The Ustilago maydis a2 Mating-Type Locus Genes lga2 and rga2 Compromise Pathogenicity in the Absence of the Mitochondrial p32 Family Protein Mrb1

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The Ustilago maydis mrb1 gene specifies a mitochondrial matrix protein with significant similarity to mitochondrial p32 family proteins known from human and many other eukaryotic species. Compatible mrb1 mutant strains were able to mate and form dikaryotic hyphae; however, proliferation within infected tissue and the ability to induce tumor development of infected maize (Zea mays) plants were drastically impaired. Surprisingly, manifestation of the mrb1 mutant phenotype selectively depended on the a2 mating type locus. The a2 locus contains, in addition to pheromone signaling components, the genes lga2 and rga2 of unknown function. Deletion of lga2 in an a2mrb1 strain fully restored pathogenicity, whereas pathogenicity was partially regained in an a2mrb1rga2 strain, implicating a concerted action between Lga2 and Rga2 in compromising pathogenicity in mrb1 strains. Lga2 and Rga2 localized to mitochondria and Mrb1 interacted with Rga2 in the yeast two-hybrid system. Conditional expression of lga2 in haploid cells reduced vegetative growth, conferred mitochondrial fragmentation and mitochondrial DNA degradation, and interfered with respiratory activity. The consequences of lga2 overexpression depended on the expression strength and were greatly exacerbated in mrb1 mutants. We propose that Lga2 interferes with mitochondrial fusion and that Mrb1 controls this activity, emphasizing a critical link between mitochondrial morphology and pathogenicity.

INTRODUCTION

Ustilago maydis is a member of the order Ustilaginiales, causing worldwide smut diseases in more than 75 plant families of the angiosperms. Among the estimated 1200 species of smut fungi, the economically significant pathogens are U. hordei, U. nuda, U. nigra, U. triticum, Urocystis agropyri, U. maydis, U. scitaminea, and species of the genus Sporisorium, causing smut diseases on barley (Hordeum vulgare), oat (Avena sativa), wheat (Triticum aestivum), maize (Zea mays), sugarcane (Saccharum officinarum), and sorghum (Sorghum bicolor), respectively, as well as Tilletia species causing bunt diseases on wheat (Martínez-Espinoza et al., 2002). In particular, it is the ability of smut fungi to develop within grain kernels that can result in drastically lowered crop yields. U. maydis and U. hordei are the most exclusively studied members of the Ustilaginiales. U. maydis infects young meristematic tissue above ground and triggers the formation of tumors, most prevalent on infected ears, tassels, stems, and nodal shoots. In these tumors, massive proliferation of the fungus occurs. A different strategy is adopted by U. hordei, which maintains the mycelium state in the apical meristem until flowering and then ramifies ovary tissue, resulting in kernels filled with teliospores (Martínez-Espinoza et al., 2002).

U. maydis is genetically tractable, and a broad spectrum of molecular methods, including an efficient gene knockout system, stage-specific and inducible promoters, reporter genes, and insertional mutagenesis, has been established over the last 15 years. Recently, the complete genome sequence of U. maydis has been released to the public database providing for novel strategies to identify pathogenicity functions based on transcriptome analysis and candidate approaches. This has rendered U. maydis a prime model organism to uncover general strategies elaborated by phytopathogenic fungi (Basse and Steinberg, 2004).

The infection process of U. maydis has been documented in previous cytological studies (Snetselaar and Mims, 1994 and references therein; Banuett and Herskowitz, 1996; Kahmann et al., 2000). Dikaryotic hyphae that emerge from fusion of compatible, haploid sporidia are able to penetrate the surface of aerial plant parts by developing appressoria-like structures at their tips. Hyphae initially grow through epidermal cells and proceed with intercellular proliferation in underlying tissue. After karyogamy, diploid spor precursors are released by fragmentation from highly branched, sporogenous hyphae and further mature to teliospores. During all these stages U. maydis maintains a biotrophic relationship with its host. Early host responses are chlorosis and anthocyanin formation (Banuett and Herskowitz, 1996), with the latter being an indicator that the fungus has entered the plant tissue. Full virulence, however,
coincides with the formation of host tumors. Pathogenicity is genetically controlled by the mating type loci a and b. The a locus specifies a pheromone/receptor system that triggers cell fusion in response to pheromone recognition by the receptor of opposite mating type (Böcker et al., 1992; Spellig et al., 1994). This locus exists in two alleles termed a1 and a2. Whereas both loci code for pheromone precursor and receptor genes, the a2 locus comprises two additional genes, lga2 and rga2, of which lga2 has been proposed to encode a putative mitochondrial protein. However, these genes are not critical for mating and their absence is not affecting pathogenic development (Urban, 1995; Urban et al., 1996a). The b locus encodes the bE and bW homeodomain proteins, which can dimerize in nonallelic combinations and then form an active transcription factor required for stability and development of the infectious filamentous dikaryon (Gillissen et al., 1992 and references therein; Kämper et al., 1995; Brachmann et al., 2001). All genes residing in the a locus are pheromone induced, and lga2 expression is additionally stimulated in the presence of an active b heterodimer (Urban et al., 1996b). More recent studies have shown that lga2 is a direct target of the b proteins (Romeis et al., 2000). Although dikaryotic hyphae represent the infectious agent in nature, haploid, solo-pathogenic strains, which bypass the requirement for a mating partner and cause infections when inoculated singly into the maize plant, can be generated in the laboratory. This is achieved by the introduction of b mating-type genes whose products can dimerize with those of resident genes. In particular, it has been demonstrated that an a1b1 strain transformed with a b2 allele is pathogenic (Kronstad and Leong, 1989; Schulz et al., 1990; Böcker et al., 1995).

By differential display analysis, we recently identified the mig2 cluster, which consists of five highly similar genes lacking homologies to database entries. All these genes are extensively upregulated after the fungus has entered the host tissue (Basse et al., 2002). The mig2 locus is flanked by the constitutively expressed U. maydis mrb1 gene (Basse et al., 2002). mrb1 encodes a protein with significant homologies to the so-called p32 family proteins and is predicted to reside in mitochondria. The founding member of this protein family is the human p32 protein, which was originally identified in association with the SR family splicing factor ASF/SF2 (Krainer et al., 1991). p32 family proteins are implicated in diverse regulatory processes, including transcriptional activation by cooperating with viral transcription factors, pre-mRNA splicing, and mitochondrial RNA editing (Krainer et al., 1991; Yu et al., 1995; Petersen-Mahrt et al., 1999; Van den Brulle et al., 1999; Van Scoy et al., 2000; Hayman et al., 2001 and references therein). Because the function of p32 family proteins has not yet been addressed in a pathogenic fungus, we became interested in analyzing a possible regulatory function of Mrb1 connected to pathogenicity.

Here, we describe that Mrb1 functions as a novel, mitochondrial virulence determinant in U. maydis. Our results indicate that Mrb1 is specifically required for pathogenicity in the presence of the a2 locus genes lga2 and rga2. We explicitly show that Lga2 interferes with cell growth, mitochondrial morphology, and fitness and that Mrb1 counteracts these Lga2 effects. Our results suggest the formation of a mitochondrial protein complex containing Mrb1, Lga2, and Rga2 and emphasize a critical connection between mitochondrial morphology and fungal development during the pathogenic growth stage.

RESULTS

mrb1 Encodes an Acidic Protein of the p32 Family

We have previously identified the U. maydis mrb1 gene based on its neighboring position to the mig2 cluster (Basse et al., 2002). The predicted mrb1 open reading frame (ORF) encodes a putative protein of 274 amino acids corresponding to a molecular mass of 30.4 kD. The protein sequence revealed highest similarity to Aspergillus nidulans SuAprgA1 (36.5% identity), Mam33p of Saccharomyces cerevisiae (22.3% identity), as well as the p32 and p22 proteins from human and Trypanosoma brucei, respectively (23.7 to 24.1% identity; Figure 1). These proteins all have a mitochondrial target sequence and are highly acidic with pI values in the range of 4 to 4.5. The predicted pI value of Mrb1 is 4.35. The majority of acidic amino acid positions are conserved among p32 family proteins, and the most pronounced sequence conservation is found in the C-terminal portion between positions 216 and 274 of Mrb1 (Figure 1). The respective region of human p32 comprises two α-helical regions implicated in oligomerization as predicted from the crystal structure (Jiang et al., 1999). In addition, Mrb1 shares several conserved acidic amino acid positions with p32 family proteins that are surface exposed in p32 and have been proposed to participate in protein–protein interactions (Figure 1; Jiang et al., 1999).

