4G and MA3 domains, respectively. **B**, The modeled structure of rice MIF4G is similar to the published structure of human MIF4G, as indicated by the predicted and observed α -helices, denoted by gray cylinders. The longer helices 3a and 3b in rice MIF4G encompass the resistance mutations, denoted by hatched squares.

which binds to eIF4A and eIF3. The C-terminus of rice eIF(iso)4G1 showed an identity score of 23% with the human MA3 domain, which contains a second eIF4A-binding site.

We focused on the central domain of rice eIF(iso)4G1 (referred to in this study as MIF4G), because it contains the *rymv1-2* resistance mutation E309K (Albar et al. 2006). The crystal structure of the MIF4G domain of human eIF4GII has been determined, revealing a crescent-like shape with five anti-parallel α -helical hairpins (HEAT repeats) arranged in a helical stack (Marcotrigiano et al. 2001). Rice MIF4G was previously modeled using the human MIF4G structure as a reference (Albar et al. 2006). The central hairpin also formed helices 3a and 3b but was predicted to be longer than that of human eIF4GII (Fig. 1B). It has been suggested that the *rymv1-2* resistance mutation does not affect the MIF4G structure (Albar et al. 2006). To investigate i) whether the differences between the human eIF4GII and rice MIF4G sequences affect the adoption of the HEAT-repeat conformation and ii) whether the *rymv1-2* resistance mutation modifies the conformation of the mutated MIF4G (referred to in this study as MIF4G*), we compared the helix contents of rice MIF4G and MIF4G*.

For this purpose, the central region comprising amino acids 206 to 454 of rice eIF(iso)4G1 was produced as a His-tagged fusion peptide in *Escherichia coli*. The recombinant protein was highly soluble under native conditions and migrated with an apparent and expected molecular mass of 32 kDa in 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). MIF4G was purified by affinity chromatography in nondenaturing conditions and was eluted in one fraction at 11.3 ml by size exclusion chromatography (Fig. 2A). In parallel, MIF4G* was produced by inserting the E309K resistance mutation using site-directed mutagenesis. Its expression in *E. coli* led to the production of a recombinant protein exhibiting solubility, electrophoretic properties, and purification behavior similar to that of wild-type MIF4G (data not shown). Far-UV circular dichroism (CD) analysis of the purified MIF4G gave a UV spectrum with a maximum at 192 nm and minima at 209 and 222 nm, consistent with the presence of an α -helical conformation (Fig. 2B). CD spectra deconvolution carried out with the K2D program (Merelo et al. 1994) indicated a total α -helix content of 37%. These data agreed with the previously published 3D model of rice MIF4G (Albar et al. 2006). The central domain of rice eIF(iso)4G1 forms a putative HEAT-repeat domain. A comparison of wild type and mutated MIF4G

by far-UV CD indicated that the E309K substitution did not induce detectable modifications of the MIF4G structure.

RYMV VPg copurifies

with wild-type MIF4G but not MIF4G* in vitro.

To test for a physical interaction between RYMV VPg and rice eIF(iso)4G1, we assessed the ability of viral VPg to bind the MIF4G domain in an in vitro assay using the recombinant proteins described above. In contrast to MIF4G, production of recombinant RYMV VPg leads to the formation of inclusion bodies (Hébrard et al. 2009). Only a small fraction of the soluble VPg was recovered from the supernatant of a native bacterial protein extract. Moreover, due to its intrinsic disorder tendency, VPg migrated at an apparent mass of 15 and 17 kDa by SDS-PAGE and size exclusion chromatography, respectively, instead of the expected molecular mass of 10.5 kDa (Hébrard et al. 2009). To overcome the technical limitation of poor VPg solubility, a copurification experiment was performed. A significant increase in VPg solubility was observed in the presence of MIF4G (data not shown). Following purification by Ni²⁺ affinity chromatography, the two proteins eluted in the same fraction during size exclusion chromatography (Fig. 3A). This result demonstrates that MIF4G and VPg copurified in an in vitro complex, suggesting a direct binary association of these proteins. It also indicates that the central domain of rice eIF(iso)4G1 is sufficient for copurification.

