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1 **Direct interaction between the *Rice yellow mottle virus* VPg and the central domain of**
2 **the rice eIF(iso)4G1 factor correlates with rice susceptibility and RYMV virulence**

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4 Eugénie Hébrard¹, Nils Poulicard¹, Clément Gérard², Oumar Traoré³, Hui-Chen Wu^{4,5},
5 Laurence Albar⁴, Denis Fargette¹, Yannick Bessin², Florence Vignols⁴

6
7 ¹UMR186 Résistance des Plantes aux Bio-agresseurs, Institut de Recherche pour le
8 Développement BP 64501, 34394 Montpellier cedex 5 (France).

9 ²UMR5048 Centre de Biochimie Structurale, 29 rue de Navacelles, 34090 Montpellier
10 (France)

11 ³Institut de l'Environnement et de Recherches Agricoles (INERA), 01 BP 476, Ouagadougou
12 (Burkina Faso)

13 ⁴UMR5096 Génome et Développement des Plantes, Université de Perpignan via Domitia –
14 CNRS – IRD, BP 64501, 34394 Montpellier cedex 5 (France)

15 ⁵Department of Life Science and Institute of Plant Biology, National Taiwan University,
16 Taipei 10617, Taiwan

17
18 Corresponding author: Eugénie Hébrard, e-mail: eugenie.hebrard@ird.fr

19
20 **ABSTRACT** 199 words

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

37
38 **INTRODUCTION**

39 Plant viruses are important agricultural pathogens worldwide. The use of resistant
40 varieties is the main method of viral disease control. Approximately half of all virus-resistant
41 genes are recessively inherited, whereas resistant genes against other plant pathogens are
42 largely dominant (Kang et al., 2005b). These recessive genes encode plant factors recruited by
43 viral proteins, and resistance mutations in these factors prevent the viral cycle. To date, all
44 cloned recessive resistance genes in crop species encode translation initiation factors
45 (Truniger & Aranda, 2009). Most are resistance genes against *Potyviridae* and encode eIF4E
46 factors. These small host proteins bind the cap structure of mRNA and form part of a
47 multimeric complex that promotes translation initiation (Browning, 2004). The virulence
48 genes have been largely identified as Viral Proteins genome-linked (VPgs). These viral
49 proteins were detected at the 5' end of the viral genomes of several families of viruses
50 (Sadowy et al., 2001). Direct interactions between eIF4Es and VPgs have been previously

1 demonstrated (Charron et al., 2008, Hwang et al., 2009, Kang et al., 2005a, Leonard et al.,
2 2000, Schaad et al., 2000, Wittmann et al., 1997). The resistant phenotypes result from
3 disruptions of the interactions between eIF4Es and VPgs (Charron et al., 2008, Kang et al.,
4 2005a, Yeam et al., 2007). However, recessive resistance can sometimes be overcome by the
5 emergence of virulent variants (Ayme et al., 2007, Moury et al., 2004). Some virulence
6 mutations in VPgs restore the interactions with proteins encoded by resistance alleles
7 (Charron et al., 2008).

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

15 Outside the *Potyviridae* family, another biological model has been used to understand
16 the adaptation of viral populations to recessive resistances. *Rice yellow mottle virus* (RYMV)
17 belongs to the unassigned *Sobemovirus* genus. RYMV adaptation to *rymv1-2*-mediated
18 resistance has been previously described. Only two cultivars of the Asiatic rice *Oryza sativa*
19 show a high resistance to RYMV, as characterized by the absence of symptoms and no effect
20 on rice yield (Ndjiondjop et al., 1999, Rakotomalala et al., 2008). The resistance durability of
21 these cultivars has been assessed by experimental evolution, and 15–20% of the isolates were
22 able to overcome this resistance (Fargette et al., 2002, Hébrard et al., 2006, Pinel-Galzi et al.,
23 2007). The virulence factor of RYMV was identified as a VPg (Hébrard et al., 2006, Pinel-
24 Galzi et al., 2007). Parallel evolution of most RYMV strains results in fixation of the same
25 VPg mutations, predominantly at codon 48 (Pinel-Galzi et al., 2007). The virulent variants
26 have been suggested to emerge from a residual multiplication of wild-type isolates in resistant
27 plants, which was estimated by qRT-PCR to be approximately 10^6 viral RNA copies/mg of
28 leaves (Poulicard et al., 2010). The origin of this residual multiplication has not yet been
29 identified. A stepwise optimization of virulent variant fitness by a competition/exclusion
30 phenomenon has been observed (Pinel-Galzi et al., 2007). The conserved site at codon 48,
31 corresponding to an arginine (R) in avirulent isolates, was substituted for different amino
32 acids in virulent isolates. The virulence mutations occurred with contrasted frequencies, and
33 several mutational pathways were followed. In the most prevalent mutational pathway, R48
34 was displaced by glycine (G) in the first step to become fixed at glutamic acid (E) in the
35 second step. By contrast, the virulence mutation R48I (isoleucine) has been identified as an
36 isolate-specific mutational pathway (Hébrard et al., 2006, Pinel-Galzi et al., 2007). The
37 virulent isolates with the 48E mutation restored the optimal multiplication level of 10^{12} viral
38 RNA copies/mg of leaves in resistant plants, whereas the 48I and 48G mutations induced an
39 intermediate level of accumulation (Poulicard et al., 2010). Increasing affinities between
40 resistance and virulence factors have been proposed to explain the stepwise optimization of
41 virulent variants, but this mechanism remains to be demonstrated (Hébrard et al., 2008).