Localization and Expression of Mrb1

For Mam33p and p32, it has been documented that these proteins are predominantly localized in the mitochondrial matrix (Muta et al., 1997; Seytter et al., 1998). The existence of a mitochondrial signal sequence was also predicted for Mrb1 (Figure 1). To assess whether the potential sorting signal is functional, we generated plasmids pMB2-1 and pMB2-2, which allow constitutive expression of enhanced green fluorescent protein (eGFP) fused to the regions coding for the N-terminal 46 and 58 amino acids of Mrb1, respectively. These plasmids were inserted ectopically into U. maydis strain FB1. As control, plasmid pMGH1 was generated, which contained the complete mrb1 ORF translationally fused to eGFP under control of the native mrb1 promoter, and inserted by homologous integration into strain FB1. In all cases, fluorescence microscopy of transformants revealed a thread-like mitochondrial pattern (Figures 2A and 2B; data not shown). This indicated that mitochondrial localization was conferred by all three constructs and that the N-terminal 46 amino acids of Mrb1 were sufficient for targeting. To determine the spatial and temporal control of mrb1 expression during pathogenic development, we used compatible FB1mrb1:eGFP and FB2mrb1:eGFP strains, which expressed the mrb1-eGFP fusion construct under the mrb1 promoter. Expression of mrb1 remained high in the initial phase of pathogenic development as reflected from mitochondrial GFP fluorescence in appressoria on the leaf surface (data not shown) as well as during biotrophic growth of hyphae in planta until the stage of hyphal fragmentation (Figures 2C and 2D). By contrast,
mature teliospores emitted only barely detectable fluorescence (Figure 2E).

Deletion of mrb1 and Complementation of mrb1 Null Mutant Strains

To assess the function of mrb1, the gene was replaced by the carboxin (cbx) resistance cassette in compatible, haploid U. maydis strains FB1 (a1b1) and FB2 (a2b2). The resulting Δmrb1 strains were unaffected in growth in either yeast extract peptone sucrose or minimal medium, and when combined, they readily formed dikaryotic filaments on solid charcoal complete medium (CM). A previous study has shown that growth of yeast mam33 mutants was retarded on solid YPG medium with glycerol as nonfermentable carbon source at 18°C (Muta et al., 1997).

However, under these conditions, mrb1 mutant strains were not affected in growth (data not shown). Remarkably, none of the six combinations of compatible FB1Δmrb1 and FB2Δmrb1 strains tested was able to trigger tumor development within 9 d after inoculation (DAI), whereas plants infected with isogenic wild-type strains produced tumors with a frequency of 92% within that period (Table 1, Figures 3A and 3B). Furthermore, the induction of anthocyanin formation was drastically reduced in

Figure 1. Multiple Alignment of Mrb1 with Related Sequences from A. nidulans (SuAprgA1), S. cerevisiae (Mam33p), T. brucei (p22), and Human (p32). Identical (≥60%) positions are boxed and shaded. Predicted N-terminal amino acids generated after cleaving the presequence are encircled. Conserved acidic positions in the Mrb1 sequence are marked by dots. Asterisks mark highly conserved amino acids of p32 proteins (Glu89, Arg118, Arg242, Gly243, Glu263, and Tyr264) assigned to the protein surface in p32 (Jiang et al., 1999).

Figure 2. Localization and Expression of Mrb1. (A) and (B) U. maydis strains FB1mrb1:eGFP (A) and FB1/pMB2-1 (B) were grown in yeast extract peptone sucrose medium and assayed for fluorescence of GFP (left) and the mitochondrial marker dye CM-H2Xros (middle). The yellow color (right) indicates coincidence. The bar refers to all panels. (C) to (E) Spatial mrb1 expression during the life cycle of U. maydis. Plants were inoculated with mixtures of compatible wild-type reporter strains FB1mrb1:eGFP (a1b1) and FB2mrb1:eGFP (a2b2), which express mrb1 under its own promoter. Maize tissue samples were assayed by differential interference contrast (DIC) (C1 to E1) and fluorescence microscopy (C2 to E2). GFP fluorescence in intracellularly growing hyphae 5 DAI (C) and in sporogenic hyphae 10 and 12 DAI (arrows in D and E), respectively, are shown. The arrow in (E) points to a mature teliospore. The bar refers to all panels.
infections with Δmrβ1 mutants (data not shown). After a period of 10 to 12 DAI, only 5% of infected plants produced tumors, and these were significantly reduced in size with diameters of ~2 mm. Combinations of compatible Δmrβ1 and wild-type strains were able to mate and cause disease symptoms in all cases; however, incidence of tumor formation was markedly diminished in all combinations with FB2Δmrβ1 strains (Table 1). To unequivocally demonstrate that the absence of mrβ1 accounted for the loss of pathogenicity, plasmid pMGH1 (see above) was introduced into strains FB1Δmrβ1 and FB2Δmrβ1. Combinations of these transformants induced wild-type disease symptoms (Figure 3C, Table 1). This shows that the pathogenicity defect of Δmrβ1 strains can be complemented and illustrates further that the mrβ1-eGFP fusion protein is functional.

The Role of Mrb1 during the Infection Cycle

To investigate at which stage during pathogenic development Mrb1 is required, we followed the fate of dikaryotic hyphae after inoculation. Mutant Δmrβ1 hyphae were able to grow on the leaf surface and differentiated appressoria that penetrated into leaf tissue (Figure 4A). However, a significant difference in proliferation between mutant and wild-type hyphae became apparent during subsequent biotrophic growth. Already 2 DAI wild-type hyphae had ramified through epidermal cells followed by proliferation in underlying tissue 3 DAI, and 9 DAI fragmenting sporogenous hyphae were detected (Figure 4B). By contrast, only few Δmrβ1 hyphae were detected in infected tissue and these grew predominantly unbranched (Figure 4C). In one of the small tumors induced by Δmrβ1 hyphae, moderate accumulation of branched hyphae was detected 14 DAI (Figure 4D). Moreover, teliospores had developed in only one of the seven small tumors that resulted from infection with FB1Δmrβ1 and FB2Δmrβ1 strains after a prolonged period (data not shown). In conclusion, the pathogenicity defect of Δmrβ1 mutants results from severely attenuated hyphal development in epidermal and underlying cells.

Δmrβ1 Is Dispensable for Pathogenicity of Solopathogenic Strains Carrying the a1 Locus

To assess whether mrβ1 was also necessary for pathogenicity of strain SG200 (a1mfa2bE1bW2), which is derived from strain FB1 and is solopathogenic because of the presence of the bE1 and bW2 genes ( Bölker et al., 1995), SG200Δmrβ1 strains were generated. Surprisingly, these strains were unaffected in pathogenicity (Table 1), suggesting a specific mrβ1 requirement for pathogenicity of dikaryotic hyphae. Therefore, we expected to render FB1Δmrβ1 and FB2Δmrβ1 strains solopathogenic by introducing either a bE2 gene (pUb2-2) or a bE1 gene (pUb1). Whereas independent FB1Δmrβ1/pUb2-2 transformants acquired the ability to induce tumor and teliospore development (Table 2), none of the six tested FB2Δmrβ1/pUb1 strains was able to trigger tumor formation in infected plants. To demonstrate functionality, pUb1 was introduced into wild-type FB2, and here all three transformants tested had become pathogenic (Table 2). These results suggested the existence of a FB2-specific factor that interfered with pathogenic development in the absence of mrβ1.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Inoculated Plants</th>
<th>Plants with Tumors/Percentagea</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1Δmrβ1 × FB2Δmrβ1 (4/6)b,c</td>
<td>199</td>
<td>0/0</td>
</tr>
<tr>
<td>FB1 × FB2 (4/1)b</td>
<td>101</td>
<td>93/92</td>
</tr>
<tr>
<td>FB1Δmrβ1#12 × FB2 (1/1)</td>
<td>20</td>
<td>20/100</td>
</tr>
<tr>
<td>FB1Δmrβ1#15 × FB2 (1/1)</td>
<td>20</td>
<td>19/95</td>
</tr>
<tr>
<td>FB1 × FB2Δmrβ1#4 (1/1)</td>
<td>15</td>
<td>7/47</td>
</tr>
<tr>
<td>FB1 × FB2Δmrβ1#9 (1/1)</td>
<td>19</td>
<td>6/32</td>
</tr>
<tr>
<td>FB1Δmrβ1#15 × FB2Δmrβ1#9 (1/1)</td>
<td>15</td>
<td>0/0</td>
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<tr>
<td>FB1Δmrβ1#12 × FB2Δmrβ1#4 (1/1)</td>
<td>16</td>
<td>0/0</td>
</tr>
<tr>
<td>FB1 × FB2 (1/1)</td>
<td>20</td>
<td>19/95</td>
</tr>
<tr>
<td>FB1Δmrβ1#1/ pMGH1 × FB2Δmrβ1#1/ pMGH1 (1/4)</td>
<td>84</td>
<td>59/70</td>
</tr>
</tbody>
</table>

a Number of plants possessing at least one tumor and indication of the respective percentage.

b (xy): x is the number of independent pathogenicity tests performed; y is the number of different strains or strain combinations.

c Plants were inspected 6 to 9 DAI. Tumors of wild-type infected plants were already present 6 DAI.