We next performed a copurification experiment of VPg with *rymv1-2*-mutated MIF4G*. The presence of MIF4G* did not increase VPg solubility but, instead, impaired the ability of the two proteins to coelute in the same fraction, yielding complete abolition of the in vitro interaction between the two proteins (Fig. 3B). VPg and MIF4G* eluted in two distinct fractions. The elution volumes corresponded to those observed separately in single purification experiments. This result indicates that the point mutation E309K in MIF4G*, mimicking the *rymv1-2* resistance allele, is sufficient to abolish the in vitro interaction with wild-type RYMV VPg.

MIF4G but not MIF4G* strongly interacts with RYMV VPg in a yeast two-hybrid system.

The direct interaction between RYMV VPg and MIF4G was further evaluated in vivo in a GAL4 yeast two-hybrid system. VPg was constitutively expressed in yeast as a GAL4 DNA-binding domain fusion protein from the ADH promoter of the

Fig. 2. The MIF4G central domain of rice eIF(iso)4G1 adopts an α -helical conformation. **A**, Recombinant His-tagged rice MIF4G was purified by affinity chromatography. Purified His-tagged rice MIF4G was eluted in one fraction from a Superdex 75 HR10/30 column (GE Healthcare) in 50 mM Tris-HCl, pH 8, and 300 mM NaCl, at a flow rate of 0.5 ml/min (F1) (inset: 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis). **B**, The far-UV circular dichroism spectra show characteristic maxima at 192 nm and minima at 209 and 222 nm, confirming the presence of an α -helical conformation. Site-directed mutagenesis was used to introduce an E309K point mutation, mimicking the *rymv1-2* resistance allele, into the His-tagged MIF4G construct. The spectra of rice MIF4G and MIF4G* are similar. The E309K mutation induced no conformational changes in the MIF4G domain.

pGBKT7 vector. MIF4G was similarly expressed as a GAL4 activation domain fusion protein from the pGADT7 plasmid. Yeast strain AH109 was cotransformed with the two plasmids and further spotted as serial dilutions on two different media. The control medium was supplemented with all amino acids and bases to validate uniform growth after transformation. A medium lacking histidine and adenine was used to select yeast cells in which *in vivo* interactions between selected protein combinations occurred. Figure 4 indicates that cotransformation with VPg- and MIF4G-containing vectors allowed AH109 to grow on the restricted medium lacking adenine and histidine, in contrast to the negative controls (each partner cotransformed with the corresponding empty vector). The interaction was also detected by analyzing LacZ reporter gene transactivation. This result indicates a direct interaction between VPg and MIF4G.

The *rymv1-2* resistance mutation E309K was introduced into pGADT7::MIF4G to precisely assess its impact on the *in*

vivo interaction in the yeast two-hybrid system. The interaction of MIF4G* with wild-type VPg was strongly reduced compared with that of wild-type MIF4G (Fig. 4), as revealed by the decrease of both the growth in the absence of auxotrophic markers and galactosidase activity. In contrast to the *in vitro* copurification assay, a residual interaction was detected in yeast. The intensity of yeast growth at an optical density at 600 nm (OD₆₀₀) of 5×10^{-2} was assessed from ten independent experiments using ImageJ software, since galactosidase activity was found to be poorly recordable and reliable for weak interactions. After normalization, the interaction between MIF4G* and wild-type VPg was estimated at approximately 15% of the level of the wild-type MIF4G-VPg interaction (Fig. 4). This experiment indicated that the yeast two-hybrid system is a sensitive method that allowed us to detect very faint interactions that could not be revealed by copurification experiments.

Fig. 3. The viral genome-linked protein (VPg) interacts *in vitro* with MIF4G but not with *rymv1-2*-mutated MIF4G* in a copurification assay. Both MIF4G or MIF4G* and VPg recombinant proteins were loaded and copurified from a Superdex 75 HR10/30 column with an elution buffer containing 50 mM Tris-HCl, pH 8, and 300 mM NaCl, at a flow rate of 0.5 ml/min. **A**, With wild-type MIF4G, the elution profile shows the presence of the two proteins in one unique fraction, F1 (inset: 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]). **B**, With MIF4G*, the elution profile shows the presence of MIF4G* and VPg in distinct fractions, F1 and F2 (inset: 15% SDS-PAGE).