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

1 diversifying selection, has been previously suggested to be involved in antagonistic epistasis
2 with the codon 48 (Pinel-Galzi et al., 2007).

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

22 23 **RESULTS**

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

25 The rice resistance gene *RYMV1* has been identified as the eukaryotic translation
26 initiation factor eIF(iso)4G1. Compared to the well-known human eIF4Gs (Lamphear et al.,
27 1995, Marintchev & Wagner, 2005), rice eIF(iso)4G1 is shortened at its two extremities,
28 similar to other plant eIF(iso)4Gs (Figure 1A). The N-terminus of rice eIF(iso)4G1 comprises
29 a conserved eIF4E-interacting domain, a Tyr-X₄-Leu-Leu motif (Mader et al., 1995) and a
30 PolyA binding protein (PABP)-interacting domain, which has not been precisely mapped. The
31 amino acid sequence of the central region of rice eIF(iso)4G1 showed an identity score of
32 37% with the central domain (named the MIF4G domain) of human eIF4GII, which binds to
33 eIF4A and eIF3. The C-terminus of rice eIF(iso)4G1 showed an identity score of 23% with
34 the human MA3 domain, which contains a second eIF4A-binding site.

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

48 For this purpose, the central region comprising amino acids 206–454 of rice
49 eIF(iso)4G1 was produced as a His-tagged fusion peptide in *E. coli*. The recombinant protein
50 was highly soluble under native conditions and migrated with an apparent and expected

1 molecular mass of 32 kDa in a 15% SDS-PAGE gel. MIF4G was purified by affinity
2 chromatography in non-denaturing conditions and eluted in one fraction at 11.3 ml by size
3 exclusion chromatography (Figure 2A). In parallel, MIF4G* was produced by inserting the
4 E309K resistance mutation using site-directed mutagenesis. Its expression in *E. coli* led to the
5 production of a recombinant protein exhibiting solubility, electrophoretic properties and
6 purification behavior similar to that of wild-type MIF4G (data not shown). Far-UV circular
7 dichroism (CD) analysis of the purified MIF4G gave a UV spectrum with a maximum at 192
8 nm and minima at 209 and 222 nm, consistent with the presence of an α -helical conformation
9 (Figure 2B). CD spectra deconvolution carried out with the K2D program (Merelo et al.,
10 1994) indicated a total α -helix content of 37%. These data agreed with the previously
11 published 3D model of rice MIF4G (Albar et al., 2006). The central domain of rice
12 eIF(iso)4G1 forms a putative HEAT-repeat domain. A comparison of wild-type and mutated
13 MIF4Gs by far-UV circular dichroism indicated that the E309K substitution did not induce
14 detectable modifications of the MIF4G structure.

15 16 **RYMV VPg co-purifies with wild-type MIF4G but not MIF4G* *in vitro***

17 To test for a physical interaction between RYMV VPg and rice eIF(iso)4G1, we
18 assessed the ability of viral VPg to bind the MIF4G domain in an *in vitro* assay using the
19 recombinant proteins described above. In contrast to MIF4G, production of recombinant
20 RYMV VPg leads to the formation of inclusion bodies (Hébrard et al., 2009). Only a small
21 fraction of the soluble VPg was recovered from the supernatant of a native bacterial protein
22 extract. Moreover, due to its intrinsic disorder tendency, VPg migrated at an apparent mass of
23 15 and 17 kDa by SDS-PAGE and size exclusion chromatography, respectively, instead of the
24 expected molecular mass of 10.5 kDa (Hébrard et al., 2009). To overcome the technical
25 limitation of poor VPg solubility, a co-purification experiment was performed. A significant
26 increase in VPg solubility was observed in the presence of MIF4G (data not shown).
27 Following purification by Ni²⁺ affinity chromatography, the two proteins eluted in the same
28 fraction during size exclusion chromatography (Figure 3A). This result demonstrates that
29 MIF4G and VPg co-purified in an *in vitro* complex, suggesting a direct binary association of
30 these proteins. It also indicates that the central domain of rice eIF(iso)4G1 is sufficient for co-
31 purification.

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

40 41 **MIF4G but not MIF4G* strongly interacts with RYMV VPg in a yeast two-hybrid 42 system**

43 The direct interaction between RYMV VPg and MIF4G was further evaluated *in vivo*
44 in a GAL4 yeast two-hybrid system. VPg was constitutively expressed in yeast as a GAL4
45 DNA-binding domain (DNA-BD) fusion protein from the ADH promoter of the pGBKT7
46 vector. MIF4G was similarly expressed as a GAL4 activation domain (AD) fusion protein
47 from the pGADT7 plasmid. The yeast strain AH109 was co-transformed with the two
48 plasmids and further spotted as serial dilutions on two different media. The control medium
49 was supplemented with all amino acids and bases to validate uniform growth after
50 transformation. A medium lacking histidine and adenine was used to select yeast cells in

1 which *in vivo* interactions between selected protein combinations occurred. Figure 4 indicates
2 that co-transformation with VPg- and MIF4G-containing vectors allowed AH109 to grow on
3 the restricted medium lacking adenine and histidine, in contrast to the negative controls (each
4 partner co-transformed with the corresponding empty vector). The interaction was also
5 detected by analyzing LacZ reporter gene transactivation. This result indicates a direct
6 interaction between VPg and MIF4G.