Comparable results were obtained with Δmrβ1 strains, in which the ip cassette was substituted by the hph cassette.

Figure 3. Complementation of Δmrβ1 Strains with pMGH1.

(A) to (C) Infection symptoms of maize plants 6 DAI with mixtures of wild-type FB1 (a1b1)/FB2 (a2b2) strains (A), FB1Δmrβ1/FB2Δmrβ1 strains (B), and FB1Δmrβ1/pMGH1/FB2Δmrβ1/pMGH1 strains (C).

(D) Leaf tumor (arrow) 11 DAI with strains FB1Δmrβ1/FB2Δmrβ1.
The Influence of the \textit{lga2} and \textit{rga2} Genes on Pathogenic Development in the Absence of \textit{mrb1}

Prompted by the observation that \textit{Mrb1} was specifically required for pathogenicity of \textit{FB2} strains, we asked whether the \textit{a2}-specific \textit{lga2} and \textit{rga2} genes interfered with pathogenic development in the absence of \textit{mrb1}. The \textit{lga2} gene encodes a putative mitochondrial matrix protein (Urban et al., 1996a) that has been shown to contain a functional N-terminal mitochondrial signal peptide (P. Müller, M. Bölker, and R. Kahmann, unpublished data). By contrast, a low probability (0.06) for a mitochondrial target sequence was predicted for \textit{rga2} (see below). The region comprising \textit{lga2} or \textit{rga2} was replaced by a hygromycin resistance cassette in the \textit{FB2\Delta mrb1} strain. As expected from previous experiments (Urban et al., 1996a), deleting \textit{lga2} and/or \textit{rga2} in a wild-type background did not affect pathogenic development (Table 3). Amazingly, mixtures of \textit{FB1\Delta mrb1} and \textit{FB2\Delta mrb1\Delta lga2\Delta rga2} strains were as pathogenic as a mixture of compatible wild-type strains (Table 3). Pathogenicity assays with \textit{\Delta mrb1} mutants deleted in either \textit{lga2} or \textit{rga2} alone revealed that a mixture of \textit{FB1\Delta mrb1} and \textit{FB2\Delta mrb1\Delta lga2\Delta rga2} strains was fully pathogenic, whereas mixtures of \textit{FB1\Delta mrb1} and \textit{FB2\Delta mrb1\Delta rga2} strains caused only weak disease symptoms (Table 3). Nevertheless, these significantly exceeded disease symptoms induced by a mixture of \textit{FB1\Delta mrb1} and \textit{FB2\Delta mrb1} strains (Table 3). Together, these results reveal that \textit{lga2} and \textit{rga2} negatively contribute to pathogenic development in the absence of \textit{mrb1}, although to different extents.

Interaction of \textit{Mrb1} with \textit{Lga2} and \textit{Rga2}

To explore whether \textit{Mrb1}, \textit{Lga2}, and \textit{Rga2} proteins interact, we conducted yeast two-hybrid assays. For this purpose and to avoid possible interference with subcellular localization, N-terminal deletion derivatives of \textit{Mrb1}, \textit{Lga2}, and \textit{Rga2} lacking putative mitochondrial targeting sequences were fused to either the \textit{LexA} DNA binding domain or the \textit{B42} activation domain specified by the vectors \textit{pLexA} and \textit{pB42AD}, respectively. In the resulting constructs (Table 4), the lower case number reflects the first amino acid position of the respective \textit{U. maydis} protein that is present in the fusion. Yeast transformants harboring the \textit{pLexA} constructs alone displayed no \textit{LacZ} activity, whereas \textit{pB42AD} constructs as well as the combination of empty \textit{pLexA} and \textit{pB42AD} vectors conferred slightly enhanced expression levels (Table 4). The combination of \textit{pLexA-\textit{mrb1}_{2237}/pB42AD-\textit{rga2}_{2237},} displayed ~100-fold elevated levels of activity and for the reciprocal combination \(	extit{\beta}-\textit{galactosidase activity elevated 20-fold (Table 4, combinations 3 and 7). This difference is likely to result from steric constraints. Among the remaining combinations, high \textit{LacZ} activity levels were only measured in the \textit{mrb1/mrb1}\textit{mrb1} (right panel in \textbf{[C]}). The arrows point to a short hyphal branch in epidermal cells (left panel in \textbf{[C]}), and a hyphae growing in the mesophyll layer (middle panel in \textbf{[C]}), and a fragmenting sporogeneous hyphae (right panel in \textbf{[C]}). d p.i., days after inoculation.
complex in the dikaryon (Urban et al., 1996b; Romeis et al.,
strongly induced in response to the formation of an active bE/bW
inoculum
harboring compatible
fused to
eGFP
coding region for the initial 61 amino acids was translationally
to assess
The genetic relationship among Mrb1, Lga2, and Rga2 suggests
Subcellular Localization of Mrb1, Lga2, and Rga2
The genetic relationship among Mrb1, Lga2, and Rga2 suggests
that all three proteins reside within mitochondria. To assess
the 
numbers of different strains.

Expression of lga2 in Strains Lacking mrb1 Reduces
Fitness, Affects Mitochondrial Morphology, and
Triggers Degradation of Mitochondrial DNA
Expression of lga2 is undetectable in haploid strains but is strongly induced in response to the formation of an active bE/bW complex in the dikaryon (Urban et al., 1996b; Romeis et al.,
2000). We therefore analyzed the shape of mitochondria in
mrb1 dikaryotic hyphae during pathogenic growth. Hyphae resulting from fusion of Mrb1/pMB2-2 strains were only rarely detected during the investigated period from 2 to 4 DAI and mainly displayed diffuse or speckled fluorescence patterns (Figures 6A and 6B). By contrast, wild-type hyphae carrying the pMB2-2 reporter contained thread-like mitochondrial structures (Figure 2C) in the majority of cells but also showed the presence of highly fragmented mitochondrial aggregates in a small percentage (Figure 6C). Prompted by this observation, we investigated the consequences of lga2 expression in haploid cells. Plasmid pCLN1 provided for conditional lga2 expression under the arabinose-inducible crg1 promoter. This plasmid was ectopically integrated into the genome of U. maydis strains FB2/
pMB2-2 and FB2/mrb1/pMB2-2 expressing eGFP targeted to mitochondria. RNA gel blot analysis showed that the resulting transformants, designated MC2 and MC2/mrb1, respectively, expressed lga2 under inducing conditions but varied in their relative expression levels up to 10-fold (Figure 7A). This variation reflects copy number differences as demonstrated by DNA gel blot analysis (data not shown). Strain FB2 showed no lga2 expression in CM/Ara medium as expected (Figure 7A). Growth in noninducing CM/Glu medium revealed no significant differences among MC2, MC2/mrb1, and their progenitor strains, and this was also apparent on solid CM/Glu plates (Figure 7C; data not shown). When shifted to CM/Ara medium, growth of all cultures was diminished, reflecting the carbon source preference (Figure 7C; data not shown). Remarkably, under these conditions, growth was negatively influenced by the extent of lga2 overexpression, and this effect was more pronounced in the absence of mrb1 (Figures 7A and 7B). Growth differences related to the level of lga2 expression were also seen when MC2/mrb1 strains were grown on solid CM/Ara medium. Growth of strains

### Table 2. Pathogenicity of Haploid Wild-Type and Δmrb1 Strains
Harboring Compatible b Alleles

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Inoculated Plants</th>
<th>Tumors/Plants</th>
<th>Percentagea</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB1/mrb1/pUb2-2 (3/2)</td>
<td>62</td>
<td>29/47</td>
<td></td>
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<tr>
<td>FB1/pUb2-2 (2/2)</td>
<td>47</td>
<td>21/45</td>
<td></td>
</tr>
<tr>
<td>FB2/mrb1/pUb1 (2/6)</td>
<td>121</td>
<td>11/1 &lt;1</td>
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</tr>
<tr>
<td>FB2/pUb1 (1/3)</td>
<td>48</td>
<td>29/60</td>
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</table>

Plants were inspected 6 to 7 DAI.

a Number of plants possessing at least one tumor and indication of the respective percentage.