Fig. 4. Wild-type MIF4G and *rymv1-2*-mutated MIF4G* interact differently with the viral genome-linked protein (VPg) in a yeast two-hybrid assay. Site-directed mutagenesis was used to introduce an E309K point mutation, mimicking the *rymv1-2* resistance allele, into the pGAD::MIF4G construct. The yeast strain AH109 was cotransformed with either pGAD::MIF4G or pGAD::MIF4G* and pGBK::VPg constructs or with a set of negative controls (empty pGAD/empty pGBK, empty pGAD/pGBK::VPg and pGAD::MIF4G/empty pGBK). Cells were plated as serial dilutions (optical density at 600 nm of 5×10^{-2} , 5×10^{-3} and 5×10^{-4}) on a control plate with (first panel) or without (second panel) the histidine amino acid and the adenine base. Yeast cells were allowed to grow at 30°C for 4 days. Neither the pGAD::MIF4G/empty pGBK nor the pGBK::VPg/empty pGAD pairs of constructs allowed yeast growth on selective media without histidine and adenine, indicating the absence of autonomous activation of the reporter genes ADE2 and HIS3 by MIF4G or VPg. A direct interaction between VPg and MIF4G was demonstrated as indicated by the ability of the cotransformed yeast cells to grow in the absence of histidine and adenine. By contrast, the VPg interaction was strongly reduced by the E309K substitution. Interactions were also recorded by their ability to transactivate the LacZ reporter gene (third panel). The direct interaction between VPg and MIF4G is visualized as a strong staining due to LacZ transactivation. The VPg/MIF4G interaction is reduced by the E309K substitution in MIF4G as revealed by a light staining. No galactosidase activity is detected in cells cotransformed by negative control constructs. Because precise LacZ activity was hardly measurable due to the weakness of some interactions, comparisons of yeast growth were estimated as percentages after quantifying spot intensities presented in the second panel using ImageJ (fourth panel), with 100% control efficiency being assigned to the susceptible MIF4G/VPg binary interaction. a, b, and c, represent groups significantly different after multiple mean comparison (analysis of variance, $P = 0.05$). Results were highly reproducible and are the means of ten independent experiments.

Restoration of the interaction with MIF4G* by virulence mutations in RYMV VPg.

Similar to the previous experiment, the impact of virulence mutations in VPg on its interaction with MIF4G* was assessed using the yeast two-hybrid assay. For this purpose, virulence mutations were introduced into the pGBKT7::VPg plasmid, creating VPg*. The arginine at position 48 (R48) of wild-type VPg was substituted for either glutamic acid (E), as in the main mutational pathway, or isoleucine (I), as in the isolate-specific mutational pathway. The glycine (G) involved in the first step of the major pathway was also introduced to test its impact on the ability of VPg to interact with MIF4G* in the S2/S3 strain context. The interaction with MIF4G* was restored when the virulence mutations were introduced in VPg (Fig. 5). The virulence mutation R48E restored the interaction with MIF4G* to approximately 80% of the level of the wild-type VPg R48-MIF4G interaction (Fig. 5). The virulence mutation R48I was less efficient, resulting in an interaction of 20% of the level of the wild-type interaction, which was not significantly different from the residual interaction with wild-type VPg. The virulence mutation R48G, which served to reproduce the S2/S3 strain context when inserted into the pGBKT7::VPg plasmid, induced an intermediate level of restoration of the interaction with mutated MIF4G* (40% of the level of the wild-type interaction). As a control, the interaction between VPg* and wild-type MIF4G was assessed. Each mutated VPg* interacted similarly with wild-type MIF4G (Supplementary Fig. 1).

In parallel with the above dot assays, yeast growth was monitored to precisely measure the efficiency and kinetics of each partnership (Fig. 6). For this purpose, cotransformed yeast cells were cultured both in a control medium containing all required amino acids and in a medium without adenine, in order to select for yeast two-hybrid interactions. While all double transformants grew similarly in control media, yeast cells producing either wild-type MIF4G/VPg or mutated

MIF4G*/VPg*E grew significantly faster than the other cells. Yeast cells producing mutated MIF4G*/VPg*G showed a delay of approximately 10 h in reaching exponential growth, while cells with mutated MIF4G* and VPg*I or wild-type VPg reached exponential growth 30 h later than cells with optimal growth.