7 The *rymv1-2* resistance mutation E309K was introduced into pGADT7::MIF4G to
8 precisely assess its impact on the *in vivo* interaction in the yeast two-hybrid system. The
9 interaction of MIF4G* with wild-type VPg was strongly reduced compared to that of wild-
10 type MIF4G (Figure 4), as revealed by the decrease of both the growth in the absence of
11 auxotrophic markers and galactosidase activity. In contrast to the *in vitro* co-purification
12 assay, a residual interaction was detected in yeast. The intensity of yeast growth at an OD₆₀₀
13 of 5×10^{-2} was assessed from 10 independent experiments using ImageJ software, since
14 galactosidase activity was found to be poorly recordable and reliable for weak interactions.
15 After normalization, the interaction between MIF4G* and wild-type VPg was estimated at
16 approximately 15% of the level of the wild-type MIF4G/VPg interaction (Figure 4). This
17 experiment indicated that the yeast two-hybrid system is a sensitive method that allowed us to
18 detect very faint interactions that could not be revealed by co-purification experiments.

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

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

37 In parallel with the above dot assays, yeast growth was monitored to precisely measure
38 the efficiency and kinetics of each partnership (Figure 6). For this purpose, co-transformed
39 yeast cells were cultured both in a control medium containing all required amino acids and in
40 a medium without adenine, in order to select for yeast two-hybrid interactions. While all
41 double transformants grew similarly in control media, yeast cells producing either wild-type
42 MIF4G/VPg or mutated MIF4G*/VPg*E grew significantly faster than the other cells. Yeast
43 cells producing mutated MIF4G*/VPg*G showed a delay of approximately 10 h in reaching
44 exponential growth, while cells with mutated MIF4G* and VPg*I or wild-type VPg reached
45 exponential growth 30 h later than cells with optimal growth.

46 **DISCUSSION**

47 In this study, *in vitro* co-purification and *in vivo* yeast two-hybrid experiments
48 demonstrated that (1) VPg of RYMV and the central domain of rice eIF(iso)4G1 interact
49
50

1 directly, (2) the *rymv1-2* resistance mutation of MIF4G* strongly diminishes the wild-type
2 VPg interaction, and (3) the VPg virulence mutations restore the MIF4G* interaction with
3 different efficiencies. We provide the first molecular explanation of *rymv1-2*-mediated
4 resistance and RYMV virulence phenotypes, and our results validate our hypothesis of a
5 direct interaction. We propose that, in a compatible interaction, the virus is able to interact
6 with the central domain of eIF(iso)4G1 and complete its infectious cycle. The *rymv1-2*
7 mutation, located in the MIF4G* domain of the eIF(iso)4G1 protein in resistant rice cultivars,
8 strongly decreases the interaction with RYMV VPg, inducing an incompatible interaction and
9 a phenotype of high resistance. However, wild-type VPg can still induce a residual interaction
10 with mutated MIF4G*. We have proposed that this interaction between wild-type VPg and
11 mutated MIF4G*, although limited, is sufficient to permit the residual multiplication of
12 avirulent isolates in resistant plants, which was detected by Q-RT-PCR (Poulicard et al.,
13 2010). Therefore, this limited interaction could be the source of virulence mutations in
14 resistant plants.

15 Residue-residue contact index preferences, derived from a set of protein-protein
16 interfaces of known structure, have been used to compare predicted affinities along the main
17 mutational pathways to virulence (Hébrard et al., 2008). The highest affinities were predicted
18 to be between E309 of wild-type eIF(iso)4G1 and R48 of wild-type VPg and between K309
19 of *rymv1-2*-mutated eIF(iso)4G1 and E48 of virulent VPg. Moreover, only the virulent isolate
20 with the R48E mutation has been shown to fully restore optimal virus multiplication in
21 resistant plants (Poulicard et al., 2010). In this study, we demonstrated that the virulent
22 mutation 48E in VPg can restore the optimal interaction with *rymv1-2*-mutated MIF4G* with
23 a higher efficiency than other virulence mutations (48I and 48G). These results explain the
24 contrasted prevalences of virulence mutations observed in experimental evolution and the
25 stepwise fitness optimization process of the main mutational pathway (R/G/E). Furthermore,
26 the major role of residue 48 in VPg in the interaction specificity with MIF4G* might explain
27 the parallel evolution observed in RYMV strains.

28 The weak ability of the widely spread West African S2/S3 strain to overcome *rymv1-*
29 *2*-mediated resistance is apparent in experimental evolution studies (Pinel-Galzi et al., 2007).
30 Moreover, artificial insertion of the main virulence mutations 48I, 48G and 48E in an S2/S3
31 isolate induced contrasted impacts on variant fitness (Poulicard et al., 2010). The virulence
32 mutation 48E induced the same optimal multiplication in resistant plants in both S1 and S2/S3
33 strain contexts. This was not the case for the virulence mutation 48I, which showed a
34 multiplication rate that was approximately 10^5 times lower when introduced into an S2/S3
35 than when introduced into an S1 context. In our two-hybrid experiments, the virulence
36 mutation 48I resulted in a weaker restoration of the mutated VPg*/MIF4G* interaction than
37 did 48E. Finally, the virulence mutation 48G was lethal when introduced into the S2/S3
38 infectious clone. However, we showed an intermediate ability of the VPg 48G two-hybrid
39 construct to restore the interaction with MIF4G*. No antagonistic epistasis was observed in
40 our conditions. This result indicates that the failure of the S2/S3 strain to use the main
41 mutational pathway is not due to an inability of VPg*G to interact with MIF4G*. In the S2/S3
42 strain context, the 48G mutation may induce a blockage of another function of VPg and/or
43 another step of the viral cycle. Thus, the impact of strain context and genetic constraints on
44 interaction efficiency should be investigated further. Yeast two-hybrid assays are currently
45 being performed to assess the influence of residue 49 under diversifying selection on this
46 interaction. Other resistance alleles have been described that harbor point mutations or short
47 deletions in the central hairpin of MIF4G (Albar et al., 2006). The virulence mutations of
48 these alleles have not yet been identified. The assessment of these resistance and virulence
49 mutations would provide deeper knowledge of the role of each amino acid in the interaction
50 domain.