### Table 3. Influence of lga2 and rga2 on Pathogenicity of mrb1 Mutants

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Inoculated Plants</th>
<th>Tumors/Plants</th>
<th>Percentagea</th>
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<tbody>
<tr>
<td>FB1/mrb1 × FB2/mrb1 (2/1)</td>
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<td>0/0</td>
<td></td>
</tr>
<tr>
<td>FB1/mrb1 × FB2/mrb1/lga2 (2/4)</td>
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<td>91/80</td>
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</tr>
<tr>
<td>FB1 × FB2/lga2 (1/2)</td>
<td>31</td>
<td>26/84</td>
<td></td>
</tr>
<tr>
<td>FB1 × FB2 (2/1)</td>
<td>62</td>
<td>46/74</td>
<td></td>
</tr>
<tr>
<td>FB1/mrb1 × FB2/mrb1 (2/2)</td>
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<td>11&lt;1.5</td>
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<tr>
<td>FB1/mrb1 × FB2/mrb1/lga2 (2/2)</td>
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<td>69/96</td>
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<tr>
<td>FB1/mrb1 × FB2/mrb1/rga2 (2/2)</td>
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<td>19/25</td>
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<tr>
<td>FB1 × FB2/lga2 (2/2)</td>
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<td>43/66</td>
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<tr>
<td>FB1 × FB2/rga2 (1/2)</td>
<td>23</td>
<td>19/83</td>
<td></td>
</tr>
<tr>
<td>FB1 × FB2 (3/1)</td>
<td>52</td>
<td>48/92</td>
<td></td>
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</tbody>
</table>

a Number of plants possessing at least one tumor and indication of the respective percentage.

b (x/y): x is the number of independent pathogenicity tests performed; y is the number of different strains.

c Plants were inspected 6 to 7 DAI.

d Plants were inspected 9 to 10 DAI.

e Tumor with a diameter <1 mm.

f The majority of tumors (68%) developed after 7 DAI and had diameters of 1 to 3 mm.
Table 4. Interaction among Mrb1, Lga2, and Rga2

<table>
<thead>
<tr>
<th>Combination</th>
<th>pLexA Construct</th>
<th>pB42AD Construct</th>
<th>β-Galactosidase Activity*</th>
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<tbody>
<tr>
<td>1</td>
<td>pLexA-mrb1.42</td>
<td>pB42AD-mrb1.42</td>
<td>295.2 ± 78.3 (6/1)</td>
</tr>
<tr>
<td>2</td>
<td>pLexA-mrb1.42</td>
<td>pB42AD-lga2.44</td>
<td>17.8 ± 11.2 (8/1)</td>
</tr>
<tr>
<td>3</td>
<td>pLexA-mrb1.42</td>
<td>pB42AD-rga2.44</td>
<td>1364.4 ± 140.2 (6/2)</td>
</tr>
<tr>
<td>4</td>
<td>pLexA-lga2.43</td>
<td>pB42AD-mrb1.42</td>
<td>8.4 ± 5.6 (6/1)</td>
</tr>
<tr>
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<td>pB42AD-lga2.44</td>
<td>16.6 ± 3.7 (9/1)</td>
</tr>
<tr>
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<td>pB42AD-rga2.44</td>
<td>10.8 ± 3.3 (6/1)</td>
</tr>
<tr>
<td>7</td>
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<td>252.8 ± 47.8 (11/1)</td>
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<tr>
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</tr>
<tr>
<td>9</td>
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<td>pB42AD-rga2.44</td>
<td>11.4 ± 2.2 (12/1)</td>
</tr>
<tr>
<td>10</td>
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</tr>
<tr>
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<td>1.2 ± 0.5 (6/1)</td>
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<tr>
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<td>–</td>
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</tr>
<tr>
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<td>–</td>
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<td>9.3 ± 5.2 (11/1)</td>
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<tr>
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<td>5.3 ± 4.4 (5/1)</td>
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<td>–</td>
<td>pB42AD-rga2.44</td>
<td>7.0 ± 4.1 (4/1)</td>
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<tr>
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<td>pGB-E1473</td>
<td>pW2546-GA</td>
<td>135.3 ± 21.1 (4/6)</td>
</tr>
<tr>
<td>17*</td>
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<td>pW2546-GA</td>
<td>5.1 ± 3.4 (4/1)</td>
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<td>pLexA</td>
<td>pB42AD</td>
<td>11.7 ± 3.7 (4/2)</td>
</tr>
</tbody>
</table>

*β-Galactosidase activity was assayed as described in Methods; β-galactosidase activity in Miller units ± SD.

During growth in glucose-containing medium, all progenitor strains as well as all transformants displayed thread-like mitochondrial networks (data not shown). Strikingly, after shifting to arabinose, all transformants highly overexpressing lga2 predominantly (96 to 98%) displayed excessive accumulation of dot-like fluorescent fragments throughout the cytoplasm (Figures 8A, 8D, and 8E1), indicative of mitochondrial fragmentation. Among the transformants that expressed lga2 to a lower extent (Figure 7A), a high proportion (90 to 92%) of MC2Δmrb1 cells displayed fragmented mitochondria (Figures 8A and 8C), whereas mitochondrial networks were seen in the majority of MC2 cells (Figures 8A and 8B). We monitored the time course of mitochondrial fragmentation in strains MC2Δ4 and MC2Δmrb1Δ8, which strongly overexpress lga2. Starting with intact mitochondria 2.5 h after shifting to inducing conditions, fragmented mitochondria were observed in 10 and 15% of MC2Δ4 and MC2Δmrb1Δ8 cells, respectively. The percentage further increased to 74 and 83%, respectively, after an additional 6 h. This indicates that lga2 expression directly affects mitochondrial morphology.

To assess respiratory activity in MC2 and MC2Δmrb1 strains expressing lga2, cells were incubated with the mitochondrial marker dye CM-H2Xros, whose fluorescence and sequestration into mitochondria is limited to actively respiring cells. In contrast with MC2Δ3 and MC2Δmrb1Δ7 strains expressing lower levels of lga2 (Figures 8B and 8C), mitochondrial fluorescence was markedly reduced in highly expressing lga2 transformants as assessed from rapid fluorescence fading of mitochondrial fragments. Upon excitation, fluorescence either diminished to a few spots (Figure 8D) or was barely detectable (Figure 8E1). Reduction in mitochondrial fluorescence was always associated with the highly fragmented mitochondrial phenotype and was not observed in the minority of cells exhibiting a thread-like mitochondrial pattern (Figure 8E2). Collectively, these results reflect the interaction between Mrb1 and Lga2 and show that overexpression of lga2 interferes with metabolic activity and leads to mitochondrial fragmentation.

The effect of lga2 overexpression on mitochondrial morphology as well as metabolic activity led us to consider mitochondrial DNA (mtDNA) degradation as cause for this phenotype. To assess DNA integrity, total DNA was isolated at various time points after transfer of strains MC2Δ3, MC2Δ5, MC2Δmrb1Δ7, and MC2Δmrb1Δ8, and control strains FB2/pMB2-2 and FB2Δmrb1/pMB2-2 to CM/Ara (Figure 8A). DNA was subjected to DNA gel blot analysis using probes from the cytochrome b (cytb) and the cbx genes to discriminate between mtDNA and genomic DNA, respectively. In strains MC2Δ5 and MC2Δmrb1Δ8 strongly overexpressing lga2 as well as in strain MC2Δmrb1Δ7, which moderately overexpressed lga2 (Figure 7A), the mtDNA signal started to disappear between 4 and 8 h after transfer to inducing conditions compared with the nuclear cbx signal (Figure 8F; data not shown). At the 4 h time point (t1), fragmentation was mainly confined to mitochondria of the strongly overexpressing
strain, whereas at the 8 h time point (t2), this was also apparent in strain MC2 Dmrb1 Dmrb1 #7 weakly overexpressing lga2. In these strains, the percentage of highly fragmented mitochondria further increased to ≥96% until 12 h after transfer (Figure 8F). By contrast, ratios of genomic to mtDNA appeared similar in respective control strains lacking pCLN1 as well as in strain MC2 Dmrb1 Dmrb1 #3 moderately overexpressing lga2 (Figure 8F; data not shown).