DISCUSSION

In this study, in vitro copurification and in vivo yeast two-hybrid experiments demonstrated that i) VPg of RYMV and the central domain of rice eIF(iso)4G1 interact directly, ii) the *rymv1-2* resistance mutation of MIF4G* strongly diminishes the wild-type VPg interaction, and iii) the VPg virulence mutations restore the MIF4G* interaction with different efficiencies. We provide the first molecular explanation of *rymv1-2*-mediated resistance and RYMV virulence phenotypes, and our results validate our hypothesis of a direct interaction. We propose that, in a compatible interaction, the virus is able to interact with the central domain of eIF(iso)4G1 and complete its infectious cycle. The *rymv1-2* mutation, located in the MIF4G* domain of the eIF(iso)4G1 protein in resistant rice cultivars, strongly decreases the interaction with RYMV VPg, inducing an incompatible interaction and a phenotype of high resistance. However, wild-type VPg can still induce a residual interaction with mutated MIF4G*. We have proposed that this interaction between wild-type VPg and mutated MIF4G*, although limited, is sufficient to permit the residual multiplication of avirulent isolates in resistant plants, which was detected by qRT-PCR (Poulicard et al. 2010). Therefore, this limited interaction could be the source of virulence mutations in resistant plants.

Residue-residue contact index preferences, derived from a set of protein-protein interfaces of known structure, have been used to compare predicted affinities along the main mutational pathways to virulence (Hébrard et al. 2008). The highest affinities were predicted to be between E309 of wild-type eIF(iso)

Fig. 5. Point mutations at residue R48, mimicking virulent viral genome-linked proteins (VPg), restore in vivo interactions between VPg and *rymv1-2*-mutated MIF4G*. Site-directed mutagenesis was used to introduce R48I, R48G, and R48E point mutations into the pGBKT7::VPg construct. These constructs were used to transform the AH109 yeast strain together with the pGAD::MIF4G* construct. The positive control is pGAD::MIF4G/pGBKT7::VPg. The negative control is empty pGAD/empty pGBKT7. The interaction strength between VPg and MIF4G* was successively reinforced by virulence mutations in VPg (VPg*I to VPg*E), as visualized by dot assays (middle panels) and growth percentage comparisons (fourth panel). While the MIF4G*/VPg interaction strength was only 10% that of the control interaction, the highest interaction score was obtained for the virulence mutation R48E, which restored the interaction with MIF4G* to approximately 80% of the wild-type efficiency. Inversely, the lowest interaction score was obtained with the virulence mutation R48I (MIF4G*/VPg*I), which interacted at only 20% of the strength of the MIF4G/VPg control interaction. The virulence mutation R48G induced an intermediate interaction strength of 40% of the control interaction. Interactions are also recorded by their ability to transactivate the LacZ reporter gene (third panel) and by ImageJ scanning (fourth panel), comparisons of yeast growth were estimated as percentages after quantifying spot intensities presented in the second panel using ImageJ (fourth panel), with 100% control efficiency being assigned to the susceptible MIF4G/VPg binary interaction. a, b, and c, represent groups significantly different after multiple mean comparison (analysis of variance, $P = 0.05$). Results represent the means of ten independent experiments.

4G1 and R48 of wild-type VPg and between K309 of *rymv1-2*-mutated eIF(iso)4G1 and E48 of virulent VPg. Moreover, only the virulent isolate with the R48E mutation has been shown to fully restore optimal virus multiplication in resistant plants (Poulicard et al. 2010). In this study, we demonstrated that the virulent mutation 48E in VPg can restore the optimal interaction with *rymv1-2*-mutated MIF4G* with a higher efficiency than other virulence mutations (48I and 48G). These results explain the contrasted prevalences of virulence mutations observed in experimental evolution and the stepwise fitness optimization process of the main mutational pathway (R/G/E). Furthermore, the major role of residue 48 in VPg in the interaction specificity with MIF4G* might explain the parallel evolution observed in RYMV strains.