1 In this study, we provided the first demonstration for a direct interaction between plant
2 MIF4G and a phyto-viral VPg. In order to determine if this strategy is specific to RYMV, the
3 MIF4G interaction ability of VPgs from other viruses should be investigated. A point
4 mutation downstream the MIF4G domain of the eIF4G gene has been associated to the loss of
5 susceptibility of *Arabidopsis thaliana* mutant *cum2* to *Turnip crinkle virus* (genus
6 Carmovirus, family *Tombusviridae*) and *Cucumber mosaic virus* (genus *Cucumovirus*, family
7 *Bromoviridae*) (Yoshii et al., 2004). However, without VPg genes, these viruses must use
8 another strategy to interact with eIF4G. Recently, single nucleotide polymorphisms upstream
9 of the MIF4G domain of the rice eIF4G gene have been associated to the recessive resistance
10 *Rts1* to *Rice tungro spherical virus* (RTSV, genus *Waikavirus*, family *Sequiviridae*) (Lee et
11 al., 2010). Although VPg is detected at the 5' extremity of *Sequiviridae* genome, the VPg
12 domain has not been defined yet. Further studies on *Rts1*-mediated resistance should describe
13 the relationships between RTSV VPg and eIF4G gene. The involvement of eIF4Gs was rarely
14 reported for potyvirus. The incorporation of eIF4G has been shown to strengthen *in vitro*
15 interaction between eIF4E and VPg of *Turnip mosaic virus* (TuMV), *Lettuce mosaic virus*
16 (LMV) and *Potyvirus Y* (Grzela et al., 2006, Michon et al., 2006, Miyoshi et al., 2006).
17 Furthermore, *Arabidopsis thaliana* knock-out mutants for eIF4G and eIF(iso)4G genes lost
18 their susceptibility to LMV, TuMV, *Plum pox virus* and *Clover yellow vein virus* (Nicaise et
19 al., 2007). However, the direct or indirect role of eIF4G during interactions with VPgs
20 remains to be determined. We propose that for *Potyviridae*, the coordinated recruitment of
21 eIF4E/eIF4G or eIF(iso)4E/eIF(iso)4G is an indirect consequence of the direct involvement of
22 the factors 4E and of their interaction specificity with the factors 4G rather than a direct
23 interaction between VPg and factors 4G. Notably, eIF4G is indirectly recruited by *Feline*
24 *calicivirus* VPgs via interactions with eIF4E (Chaudhry et al., 2006). However, different
25 strategies for eIF recruitments coexist in the same viral family, as demonstrated by the finding
26 that the *Murine norovirus* VPg recruits eIF4G via eIF3 (Chaudhry et al., 2006). Despite their
27 common structural feature of being intrinsically flexible, RYMV VPg does not show
28 sequence homology with caliciviral or potyviral VPgs (Hébrard et al., 2009). Its direct
29 interaction with eIF4G could be another strategy to recruit the translation initiation complex.
30 Although the fact that *Potyviridae* interacts with eIF4Es is well established, the functional
31 relevance of this interaction in translation is still unclear. The role of internal ribosome entry
32 sites in the 5' untranslated regions of the potyviral genomes in supporting translation
33 initiation raised the possibility that VPg has redundant functions (Carrington & Freed, 1990,
34 Levis & Astier-Manificier, 1993). Other functions in RNA replication and viral movement
35 have been previously suggested (for review, see (Robaglia & Caranta, 2006). Even though the
36 function of eIF recruitment has not yet been determined for *Potyviridae*, the interaction of
37 VPg with MIF4G could play a similar role in RYMV.

38 39 MATERIAL AND METHODS

40 Construction of recombinant proteins

41 The complete coding sequence of eIF(iso)4G1, obtained from the susceptible *Oryza*
42 *sativa indica* cultivar IR64, was available in a pGEMT plasmid (Albar et al., 2006). The
43 MIF4G-encoding region was amplified by PCR from the pGEMT::eIF(iso)4G1 construct
44 using the *Bam*HI-containing primers F16 5'-GGATCCTTGGTCAGCTAGAAGAGGCA-3'
45 (including MIF4G nucleotides 619-637) and R15 5'-
46 GGATCCTTAATCTATTTCACGTCGAGGCA-3' (including a sequence complementary to
47 MIF4G nucleotides 1350-1371) and subcloned into the pCR®8/GW/TOPO®TA Cloning®
48 vector (Invitrogen). The *Bam*HI fragment, obtained by digestion of the resulting
49 pCR®8/GW/TOPO®TA::MIF4G construct, was sub-cloned into a pET28 vector at the
50 corresponding *Bam*HI sites as a 6-His N-terminal fusion peptide (Novagen), giving rise to a

1 pET28::MIF4G construct. Construction of the bacterial expression plasmid pQE60::VPg,
2 corresponding to the RYMV VPg of the isolate CIa (strain S2/S3) with a 6-His C-terminal
3 fusion, was previously described (Hébrard et al., 2009).