These results demonstrate a relationship between mitochondrial fragmentation and loss of mtDNA.

Role of lga2 and rga2 in Uniparental Mitochondrial Inheritance?

The observation that overexpression of lga2 induced loss of mtDNA prompted us to investigate whether lga2 plays a role in mitochondrial inheritance as previously suggested (Urban et al., 1996a). The recent release of the mtDNA sequence from U. maydis strain S21 (Whitehead Institute) served as a basis for a comparison of the mitochondrial genomes of strains FB2 (a2b2) and BUB7 (a1b3) and led to the identification of a HindIII restriction fragment length polymorphism (2.3 kb in strain FB2 and 4.5 kb in strain BUB7). To investigate mitochondrial inheritance, we infected plants with mixtures of strain BUB7 and strain FB2 as well as mixtures of BUB7 and two independent FB2 Δlga2 Δrga2 strains. For strain combinations including Δlga2 Δrga2 mutants, six tumors were collected each, and for the wild-type combination, 12 tumors were collected from independently infected plants 7 DAI. DNA gel blot analysis with a probe detecting the polymorphic fragment revealed in all 24 DNA preparations that the band specific for strain BUB7 predominated; in only three probes from wild-type infected tumors an additional faint band specific for FB2 was detected (data not shown). This provides evidence for uniparental mitochondrial inheritance in U. maydis.

Figure 5. Subcellular Localization of Rga2 and Immunodetection of Mrb1, Lga2, and Rga2 in Mitochondrial Fractions.

(A) and (B) Localization of Rga2. U. maydis strains FB2/pRAGC1 (A) and FB2/pRIYC1 (B) were grown in liquid CM/Glu medium and assayed for GFP or YFP fluorescence ([A1] and [B1]) and distribution of the marker dye CM-H2Xros ([A2] and [B2]). Bars in all panels = 10 μm.

(C) Proteins from U. maydis strains (1) FB2/pMGH1, (2) FB2/pRIYC1, (3) FB2 Δmrb1/pRIYC1, (4) FB2/pLIGC1, and (5) CB35 were loaded. Strain CB35 expresses eGFP under the constitutive 9t multifunction promoter (Basse et al., 2000). Cytoplasmic (each 50 μg) and mitochondrial protein fractions (each 5 μg) were loaded and immunostained using a monoclonal GFP IgG mouse antibody. The predicted sizes (kD) of fusion proteins are 52.0 (1), 45.8 (2,3), 46.6 (4), and 26.9 (5). Predicted mitochondrial target sequences were subtracted from the Mrb1 and Lga2 sequences for molecular weight calculation. Numbers at the left refer to positions of molecular mass standards in kD. The existence of a cytoplasmic portion of the Mrb1-eGFP fusion was verified in three independent experiments.

Figure 6. Mitochondrial Structures in Dikaryotic Hyphae during Pathogenic Growth.

(A) to (C) Maize plants were inoculated with mixtures of FB1 Δmrb1/pMB2-2 and FB2 Δmrb1/pMB2-2 ([A] and [B]) or FB1/pMB2-2 and FB2/pMB2-2 ([C]). Tissue samples collected at the time points indicated from the 3rd maize leaf blade beneath the inoculation site were assayed by epifluorescence to detect mitochondrial-localized GFP ([A1], [B], and [C]). The bar refers to all panels. d p.i., days after inoculation. (A2) DIC microscopy from (A1). The arrow marks the hyphal tip.
DISCUSSION

In this study, we have shown that Mrb1 is an important mitochondrial virulence determinant in U. maydis. Most intriguingly, our data have revealed that Mrb1 is only required for pathogenicity in the presence of the a locus genes lga2 and rga2 and to a lesser extent when rga2 is absent. Lga2 and Rga2 proteins were also shown to reside in mitochondria, and Mrb1 and Rga2 were able to interact in the two-hybrid system of yeast. Expression of lga2 from a regulated promoter resulted in fragmentation of tubular structures of mitochondria, a reduction of mitochondrial metabolism, and loss of mtDNA, suggesting a mitochondrial maintenance function for Mrb1.

Mrb1 Is a Member of p32 Family Proteins

Based on its primary sequence and localization, Mrb1 is a member of the p32 protein family that localize predominantly to mitochondria. Despite common structural motifs (Jiang et al., 1999; Hayman et al., 2001), p32 family proteins seem to function in different processes, of which many are attributed to extramitochondrial locations (Krainer et al., 1991; Yu et al., 1995; Petersen-Mahrt et al., 1999; Braun et al., 2000; Van Scoy et al., 2000).

Figure 7. Influence of lga2 Expression on Cellular Growth in the Presence or Absence of mrb1 and Comparison with lga2 Expression under Natural Conditions.

(A) Expression analysis of MC2 (a2b2) and MC2△mrb1 strains after shifting from CM/Glu to CM/Ara medium. An RNA gel blot of total RNA (7 μg per lane) was hybridized with a 32P-labeled lga2 fragment. Radioactive signals (both for lga2) were quantified and standardized by comparison with signals obtained from the ppi gene.

(B) Growth comparison of MC2 and MC2△mrb1 strains in liquid CM/Ara medium at 28°C. The time scale (x axis) refers to the time points after shifting from CM/Glu to CM/Ara medium. MC2#3, closed circle; MC2#5, closed square; MC2△mrb1#7, open circle; MC2△mrb1#8, open square; FB2/pMB2-2, closed triangle; FB2△mrb1/pMB2-2, open triangle. Very similar results were obtained with strains MC2#2, 4 and MC2△mrb1#6, 9 (data not shown).

(C) Individual strains were streaked out from nourseothricin containing solid PD medium on solid CM/Ara (top) or CM/Glu (bottom) medium and incubated for 64 h at 28°C. Control (left), FB2/pMB2-2; control (right), FB2△mrb1/pMB2-2.

(D) Comparison of lga2 expression in MC2 and MC2△mrb1 strains with expression during distinct stages of development. The strains and strain combinations indicated at the top (lanes 1 and 2) were grown on CM-charcoal plates for 48 h for RNA preparation. Lanes 3 and 4, RNA was isolated from infected (FB1×FB2) leaves and leaf tumors 2 and 5 DAI, respectively. Lanes 5 to 7, see (A); 7 μg (lanes 1 and 5 to 7), 15 μg (lane 2), and 80 μg (lanes 3 and 4) RNA were loaded. Two identical RNA gel blots were hybridized with 32P-labeled lga2 and ppi fragments, respectively. Radioactive signals were quantified and the ratios of lga2/ppi hybridization signals were calculated with the most intensive signal (lane 7) set to 100. For lanes 5 to 7, both lga2 signals were used for quantification. Different transcript sizes are probably because of variations in transcription start sites.
2000 and references therein). Only few studies address mitochondrial functions. Among these, a role of p32 in calcium homeostasis was proposed based on structural similarity to calsequestrin (Jiang et al., 1999). Additionally, p32 was found to be associated with mitochondrial protein kinase C and implicated in its activity (Storz et al., 2000). p22 of T. brucei is involved in mitochondrial RNA editing and was shown to stimulate the binding properties of the mitochondrial guide RNA binding protein RBP16 (Hayman et al., 2001). Human p32 and Mam33p of yeast are primarily localized in mitochondria and form oligomeric complexes (Muta et al., 1997; Seytter et al., 1998; Jiang et al., 1999). The demonstration of Mrb1 self-interaction suggests that Mrb1 also adopts an oligomeric structure. However, a mitochondrial function of p32 and Mam33p has not been established conclusively. For Mam33p, it has been demonstrated that it is functionally important for maintaining oxidative phosphorylation in yeast (Muta et al., 1997), but such a role was not detected in an independent study (Seytter et al., 1998). Comparable analysis of Δmrb1 mutants did not reveal growth differences on either fermentable or nonfermentable carbon sources, ruling out a physiologically important role of Mrb1 in mitochondrial functions of haploid cells.

