The a2 Mating-Type Locus Genes lga2 and rga2 Direct Uniparental Mitochondrial DNA (mtDNA) Inheritance and Constrain mtDNA Recombination During Sexual Development of Ustilago maydis

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ABSTRACT

Uniparental inheritance of mitochondria dominates among sexual eukaryotes. However, little is known about the mechanisms and genetic determinants. We have investigated the role of the plant pathogen Ustilago maydis genes lga2 and rga2 in uniparental mitochondrial DNA (mtDNA) inheritance during sexual development. The lga2 and rga2 genes are specific to the a2 mating-type locus and encode small mitochondrial proteins. On the basis of identified sequence polymorphisms due to variable intron numbers in mitochondrial genotypes, we could demonstrate that lga2 and rga2 decisively influence mtDNA inheritance in matings between a1 and a2 strains. Deletion of lga2 favored biparental inheritance and generation of recombinant mtDNA molecules in combinations in which inheritance of mtDNA of the a2 partner dominated. Conversely, deletion of rga2 resulted in predominant loss of a2-specific mtDNA and favored inheritance of the a1 mtDNA. Furthermore, expression of rga2 in the a1 partner protected the associated mtDNA from elimination. Our results indicate that Lga2 in conjunction with Rga2 directs uniparental mtDNA inheritance by mediating loss of the a1-associated mtDNA. This study shows for the first time an interplay of mitochondrial proteins in regulating uniparental mtDNA inheritance.

MITOCHONDRIA play vital roles in eukaryotic bioenergetics, and processes mediating mitochondrial genome integrity and inheritance are essential for life (Barr et al. 2005). In sexual eukaryotes, mitochondrial and chloroplast genomes are almost exclusively transmitted from only one parent. Mitochondria or their DNA can be eliminated from one gamete prior to fertilization, excluded from entering the zygote during fertilization, or selectively degraded after fertilization (Birky 2001). Uniparental organelle inheritance has been intensively studied in the slime mold Physarum polycephalum, the basidiomycete fungus Cryptococcus neoformans, and the green alga Chlamydomonas reinhardtii. In these species, organelle inheritance is influenced by the mating-type loci. In C. neoformans, mitochondrial DNA (mtDNA) is typically inherited from the MATa parent (Xu 2005). A complex hierarchy in the transmission of mtDNA is found in P. polycephalum and determined by the >13 matA alleles. In C. reinhardtii, mtDNA is inherited from the mating-type minus (mt−) parent, whereas chloroplast DNA is inherited from the mating-type plus (mt+) parent. Despite intensive research on sexual inheritance of organelles over the past 40 years, very little is known about the underlying mechanisms and the responsible genes (Birky 2001; Barr et al. 2005; Xu 2005). In C. neoformans, uniparental mtDNA inheritance is governed by the MAT locus-encoded SXI1α and SXI2α homeodomain proteins. Disruption of either of these genes resulted in biparental mtDNA inheritance and significant heteroplasmy; i.e., the existence of different mitochondrial genotypes (mitotypes) in an individual cell (Yan et al. 2004, 2007; Hull et al. 2005). However, the downstream targets of the presumed SXI1α/SXI2α complex have not yet been identified.

The basidiomycete fungus Ustilago maydis served as a genetics model >4 decades ago when used in seminal studies on recombination and repair by Robin Holliday (Holliday 2004). U. maydis represents one of the most intensively studied fungal pathogens, and research profits from its molecular tractability and the annotated genome sequence (Kahmann and Kämper 2004; Kämper et al. 2006). Completion of the sexual life cycle of U. maydis is intimately coupled to biotrophic growth in its host plant maize and is accompanied with host tumor formation. These tumors promote massive fungal proliferation and subsequent differentiation into the diploid teliospores (Bannett and Herskowitz 1996; Kahmann et al. 2000).