4 To build the yeast two-hybrid plasmids, two subclones, pGEMT::MIF4G and
5 pGEMT::VPg, were constructed to introduce restriction enzyme sites. The MIF4G-encoding
6 region was amplified by PCR using the primers F4G4*NdeI* 5'-
7 CGCATATGCCTTGGTCAGCTAGAAGAGG-3' and R4G4*BamHI* 5'-
8 CGGGATCCATTATTCACGTCGAGGC-3', while the VPg-encoding region was amplified
9 using the primers FVPg*NdeI* 5'-CGCATATGTCTCCATTTGAGATTTACGGC-3' and
10 RVPg*BamHI* 5'-CGGGATCCATTACTCGATATCAACATCC-3'. After digestion, the
11 resulting fragments were cloned into *NdeI/BamHI*-digested pGADT7 and *NcoI/BamHI*-
12 digested pGBKT7, respectively, giving rise to the pGADT7::MIF4G and pGBKT7::VPg
13 constructs.

14 The pET28::MIF4G*, pGADT7::MIF4G*, pGBKT7::VPg* (with I, G or E
15 substitutions, respectively) vectors carrying MIF4G and VPg variants were obtained by site-
16 directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). All
17 primers used in this study are listed in Supplemental Table 1. All constructs were
18 systematically confirmed by sequencing.

19 **Single and co-purification of recombinant proteins**

20 The expression constructs pET28::MIF4G and pET28::MIF4G* were used to separately
21 transform the *E. coli* strain BL21 (DE3) (Novagen). After induction with 0.2 mM isopropyl-1-
22 thio- β -D-galactopyranoside at 37°C for 4 h, cells from 700-ml cultures in LB medium were
23 harvested by centrifugation and frozen at -80°C. Cells were thawed, resuspended in 30 ml of
24 purification buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5% glycerol), disrupted with a
25 French press (Thermo) and centrifuged at 18,000 rpm for 30 min. The supernatant was
26 filtered (0.45- μ m filters), and purification of MIF4G and MIF4G* under native conditions
27 was carried out using a nickel-loaded HisTrap IMAC HP column (GE Healthcare) followed
28 by gel filtration onto a HR10/300GL Superdex 75 column (GE Healthcare) in 20 mM Tris-
29 HCl, pH 8.0, 300 mM NaCl, and 5% glycerol.

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

35 **Far-UV circular dichroism**

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

45 **Yeast two-hybrid test**

46 The Matchmaker GAL4 Two-Hybrid System 3 (Clontech Laboratories, Inc.) was used
47 according to the manufacturer's protocols. pGADT7::MIF4G and pGBKT7::VPg constructs

1 were introduced into the AH109 yeast strain (Clontech). Double-transformed cells were
2 grown on minimal YNB medium (0.7% yeast extract without amino acids, 2% glucose, 2%
3 agar) with all required amino acids including (control) or omitting (Y2H assay) histidine and
4 adenine. Between three and ten clones from four independent transformations were used for
5 the yeast two-hybrid assays. Cells expressing interacting proteins were selected on media
6 lacking leucine and tryptophan (SD-LW) or leucine, tryptophan, histidine, and adenine (SD-
7 LWHA). Cells were grown to stationary phase and adjusted to an OD₆₀₀ of 5.10⁻², 5.10⁻³ or
8 5.10⁻⁴ before spotting onto the appropriate plates. Plates were incubated at 30°C, and growth
9 was checked 3–5 days after spotting. Empty pGADT7 and pGBKT7 vectors were used as
10 negative controls.

11 Growth intensities were monitored with ImageJ software (Abramoff et al., 2004), and
12 row data were normalized to positive and negative control and expressed as a percentage of
13 the “initial” interaction between wild-type MIF4G and avirulent VPg. Data were analyzed by
14 ANOVA (Statistica software version 6.0).

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

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

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31

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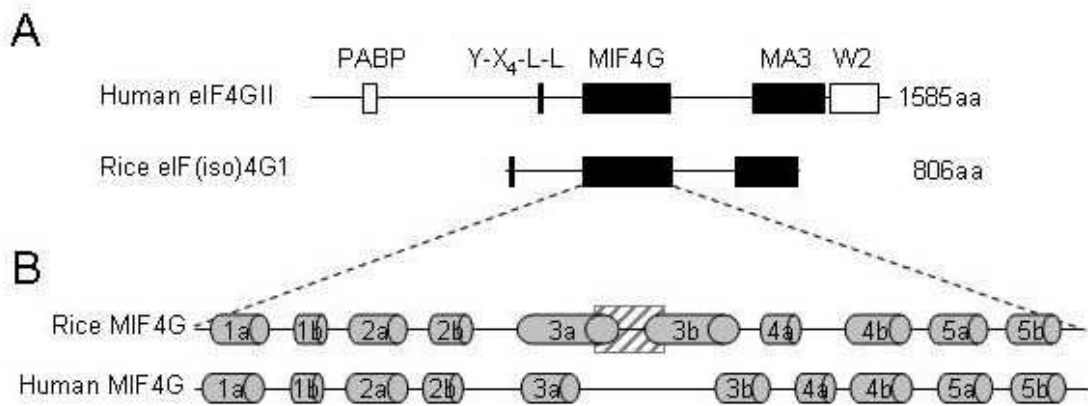
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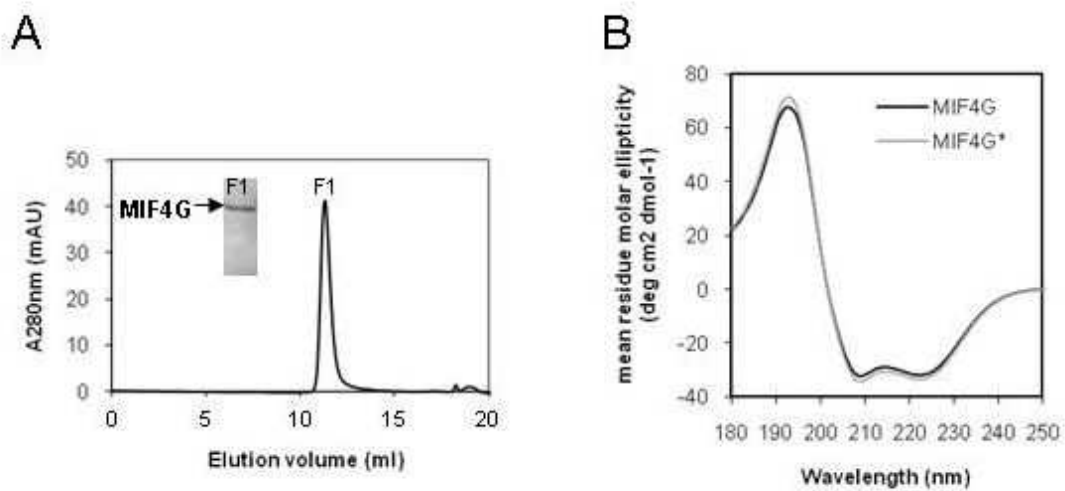
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2 **Figure 1: Organization of the MIF4G central domain of rice eIF(iso)4G1.** (A) Human
3 eIF4GII is a modular protein containing three regions separated by flexible hinges. The N-
4 terminal region contains the binding site for PABP and the eIF4E-binding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