The weak ability of the widely spread West African S2/S3 strain to overcome *rymv1-2*-mediated resistance is apparent in experimental evolution studies (Pinel-Galzi et al. 2007). Moreover, artificial insertion of the main virulence mutations 48I, 48G, and 48E in an S2/S3 isolate induced contrasted impacts on variant fitness (Poulicard et al. 2010). The virulence mutation 48E induced the same optimal multiplication in resistant plants in both S1 and S2/S3 strain contexts. This was not the case for the virulence mutation 48I, which showed a multiplication rate that was approximately 10^5 times lower when introduced into an S2/S3 than when introduced into an S1 context. In our two-hybrid experiments, the virulence mutation 48I resulted in a weaker restoration of the mutated VPg*/MIF4G* interaction than did 48E. Finally, the virulence mutation 48G was lethal when introduced into the S2/S3 infectious clone. However, we showed an intermediate ability of the VPg 48G two-hybrid construct to restore the interaction with MIF4G*. No antagonistic epistasis was observed in our conditions. This result indicates that the failure of the S2/S3 strain to use the main mutational pathway is not due to an inability of VPg*G to interact with MIF4G*. In the S2/S3 strain context, the 48G mutation may induce a blockage of another function of VPg or another step of the viral cycle or both. Thus, the impact of strain context and genetic constraints on interaction efficiency should be investigated further. Yeast two-hybrid assays are currently being performed to assess the influence of residue 49 under diversifying selection on this interaction. Other resistance alleles have been described that harbor point mutations or short deletions in the central hairpin of MIF4G (Albar et al.

2006). The virulence mutations of these alleles have not yet been identified. The assessment of these resistance and virulence mutations would provide deeper knowledge of the role of each amino acid in the interaction domain.

In this study, we provided the first demonstration for a direct interaction between plant MIF4G and a phyto-viral VPg. In order to determine if this strategy is specific to RYMV, the MIF4G interaction ability of VPg from other viruses should be investigated. A point mutation downstream from the MIF4G domain of the eIF4G gene has been associated to the loss of susceptibility of *Arabidopsis thaliana* mutant *cum2* to *Turnip crinkle virus* (genus *Carmovirus*, family *Tombusviridae*) and *Cucumber mosaic virus* (genus *Cucumovirus*, family *Bromoviridae*) (Yoshii et al. 2004). However, without VPg genes, these viruses must use another strategy to interact with eIF4G. Recently, single nucleotide polymorphisms upstream of the MIF4G domain of the rice eIF4G gene have been associated to the recessive resistance *Rts1* to *Rice tungro spherical virus* (RTSV; genus *Waikavirus*, family *Sequiviridae*) (Lee et al. 2010). Although VPg is detected at the 5' extremity of the *Sequiviridae* genome, the VPg domain has not been defined yet. Further studies on *Rts1*-mediated resistance should describe the relationships between RTSV VPg and the eIF4G gene. The involvement of eIF4G factors was rarely reported for potyvirus. The incorporation of eIF4G has been shown to strengthen in vitro interaction between eIF4E and VPg of *Turnip mosaic virus* (TuMV), *Lettuce mosaic virus* (LMV), and *Potyvirus Y* (Grzela et al. 2006; Michon et al. 2006; Miyoshi et al. 2006). Furthermore, *Arabidopsis thaliana* knock-out mutants for eIF4G and eIF(iso)4G genes lost their susceptibility to LMV, TuMV, *Plum pox virus*, and *Clover yellow vein virus* (Nicaise et al. 2007). However, the direct or indirect role of eIF4G during interactions with VPg remains to be determined. We propose that for members of family *Potyviridae*, the coordinated recruitment of eIF4E/eIF4G or eIF(iso)4E/eIF(iso)4G is an indirect consequence of the direct involvement of the factors 4E and of their interaction specificity with the factors 4G rather than a direct interaction between VPg and factors 4G. Notably, eIF4G is indirectly recruited by *Feline calicivirus* VPg via interactions with eIF4E (Chaudhry et al. 2006). However, different strategies for eIF recruitments coexist in the same viral family, as demonstrated by the finding that the *Murine norovirus* VPg recruits eIF4G via eIF3 (Chaudhry et al.