The Interplay among Mrb1, Lga2, and Rga2

We observed that mrb1 was dispensable for pathogenicity of haploid, solopathogenic strains derived from FB1 (a1b1), whereas pathogenicity was abolished in solopathogenic strains derived from FB2 (a2b2). This effect is reminiscent of a recent

Mitochondrial fragmentation was assessed from distribution of mitochondrial GFP fluorescence (see below). At least 200 cells were included for each calculation.

(B) to (E) Strains MC2 (a2b2) and MC2Δmrb1 were assayed 20 h after transfer to liquid CM/Ara medium for GFP fluorescence (GFP) and distribution of the marker dye CM-H2Xros (Mito) by fluorescence and DIC light microscopy. Representative examples of mitochondrial patterns are shown. MC2#2 (B), MC2Δmrb1#7 (C), MC2#6 (D), and MC2Δmrb1#9 ([E1] and [E2]). Arrowheads in (D) point to spot-like fluorescence. The strong mitotracker fluorescence in thread-like mitochondria ([E2]) is compared with the hardly detectable fluorescence of fragmented mitochondria ([E1]) in the same strain. Exposure time for fluorescence photography: 600 ms ([B] and [C]); 2 s ([D] and [E]). All pictures ([B] to [E]) were prepared using Adobe Photoshop, applying identical processing functions and only those that could be applied equally to all pixels of the image being used. Bars in all panels = 10 μm.

(F) Strains indicated on top were precultured in liquid CM/Glu medium, transferred to liquid CM/Ara, and incubated for additional 4 (t1), 8 (t2), 12 (t3), and 14 h (t4). Samples were removed at these time points for analysis of mtDNA degradation. An additional sample was collected from cells immediately before transfer in CM/Ara (t0). Total DNA was digested with BamHI/StuI and simultaneously hybridized with digoxigenin-labeled cyt b and cbx (isolated from pSL-Cbx) fragments. The lower band represents the 1823-bp StuI/BamHI fragment from mitochondrial cyt b. The 4330-bp band (arrowhead) represents the genomic StuI/BamHI cbx fragment. Additional bands stem from plasmid pMB2-2 (cbx) integrated in one or two copies. The percentage of fragmented mitochondria (determined from at least 100 cells) at the various time points is indicated at the bottom.

Figure 8. Overexpression of lga2 Influences Mitochondrial Morphology, Activity, and Integrity of mtDNA.

(A) Mitochondrial fragmentation (%) in the indicated strains during logarithmic growth 17 to 19 h after transfer to liquid CM/Ara medium.
investigation, which showed that a ste20 homolog in U. maydis was particularly required in an a2 background for pheromone-induced mfa2 gene expression and mating (Smith et al., 2004). Most intriguingly, our data have revealed that Mrb1 is required for pathogenicity in normal crosses of compatible strains, but this requirement ceases when the a2 mating partner is mutant in the a2 locus genes lga2 and rga2. The contribution of lga2 and rga2 to this effect is not equal, and Lga2 acts as major player in preventing pathogenic development of Δmrb1 mutants. Whereas Rga2 alone is unable to interfere with pathogenic development of Δmrb1 strains, a concerted action between Lga2 and Rga2 is suggested by the finding that the Δmrb1Δrga2 mutants are less severely affected in pathogenicity than Δmrb1 mutants, in which lga2 and rga2 are intact. Lga2 and Rga2 were also shown to reside in mitochondria, and Mrb1 and Rga2 interacted in the yeast two-hybrid system. Rga2 represents a small, hydrophilic protein of 18.9 kD with a high content of basic amino acids (20.3%) that are predicted to adopt α-helical structures. In line with the basic nature of Rga2, many of the described interaction partners of p32 family proteins are characterized by basic regions (T. brucei RBP16, human ASF/SF2, and Epstein-Barr nuclear antigen-1), suggesting the participation of electrostatic interactions (Hayman et al., 2001 and references therein). The fact that Rga2 localizes to mitochondria in the absence of an N-terminal targeting sequence implicates the existence of an alternative targeting mechanism for which there is precedence (Biswas and Getz, 2002; Stan et al., 2003). At present we have no data concerning functional domains for the mitochondrial localization of Rga2, but its presence in mitochondria does not require Mrb1.

BLAST and PFAM database searches provided no clues to the function of either lga2 or rga2. The tight linkage of pheromone precursor and receptor genes in mating type (MAT) loci is common in basidiomycetes and has also been described in Coprinus cinereus and Schizosaccharomyces pombe (Halsall et al., 2000; Kothe et al., 2003). However, these species do not contain genes related to lga2 and rga2 in their MAT loci. BLAST searches (TBLASTN) of the first release of the C. cinereus genome from the Broad Institute revealed no homologous sequences to either lga2 or rga2 in other parts of the genome but detected a protein sequence similar to Mrb1 (E value, 3e-19). It is thus an interesting possibility that this system for control of mitochondrial function is specific to the plant pathogenic basidiomycetes.

Expression of lga2 Affects Mitochondrial Morphology

Experiments in which lga2 was expressed from the inducible crg1 promoter in wild-type and Δmrb1 mutant cells demonstrated an interference with growth and mitochondrial function. Ectopic expression of lga2 changed mitochondrial morphology from a tubular structure to a dotted appearance, and the extent of mitochondrial fragmentation was determined by the level of lga2 expression and appeared exaggerated in Δmrb1 mutants, corroborating the interaction between Mrb1 and Lga2. This points to the existence of a mitochondrial target of Lga2. The mitochondrial network is a highly dynamic structure and is maintained by balanced membrane fusion and fission events. In S. cerevisiae, mitochondrial fusion and tubular morphology are essential for correct mitochondrial segregation during vegetative growth and sexual development (Nunnari et al., 1997; Yaffe, 1999; Berger and Yaffe, 2000; Westermann, 2002). A component of the yeast mitochondrial fusion machinery is Fzo1, encoding a putative GTPase located in the mitochondrial outer membrane (Rapaport et al., 1998). Yeast fzo1 mutants are deficient in mitochondrial fusion and contain fragmented mitochondria that lose their DNA (Hermann et al., 1998; Rapaport et al., 1998). Recently, it has been determined that Fzo1 levels are regulated by MDM30 (Fritz et al., 2003). MDM30 encodes a mitochondrial protein that lacks significant homologies to other proteins in the database; however, it contains at its N terminus an F-box domain. The F-box is a motif of ~45 amino acids that serves as an interaction site for the Skp1 protein, a component of the Skp1-Cdc53 (cullin)-F-box complex involved in ubiquitin-dependent protein degradation (Patton et al., 1998). Intriguingly, the Lga2 sequence from amino acids 103 to 142 matches the F-box consensus in 17 positions and shows significant similarity to the Mdm30 F-box sequence (Figure 9C). This similarity is consistent with a role of Lga2 in controlling mitochondrial fusion. The existence of an Fzo1 homolog in U. maydis (E value, 2e-77) opens a number of testable predictions to address the function of Lga2 in U. maydis more specifically.

The features uncovered for Lga2 may also relate to apoptosis (Bossy-Wetzel et al., 2003; Priault et al., 2003), and it is conceivable that the fragmentation of mitochondria observed after induction of lga2, as well as its cytotoxicity, are secondary effects and result from permeabilization of mitochondrial membranes. Furthermore, based on mtDNA degradation in response to lga2 overexpression, we expect a reduction in cytochrome c oxidase activity, which could result in increased reactive oxygen species and a decrease in membrane potential as suggested for S. cerevisiae during apoptosis (Ludovico et al., 2002).

A Model for the Role of mrb1, lga2, and rga2 during the Sexual Life Cycle

Although it is clear that Lga2, Rga2, and Mrb1 localize to mitochondria and are likely to be interconnected in their functions, the expression of the respective genes is quite distinct: mrb1 is constitutively expressed (except in teliospores) and rga2 shows a low basal level that is induced after pheromone stimulation and remains at this level in the dikaryon, whereas lga2 expression is undetectable in haploid cells, is induced by pheromone, and is strongly upregulated by the bE/bW heterodimer (Urban et al., 1996b; Romeis et al., 2000).