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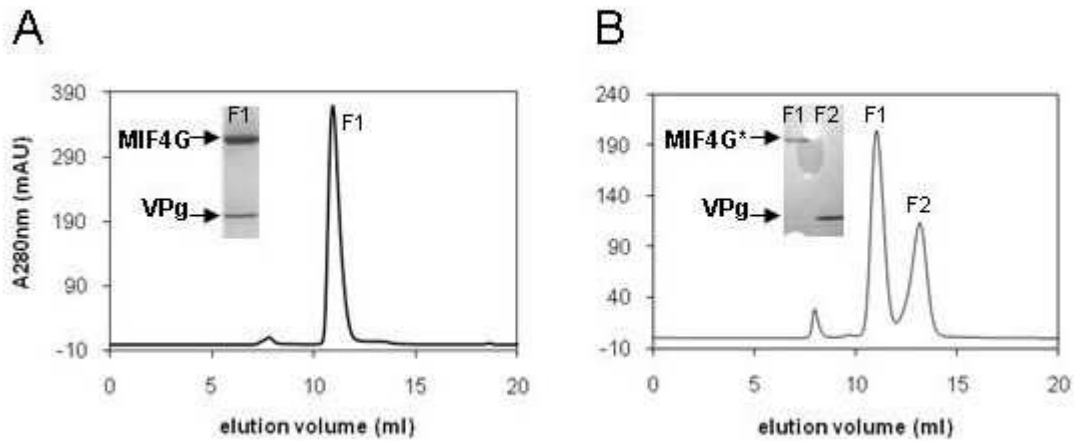
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Figure 2: The MIF4G central domain of rice eIF(iso)4G1 adopts an α -helical conformation. (A) Recombinant His-tagged rice MIF4G was purified by affinity chromatography. Purified His-tagged rice MIF4G was eluted in one fraction from a Superdex 75 HR10/30 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% SDS-PAGE). (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.