Fig. 6. Time course of cotransformed yeast growth in control or selective liquid medium. The growth rates of cotransformed cells were monitored by optical density at 600 nm measurements of liquid cultures either containing all required amino acids for proper growth in the absence of an interaction (left panel) or lacking adenine to select for an interaction (right panel). While all cotransformed cells grew similarly in the control medium, cells producing susceptible MIF4G/avirulent VPg (viral genome-linked protein) and resistant MIF4G*/virulent VPg*E partner proteins grew faster than the other cells, and cells producing resistant MIF4G*/virulent VPg*G showed a 10-h delay in reaching exponential growth. Cells carrying resistant MIF4G* and virulent VPg*I or avirulent VPg exhibited a 30-h delay in reaching the optimal growth. Values represent the means of three individual cells per pair of partners and from two independent transformation events.

2006). Despite their common structural feature of being intrinsically flexible, RYMV VPg does not show sequence homology with caliciviral or potyviral VPg (Hébrard et al. 2009). Its direct interaction with eIF4G could be another strategy to recruit the translation initiation complex. Although the fact that family *Potyviridae* interacts with eIF4E factors is well established, the functional relevance of this interaction in translation is still unclear. The role of internal ribosome entry sites in the 5' untranslated regions of the potyviral genomes in supporting translation initiation raised the possibility that VPg has redundant functions (Carrington and Freed 1990; Levis and Astier-Manificier 1993). Other functions in RNA replication and viral movement have been previously suggested (Robaglia and Caranta 2006). Even though the function of eIF recruitment has not yet been determined for family *Potyviridae*, the interaction of VPg with MIF4G could play a similar role in RYMV.

MATERIALS AND METHODS

Construction of recombinant proteins.

The complete coding sequence of eIF(iso)4G1, obtained from the susceptible *Oryza sativa indica* cultivar IR64, was available in a pGEMT plasmid (Albar et al. 2006). The MIF4G-encoding region was amplified by PCR from the pGEMT::eIF(iso)4G1 construct using the *Bam*HI-containing primers F16 5'-GGATCCTTGGTCAGCTAGAAGAGGCA-3' (including MIF4G nucleotides 619 to 637) and R15 5'-GGATCCTTAATCTATTCACGTCGAGGCA-3' (including a sequence complementary to MIF4G nucleotides 1,350 to 1,371) and subcloned into the pCR8/GW/TOPO TA cloning vector (Invitrogen, Thermo Fischer Scientific, Illkirch, France). The *Bam*HI fragment, obtained by digestion of the resulting pCR8/GW/TOPO TA::MIF4G construct, was subcloned into a pET28 vector at the corresponding *Bam*HI sites as a 6-His N-terminal fusion peptide (Novagen, Merck Chemicals LTD, Beeston, U.K.), giving rise to a pET28::MIF4G construct. Construction of the bacterial expression plasmid pQE60::VPg, corresponding to the RYMV VPg of the isolate ClA (strain S2/S3) with a 6-His C-terminal fusion, was previously described (Hébrard et al. 2009).

To build the yeast two-hybrid plasmids, two subclones, pGEMT::MIF4G and pGEMT::VPg, were constructed to introduce restriction enzyme sites. The MIF4G-encoding region was amplified by PCR using the primers F4G4*Nde*I, 5'-CGCATATGCCTTGGTCAGCTAGAAGAGG-3', and R4G4*Bam*HI, 5'-CGGGATCCATTATTCACGTCGAGGC-3', while the VPg-encoding region was amplified using the primers FVPg*Nde*I, 5'-CGCATATGTCTCCATTTGAGATTTACGGC-3', and RVPg-*Bam*HI, 5'-CGGGATCCATTACTCGATATCAACATCC-3'. After digestion, the resulting fragments were cloned into *Nde*I/*Bam*HI-digested pGADT7 and *Nco*I/*Bam*HI-digested pGBKT7, respectively, giving rise to the pGADT7::MIF4G and pGBKT7::VPg constructs.

The pET28::MIF4G*, pGADT7::MIF4G*, and pGBKT7::VPg* (with I, G, and E substitutions, respectively) vectors carrying MIF4G and VPg variants were obtained by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, Agilent Technologies, Waghäusel-Wiesental, Germany). All primers used in this study are listed in Supplementary Table 1. All constructs were systematically confirmed by sequencing.

Single- and copurification of recombinant proteins.