The reduction in tumor formation seen in combinations of FB1 and FB2Δmrb1 strains, but not in reciprocal crosses, implicates that the lga2 levels attained before fusion might be sufficiently high to interfere with mitochondrial integrity when mrb1 is absent. During subsequent pathogenic development, lga2 levels are still high enough (Figure 7D) to cause mitochondrial fragmentation and mtDNA degradation in the absence of mrb1, consequently reducing metabolic activity, whereas mrb1 would counteract these detrimental effects in infections with wild-type strains. Based on microscopic investigation of pathogenic development of mrb1 mutants, we suggest that the interference of lga2 expression with mitochondrial function specifically affects...
hyphal proliferation in planta. Because pathogenicity was compromised in dikaryotic hyphae resulting from a mixture of FB1Δmrb1 and FB2Δmrb1 strains, both mitochondria types should suffer from the absence of mrb1 (Figure 9).

Why would a fungus develop such a system in which expression of the mitochondrial protein Lga2 can become cytotoxic and interfere with subsequent pathogenic development? The lga2 and rga2 genes map to the a2 mating-type locus, an 8.5-kb region that is dissimilar from the a1 locus and is thus inherited as one unit (Urban et al., 1996a). This assures their presence in all mating events. Based on this linkage and a presumed mitochondrial function of Lga2, a role in mitochondrial inheritance was suggested (Urban et al., 1996a). Previous cytoduction assays, which relied on mitochondrial segregation in the progeny of dikaryotic cells that emerged from haploid U. maydis cells differing in their a loci, provided no evidence for preferred mitochondrial inheritance under axenic culture conditions (Trueheart and Herskowitz, 1992). Data provided in this communication suggest that uniparental inheritance of mitochondria does occur during the biotrophic growth phase, and it will be interesting to elucidate when the decision is made. However, lga2 and rga2 did not basically affect the outcome of this segregation process. Although we cannot formally exclude the possibility of Lga2/Rga2 effects on mitochondrial inheritance in other strain combinations, we consider this possibility unlikely.

Owing to the fact that all phenotypic consequences of lga2 expression are counteracted by Mrb1, we suggest the existence of a complex containing Mrb1, Lga2, and Rga2. In light of the finding of Mrb1 self-interaction and interactions between Rga2 and Mrb1, the complex is expected to be of higher order and may contain additional proteins not yet identified (Figure 9). This complex is predicted to control mitochondrial fusion. Mitochondrial morphology has been shown to be important in response to new energy demands and during toxin exposure (Bereiter-Hahn and Völö, 1994; Bossy-Wetzel et al., 2003 and references therein). In addition, it has been shown that intracellular mitochondrial networks allow the dissipation of energy between remote parts of the cell and can compensate for uneven oxygen supply (Skulachev, 2001). Increasing evidence suggests that mitochondrial structure is regulated during development, and this can involve transcriptional regulation of genes encoding factors of the fission/fusion machinery (Yaffe, 1999; Honda and Hirose, 2003 and references therein). The existence of such a system in U. maydis and its tight control through the mating type loci suggests that mitochondrial morphology needs adaptation during the switch from saprophytic to biotrophic growth. It will be a challenge to find out what special demands the fungus has to cope with in the apoplast of the host plant and how this is linked to fungal fitness via the control of mitochondrial morphology and function.

Recently, the mitochondrial Fow1 protein of Fusarium oxysporum was identified as a first mitochondrial virulence determinant of a plant pathogenic fungus (Inoue et al., 2002). Fow1 displays high similarity to mitochondrial carrier proteins of yeast. Reminiscent to mrb1 mutant strains, fow1 mutants were specifically affected during colonization of plant tissue, and it was suggested that Fow1 might have functions uncoupled from primary mitochondrial functions in response to the host environment.

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**Figure 9.** Model for the interplay among Mrb1, Rga2, and Lga2.  
(A) Arrangement of the lga2 and rga2 genes on the a2 mating-type locus and direct control of lga2 expression by an active bE/bW complex formed in the dikaryon.  
(B) In Δmrb1 mutants, Lga2 and Rga2 interfere with mitochondrial fusion in an uncontrolled manner and thus affect cellular fitness and fungal virulence. The extent of negative contribution relies on the observed genetic relationship and is reflected by the different thickness of the arrows. Although the presence of Lga2 alone is sufficient to compromise pathogenicity, Rga2 can only enhance this effect in an Lga2-dependent manner. In wild-type cells, Mrb1 directly controls Rga2, whereas Lga2 is indirectly controlled, possibly by complex formation with a yet unidentified protein. The complex is presumed to control mitochondrial fusion and to be needed for adaptation of mitochondrial morphology and function in response to special demands during the host interaction. It may thus provide for control of fungal fitness in planta.  
(C) The putative F-Box motif of Lga2 is aligned with the respective sequence of Mdm30 (S. cerevisiae) and an F-box consensus sequence (Patton et al., 1998). Residues that match F-box sequences are shaded. Identical or highly similar amino acids between Lga2 and Mdm30 are in boldface. Gaps have been introduced at a variable position of the F-box consensus sequence to increase the alignment. Small letters refer to amino acids that are not predominant in a position. Letters beneath the consensus refer to amino acids that fit the Lga2 or Mdm30 motifs and are found in at least two other F-boxes (Bai et al., 1996; Patton et al., 1998).
This study is also reminiscent of a recent investigation, which showed that the virulence of hybrid species of the basidiomycete fungus *Heterobasidion annosum*, a pathogen of conifers, is controlled by the mitochondrial genome (Olson and Sterlind, 2001). However, in this system, the nature of the mitochondrial factor involved remains to be determined. Our observation that Mrb1 is specifically required for pathogenicity in the presence of the a2 mating-type locus emphasizes the importance of mitochondrial proteins in controlling virulence in a given nuclear background. In addition, this study has uncovered that products of mating-type genes affect mitochondrial morphology and has documented a critical connection between mitochondrial morphology and fungal development during the pathogenic growth phase.

**METHODS**

### Strains and Growth Conditions

*Escherichia coli* strain TOP10 (Invitrogen, Carlsbad, CA) was used as host for plasmid amplification. *Ustilago maydis* strains, growth conditions, and mating tests were described previously (Basse et al., 2000, 2002). For arabinose induction, precultures grown in CM/Glu (1% glucose [w/v]) were washed twice in water and resuspended at equal densities before arabinose induction, precultures grown in CM/Ara (1% arabinose). Cultures were shaken at 200 rpm at 28°C. Solid CM medium also contained 2% Bacto-agar. Solid YPG medium consisted of 2% Bacto-agar, 1% yeast extract, 2% Bacto-peptone, and 3% glycerol. Plant infections were done with the variety Early Golden Bantam (Olds Seed, Madison, WI) as described (Gillissen et al., 1992). Yeast strain EGY48[p8op-LacZ], which carries *ACTTGTGC-3* under the control of the *LexA* operators, was obtained from Clontech (Palo Alto, CA) and cultivated according to the manufacturer’s instructions.

### DNA and RNA Procedures

DNA and RNA procedures were performed as described (Basse et al., 2000). Labeling and hybridization with digoxigenin followed the manufacturer’s procedure (Roche, Indianapolis, IN). The correctness of all plasmid constructs was verified by sequencing (Automatic DNA Isolation and Sequencing; Max-Planck-Institut, Köln, Germany). Integration of all plasmid constructs was verified by sequencing (Automatic DNA Isolation 2000). Labeling and hybridization with digoxigenin followed the manufacturer’s procedures (Basse et al., 1992). Yeast strain EGY48[p8op-LacZ], which carries *LEU2* and *LacZ* under the control of the *LexA* operators, was obtained from Clontech (Palo Alto, CA) and cultivated according to the manufacturer’s instructions.

### DNA Fragments and Investigation of Mitochondrial Inheritance

As selectable markers for *U. maydis*, hygromycin (*hph*) and carboxin resistance (*ip*) cassettes were isolated as *NotI* fragments as described (Basse et al., 2002). A nucleosorcin (nac) resistance cassette was described previously (Müller et al., 1999). For RNA gel blot analysis, a fragment from the constitutively expressed *ppi* gene (Bohlmann, 1996) and an *iga2* cDNA fragment spanning the complete ORF were used. A 394-bp fragment was amplified from the mitochondrial *U. maydis* cytb gene using primers M18 (5'-CAGTTGGAGGACTTGCTACATG-3')/M19 (5'-AGCATAGCAGGTAGTACAC-3'). An mtDNA fragment of 554 bp was amplified from total *U. maydis* DNA using primers M18 (5'-CACGTGTGGAGGTTGCTAATCC-3')/M29 (5'-GGCTACCTC-ATGTTGGATTAGGAG-3') to detect a HindIII fragment length polymorphism between *U. maydis* strains FB2 (*a2b2*) and BUB7 (*a1b3*).