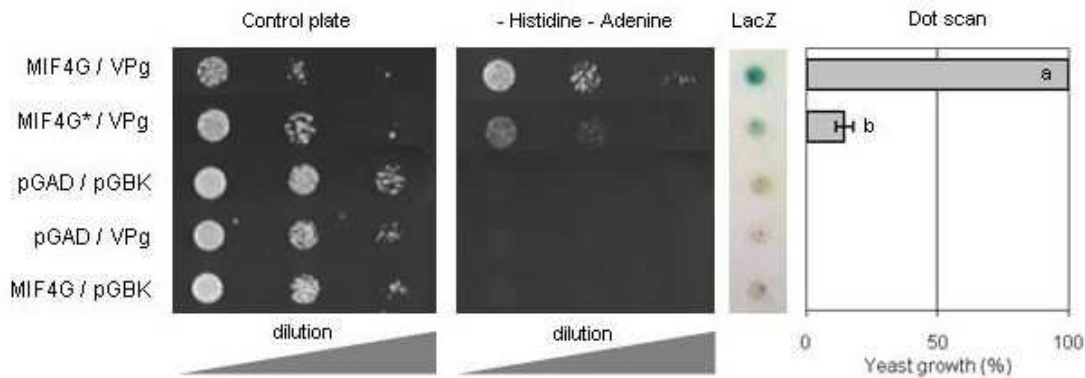
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1 **Figure 3: The viral VPg protein interacts *in vitro* with MIF4G but not with *rymv1-2-***
2 **mutated MIF4G* in a co-purification assay.** Both MIF4G or MIF4G* and VPg
3 recombinant proteins were loaded and co-purified from a Superdex 75 HR10/30 column with
4 an elution buffer containing 50 mM Tris-HCl, pH 8, and 300 mM NaCl, at a flow rate of 0.5
5 ml/min. (A) With wild-type MIF4G, the elution profile shows the presence of the two proteins
6 in one unique fraction, F1 (inset: 15% SDS-PAGE). (B) With MIF4G*, the elution profile
7 shows the presence of MIF4G* and VPg in distinct fractions, F1 and F2 (inset: 15% SDS-
8 PAGE).
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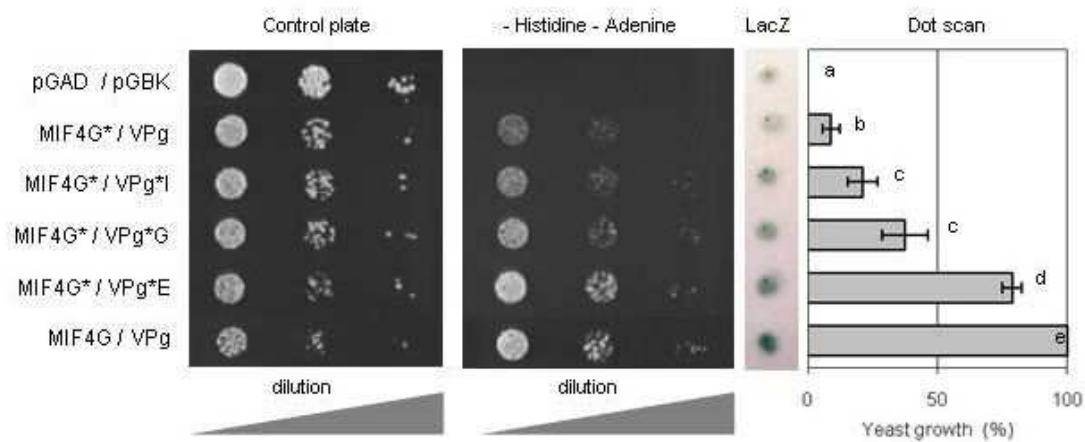
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1 **Figure 4: Wild-type MIF4G and *rymv1-2*-mutated MIF4G* interact differently with the**
2 **viral VPg protein in a yeast two-hybrid assay.** Site-directed mutagenesis was used to
3 introduce an E309K point mutation, mimicking the *rymv1-2* resistance allele, into the
4 pGAD::MIF4G construct. The yeast strain AH109 was co-transformed with either
5 pGAD::MIF4G or pGAD::MIF4G* and pGBK::VPg constructs or with a set of negative
6 controls (empty pGAD/empty pGBK, empty pGAD/pGBK::VPg and pGAD::MIF4G/empty
7 pGBK). Cells were plated as serial dilutions (OD₆₀₀ of 5x10⁻², 5x10⁻³ and 5x10⁻⁴) on a
8 control plate with (first panel) or without (second panel) the histidine amino acid and the
9 adenine base. Yeast cells were allowed to grow at 30°C for 4 days. Neither the
10 pGAD::MIF4G/empty pGBK nor the pGBK::VPg/empty pGAD pairs of constructs allowed
11 yeast growth on selective media without histidine and adenine, indicating the absence of
12 autonomous activation of the reporter genes ADE2 and HIS3 by MIF4G or VPg. A direct
13 interaction between VPg and MIF4G was demonstrated as indicated by the ability of the co-
14 transformed yeast cells to grow in the absence of histidine and adenine. By contrast, the VPg
15 interaction was strongly reduced by the E309K substitution. Interactions were also recorded
16 by their ability to transactivate the LacZ reporter gene (third panel). The direct interaction
17 between VPg and MIF4G is visualized as a strong blue staining due to LacZ transactivation.
18 The VPg/MIF4G interaction is reduced by the E309K substitution in MIF4G as revealed by a
19 light blue staining. No galactosidase activity is detected in cells co-transformed by negative
20 control constructs. Because precise LacZ activity was hardly measurable due to the weakness
21 of some interactions, comparisons of yeast growth were estimated as percentages after
22 quantifying spot intensities presented on second panel using ImageJ (fourth panel), with
23 100% control efficiency being assigned to the susceptible MIF4G/VPg binary interaction. a,
24 b, c, represent groups significantly different after multiple mean comparison (ANOVA, P =
25 0.05). Results were highly reproducible and are the means of 10 independent experiments.