The expression constructs pET28::MIF4G and pET28::MIF4G* were used to separately transform *E. coli* BL21 (DE3) (Novagen). After induction with 0.2 mM isopropyl-1-thio- β -D-galactopyranoside at 37°C for 4 h, cells from 700-ml cultures

in Luria Bertani medium were harvested by centrifugation and were frozen at -80°C. Cells were thawed and resuspended in 30 ml of purification buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5% glycerol), and then, disrupted with a French press (Thermo Fischer Scientific) and centrifuged at 18,000 rpm for 30 min. The supernatant was filtered (0.45- μ m filters), and purification of MIF4G and MIF4G* under native conditions was carried out using a nickel-loaded HisTrap IMAC HP column (GE Healthcare) followed by gel filtration onto a HR10/300GL Superdex 75 column (GE Healthcare, Amersham, Orsay, France) in 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 5% glycerol.

The expression construct pQE60::VPg was used to transform *E. coli* M15-pRep4 (Qiagen, Courtaboeuf, France) and to produce the recombinant protein at 25°C, as described previously (Hébrard et al. 2009). For copurification experiments, *E. coli* cultures expressing either MIF4G or VPg were grown independently, and the cell pellets were later mixed together before purification, using the protocol mentioned above.

Far-UV CD.

Freshly purified protein samples were used for CD analyses. The sample buffer was changed by eluting the protein from a PD10 desalting column (GE Healthcare), using 10 mM sodium phosphate buffer (pH 8.0) supplemented with 150 mM NaF. After centrifugation, the protein concentration was determined using an ND-1000 spectrophotometer (Thermo Fischer Scientific) and an extinction coefficient of 14,000 M⁻¹ cm⁻¹ for MIF4G and MIF4G*. Far-UV CD spectra were recorded with a Chirascan dichrograph (Applied Photophysics, Surrey, U.K.) in a thermostated (20°C) quartz circular cell with a 0.2-mm path length, in steps of 0.5 nm. All protein spectra were corrected by subtracting the respective buffer spectra. The mean molar ellipticity values per residue were calculated using software provided by the manufacturer.

Yeast two-hybrid test.

The Matchmaker GAL4 two-hybrid system 3 (Clontech Laboratories, Takara Bio Europe, Saint Germain-en-Laye, France) was used according to the manufacturer's protocols. pGADT7::MIF4G and pGBKT7::VPg constructs were introduced into the AH109 yeast strain (Clontech Laboratories, Inc.). Double-transformed cells were grown on minimal YNB medium (0.7% yeast extract without amino acids, 2% glucose, 2% agar) with all required amino acids including (control) or omitting (Y2H assay) histidine and adenine. Between three and ten clones from four independent transformations were used for the yeast two-hybrid assays. Cells expressing interacting proteins were selected on media lacking leucine and tryptophan (SD-LW) or leucine, tryptophan, histidine, and adenine (SD-LWHA). Cells were grown to stationary phase and were adjusted to an OD₆₀₀ of 5.10⁻², 5.10⁻³, or 5.10⁻⁴ before spotting onto the appropriate plates. Plates were incubated at 30°C, and growth was checked 3 to 5 days after spotting. Empty pGADT7 and pGBKT7 vectors were used as negative controls.

Growth intensities were monitored with ImageJ software (Abramoff et al. 2004), and row data were normalized to positive and negative control and expressed as a percentage of the 'initial' interaction between wild-type MIF4G and avirulent VPg. Data were analyzed by analysis of variance (Statistica software version 6.0; Statsoft, Tulsa, OK, U.S.A.).

The differences in interaction intensities between virulent VPg cotransformants were estimated in an independent experiment. The yeast growth of each cotransformant was kinetically followed as the OD₆₀₀ at 30°C in 10 ml of liquid media for 4 days. Two independent experiments were performed with three clones of each construct.

For LacZ assays, transformed AH109 cells (7 µl) were plated at OD₆₀₀ = 2 on a 3MM Whatman paper, were frozen in liquid nitrogen for 10 s, and were incubated onto a layer paper imbibed with Z buffer containing B-mercaptoethanol and X-Gal, as described by AH109 furnisher (Clontech Laboratories, Inc.).

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