### Deletion of mrb1

The *NheI-NotI* fragment, comprising *mrb1* isolated from pS (Basse et al., 2002), was introduced into the EcoRI and HindIII sites of pUC18 to yield pXM. The 608-bp genomic *BssHI-KpnI* fragment was replaced in pXM by the *ip* and *hph* cassettes, respectively. The deletion comprised the complete *mrb1* ORF except 85 bp at the 5' end and 123 bp at the 3' end. To replace the resident *mrb1* gene with the *mrb1* allele, pXM*dmrb1-ip* and pXMD*mrb1-hph* linearized at the *NdeI* and Eco47III sites, respectively, were introduced into *U. maydis* strains FB1, FB2, and SG200.

### Deletion of iga2 and rga2

The generation of deletions spanning the *iga2* and *rga2* genes was performed by a PCR-based approach (Kämpfer, 2004). The *iga2* and *rga2* double deletion comprised positions 5780 to 7908 of the *mfsl* locus (GenBank accession number U37796). The individual *iga2* and *rga2* deletions comprised positions 5780 to 6678 and 7385 to 7908, respectively, of the *mfsl* locus.

### Fusion Constructs

For construction of eGFP reporter constructs pMB2-1 and pMB2-2, pXM was amplified with the primers xs1 5'-ATAGTATGATGACGACGCTGCGGCAATGTCGCTATTC-3' and xs2 5'-GAGGCTACCTGAGCGAGGCTGAGAT-3' and xs1/xs3 5'-GCCACCATGGCGTGGTATTCGCCTGACC-3', respectively. PCR products were cleaved with *BspHI* and/or *NcoI* and introduced into the *NcoI* site of pHD3 (Basse et al., 2002). pMB2-1 and pMB2-2 were linearized with *AatII* and integrated ectopically in the genome of *U. maydis*. For construction of the *mrbl>eGFP* fusion construct pMGH1, pXM was amplified using the *mrbl*-specific primers 5'-CAATGATGATGACGACGCTGCGGCAATGTCGCTATTC-3'/5'-GATTCCATGACGCGGAC-3'. The PCR product was cleaved with *BspHI* and inserted into the *NcoI* site of pHD3. For construction of pLI031, genomic FB2 DNA was amplified with primers 5'-GAGGCTACCTGAGCGAGGCTGAGAT-3'/5'-GAGCTGAGCAGAGTGCCTGACC-3'. The PCR product was cleaved with *BspHI* and inserted into the *NcoI* site of pHD3. For construction of pRIY01, genomic FB2 DNA was amplified with primers RG1/RG2 (5'-CATGATGATGACGACGCTGCGGCAATGTCGCTATTC-3'/5'-GATGCCTGAGCAGAGTGCCTGACC-3'). The PCR product was cleaved with *BspHI* and inserted into the *NcoI* site of pHD3. All plasmids were linearized with *BspHI* and inserted into the *NcoI* site of pHD3. All plasmids were linearized with *AatII* for homologous integration into the *ip loci* (Basse et al., 2000).

### Construction of pCLN1

A genomic *iga2* fragment extending the complete ORF sequence at the 3' site was amplified from FB2 DNA using primers 5'-GAAATTCATG-CTGCTCTTGCTATATCC-3'/5'-GCAAGATATGCTTAGCTGAGAGTACACGACC-3'. The PCR product was cleaved with *NdeI*/EcoRV and...
inserted into the HindIII/NcoI sites of plasmid pRU11, which contains the 
crg1 promoter (Bottin et al., 1996; Brachmann et al., 2001). pCLN1 was
obtained from this plasmid by inserting a rat cassette into the PmlI site.
pCLN1 was linearized with Scal for transformation into U. maydis.

Transformation of b1 and b2 Alleles
Plasmids pUB2-2 and pUB1 contain 8-keb BamHI fragments encompassing
the complete be2 and be1 genes, respectively (Schulz et al., 1990). Both plasmids carry the hph gene. pUB2-2 linearized with NofI was
introduced into U. maydis strains FB1 and FB1/mrb1. pUB1 was
linearized with MluI and introduced into strains FB2 and FB2/mrb1. Integration of plasmids was confirmed by PCR using primers that bind in
the variable portions of bW2 and bW1/be1, respectively. The presence of
an active be1/bW2 complex in pUB1 transformants was verified by
phorome stimulation with the compatible donor strain FB6b (a1b2), and
the presence of an active be2/bW1 complex was verified by phorome
stimulation assays with strain FB6a (a2b1).

Interaction among Mrb1, Lga2, and Rga2
For construction of pLexA-mrb142, a mrb1 fragment was amplified from
positions 124 to 825 of the mrb1 ORF and introduced into the BamHI and
NcoI sites of pLexA (Gyuris et al., 1993). For construction of pLexA-iga243
and pLexA-rga245, iga2 and rga2 fragments were amplified from a FBD11
cDNA preparation (Basse et al., 2000) from positions 127 to 648 (iga2)
and 64 to 477 (rga2) and introduced into the BamHI/NcoI and EcoRI/NcoI sites of
pLexA. For construction of pB42AD-mrb146, pB42AD-iga244, and
pB42AD-rga245, fragments were amplified from positions 121 to 825,
130 to 648, and 64 to 477 from the ORG regions of mrb1, iga2, and
rga2, respectively, and introduced into the XhoI site of pB42AD (Gyuris,
1993). Plasmids pGB-E1473, pGB-E2473, and pW2546-GA have
been described (Kämper et al., 1995). Transformation of yeast strain
EGY48[p80p-LacZ] followed Clontech’s instructions. For quantification of
β-galactosidase activity according to Miller (1972), cells were grown in
galactose/raffinose-containing selective medium to an OD600 of 0.8
to 1.4.

Isolation of Mitochondria from U. maydis
U. maydis strains were grown in CM/Glu (1%) medium. Protoplasts were
prepared as described (Schulz et al., 1990) using an STC solution lacking
CaCl2 (20 mM Tris-HCl, pH 7.5, and 1 M sorbitol). Mitochondria were
prepared from protoplasts according to the yeast protocol (Yaffe, 1991).
The cytoplasmic fraction was collected from the 9500
g centrifugation according to the yeast protocol (Yaffe, 1991).

Immunodetection
Immunoblot analysis was performed as described (Basse et al., 2000)
using a monoclonal GFP IgG mouse antibody (Roche).

Microscopy
Microscopy and processing of images was performed as described
(Basse et al., 2000). Cell wall components were stained with 2 μg/mL
of Calcofluor-white (Sigma, St. Louis, MO) in PBS. The existence of
dikaryotic hyphae was verified by 4′,6-diamidino-2-phenylindole (DAPI)
staining: U. maydis cells grown on solid medium were fixed with
4% (v/v) formaldehyde and stained with 0.5 μg/mL of DAPI (Sigma)
in PBS, pH 7.2, at 60°C. Samples were observed with DiC optics or under
fluorescence microscopy (excitation/emission for DAPI or Calcofluor-
white, eGFP, YFP: 365/397 nm; 450 to 490/515 to 565 nm; 490 to 520/
505 to 565 nm). To visualize mitochondria, U. maydis cells were incubated
with 0.5 μM Mito-Tracker CM-H2Xros (Molecular Probes, Eugene, OR) in
the same medium as used for cell culture for 20 min at 25°C and observed
under fluorescence microscopy (excitation/emission: 546/590 nm).
Staining with CBE followed the method of Brundrett et al. (1996).

The nucleotide sequence data referred to the mig2 locus from position
8423 to 10,542 (Basse et al., 2002), comprising mrb1 coding and 5′/3′
noncoding sequences, have been submitted to the GenBank database
under accession number AY574042. GenBank accession numbers for the
sequences of the U. maydis mfa2 locus and the cyt b gene are U37796
and AB040663, respectively.

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Absence of the Mitochondrial p32 Family Protein Mrb1 Compromise Pathogenicity in the rga2 and lga2 Mating-Type Locus Genes of Ustilago maydis a2

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