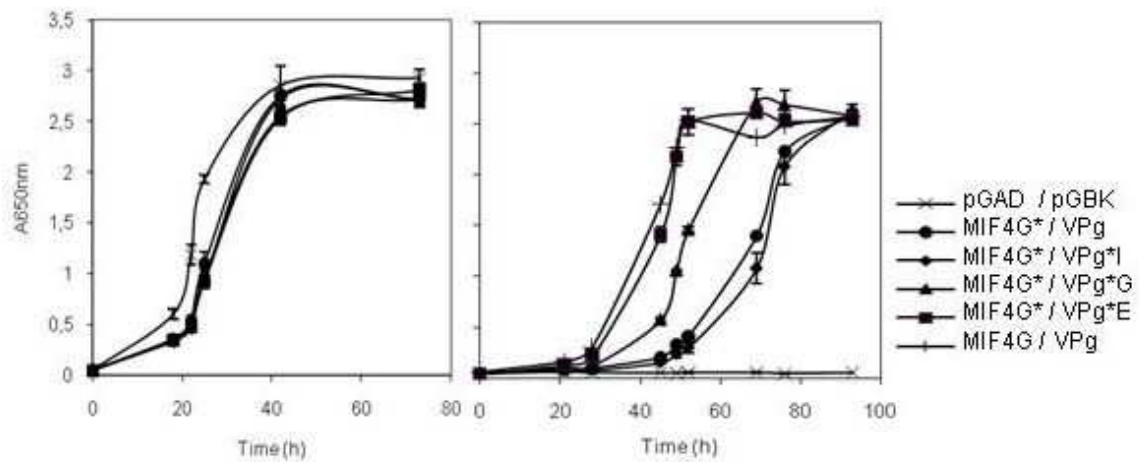
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1 **Figure 5: Point mutations at residue R48, mimicking virulent VPgs, restore *in vivo***
2 **interactions between VPg and *rymv1-2*-mutated MIF4G*.** Site-directed mutagenesis was
3 used to introduce R48I, R48G and R48E point mutations into the pGBK::VPg construct.
4 These constructs were used to transform the AH109 yeast strain together with the
5 pGAD::MIF4G* construct. Transformed yeast cells were cultured as described in Figure 4.
6 The interaction strength between VPg and MIF4G* was successively reinforced by virulence
7 mutations in VPg (VPg*I to VPg*E), as visualized by dot assays (middle panels) and growth
8 percentage comparisons (fourth panel). While the MIF4G*/VPg interaction was only 10% of
9 the strength of the control interaction, the highest interaction score was obtained for the
10 virulence mutation R48E, which restored the interaction with MIF4G* to approximately 80%
11 of the wild-type efficiency. Inversely, the lowest interaction score was obtained with the
12 virulence mutation R48I (MIF4G*/VPg*I), which interacted at only 20% of the strength of
13 the MIF4G/VPg control interaction. The virulence mutation R48G induced an intermediate
14 interaction strength of 40% of the strength of the control interaction. Interactions are also
15 recorded by their ability to transactivate the LacZ reporter gene (third panel) and by ImageJ
16 scanning (fourth panel) as described in Figure 4. Results represent the means of 10
17 independent experiments.



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1 **Figure 6: Time course of co-transformed yeast growth in control or selective liquid**
2 **medium.** The growth rates of cells that were co-transformed as described in Figure 5 were
3 monitored by OD₆₀₀ measurements of liquid cultures either containing all required amino
4 acids, for proper growth in the absence of an interaction (left panel), or lacking adenine, to
5 select for an interaction (right panel). While all co-transformed cells grew similarly in the
6 control medium, cells producing susceptible MIF4G/avirulent VPg and resistant
7 MIF4G*/virulent VPg*E partner proteins grew faster than the other cells, and cells producing
8 resistant MIF4G*/virulent VPg*G showed a 10-h delay in reaching exponential growth. Cells
9 carrying resistant MIF4G* and virulent VPg*I or avirulent VPg exhibited a 30-h delay in
10 reaching the optimal growth. Values represent the means of three individual cells per pair of
11 partners and from two independent transformation events.



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1 **Supplemental data**

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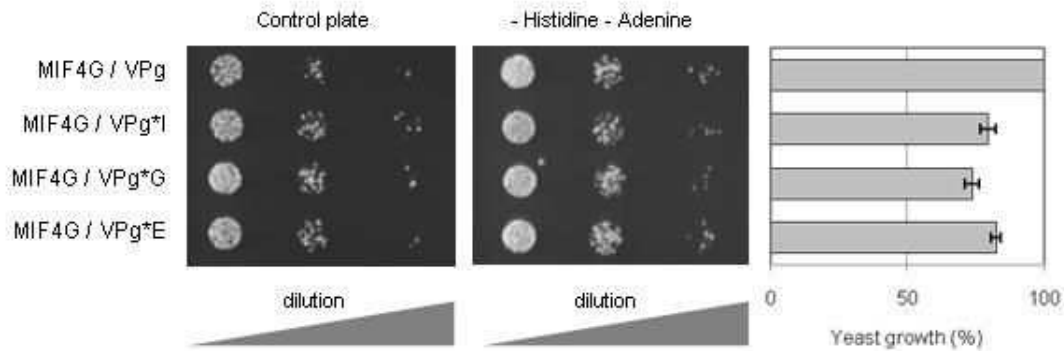
3 **Table S1: List of primers used for site-directed mutagenesis**

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5	FMIFE309K	5' GCTGAGAGCCTAAGGGCTAAAATAGCAAATTGACTGG 3'
6	RMIFE309K	5' CCAGTCAATTTTGCTATTTTAGCCCTTAGGCTCTCAGC 3'
7	FVPgR48I	5' CTGGGTGCGTGAGATAACGAAGTACCACGCTGAG 3'
8	RVPgR48I	5' CTCAGCGTGGTACTTCGTTATCTCACGCACCCAG 3'
9	FVPgR48G	5' GGGTGCGTGAGGGAACGAAGTACCACGCTGAG 3'
10	RVPgR48G	5' CTCAGCGTGGTACTTCGTTCCCTCACGCACCC 3'
11	FVPgR48E	5' CTGGGTGCGTGAGGAAACGAAGTACCACGCTGAGG 3'
12	RVPgR48E	5' CCTCAGCGTGGTACTTCGTTTCCTCACGCACCCAG 3'

13

14 **Figure S1: Point mutations at residue R48, mimicking virulent VPgs, interact with wild-**
 15 **type MIF4G.** VPg mutants mimicking virulent isoforms at residue R48 that interacted with
 16 wild-type MIF4G constructs were used to transform the AH109 yeast strain together with the
 17 pGAD::MIF4G construct. Interaction strength values between virulent VPgs and wild-type
 18 MIF4G were similar and were approximately 70–80% of control interaction values, as
 19 visualized by dot assays (middle panel) and growth percentage comparisons (right panel).



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