Sexual development of U. maydis is governed by the a and b mating-type loci. The a locus exists in two alleles, a1 and a2, which provide for pheromone-based recognition.
between compatible haploid sporidia followed by fusion (Bölker et al. 1992;Spellig et al. 1994). The multiallelic b locus contains the divergently transcribed homeodomain-encoding bE and bW genes. The combination of different b alleles in the dikaryon leads to formation of the bE/bW complex, which functions as a transcriptional regulator governing the switch from yeast-like to filamentous growth and subsequent pathogenic development (Kronstad and Leong 1989; Kämper et al. 1995 and references therein; Kahmann et al. 2000; Kahmann and Kämper 2004). The a2 locus contains two additional unique genes, lga2 and rga2, which encode small mitochondrial proteins among which Lga2 harbors a F-box-like motif. Genes related to lga2 and rga2 are absent in a1 strains (Urbán et al. 1996a; Bortfeld et al. 2004). Expression of both genes is tightly linked to sexual development. While lga2 and rga2 are upregulated in response to pheromone stimulation, lga2 is a direct target of the bE/bW complex (Urbán et al. 1996b; Romes et al. 2000; Brachmann et al. 2001). Previous cytoduction experiments have revealed that mitochondria can freely segregate from dikaryotic cells, irrespective of their associated mating genotype. The requirement of compatible loci for cytoduction further raised the question of whether the a locus plays a role in mitochondria segregation (Trueheart and Herskowitz 1992). A role of lga2 and rga2 in uniparental mtDNA inheritance was suggested on the basis of their linkage to the a2 locus, stage-specific expression and mitochondrial localization of the products (Urbán et al. 1996a; Bortfeld et al. 2004). Comparison of mitochondrial genomes of the U. maydis strains BUB7 (a1) and FB2 (a2) led to the identification of a restriction fragment length polymorphism (RFLP), which allowed investigation of mtDNA inheritance during pathogenesis. This demonstrated efficient uniparental mtDNA inheritance in favor of the a1-associated mitotype (termed m1); however, mtDNA inheritance was apparently not influenced by the Δlga2Δrga2 deletion in this strain combination (Bortfeld et al. 2004).

On the basis of the identification of distinct mitotypes in U. maydis isolates and analysis of transmission patterns of mtDNA in a multitude of crosses, we can now demonstrate a decisive influence of lga2 and rga2 on uniparental mtDNA inheritance and the generation of recombinant mitotypes during sexual progression in the host. This has allowed us to make further distinctions between the roles of a and b in the life cycle of U. maydis.

**MATERIALS AND METHODS**

**Strains and growth conditions:** Origin and genotypes of U. maydis strains are listed in Table 1. U. maydis strains were grown at 28°C in YEPSl medium [1% (w/v) yeast extract, 0.4% (w/v) peptone, and 0.4% (w/v) sucrose modified from Tsukuba et al. (1988)], complete medium (CM; Holliday 1974), on solid potato dextrose (PD) medium [2.4% (w/v) PD, 2% (w/v) Bacto agar (Difco, Sparks, MD)], or on solid minimal medium (Holliday 1974) containing 2% (w/v) acetate (pH 7.0), 2% (w/v) glutamate (pH 7.0), or 1% (w/v) glucose as the carbon source. CM contains 1% (w/v) glucose. CM/Ara contains 1% (w/v) arabinose instead of glucose. U. maydis teliospores were allowed to germinate on solid PD medium containing 0.1% (w/v) of a saturated tetracycline solution. Resulting colonies were spread on solid PD medium to yield single meiotic products. Mating assays and infection of 6- to 7-day-old maize (Zea mays) plants (var. Early Golden Bantam; Olds Seed, Madison, WI) was done as described (Holliday 1974; Banuett and Herskowitz 1989; Basse 2005). For pheromone stimulation, synthetic Mfa2 pheromone (Köppitz et al. 1996; kindly provided by M. Feldbrügge, Max-Planck-Institute, Marburg, Germany) was added at a final concentration of 2.5 μg/ml to a logarithmically growing culture transferred to fresh CM and then incubation proceeded on a vertical rotary platform (10 rpm) at 28°C for 6 hr. The antibiotics hygromycin B (hyg), carboxin (cxb), and nourseothricin (ntr) were purchased from Roche (Mannheim, Germany), Riedel-de Haën (Hannover, Germany), and Werner BioAgents (Jena-Cospeda, Germany), respectively. Chemicals were of analytical grade and obtained from Sigma (Taufkirchen, Germany) or Roth (Karlsruhe, Germany).

**DNA and RNA procedures:** Escherichia coli K12 strain TOP10 (Invitrogen, Karlsruhe, Germany) was used as host for plasmid amplification. Nucleic acid procedures and quantification of radioactive signals were performed as described (Basse et al. 2000). Only cloned fragments were used for DNA and RNA gel blot analysis. The mtDNA fragment (554 bp) used for RFLP analysis and the pfs probe have been described (Bortfeld et al. 2004). A 532-bp rga2 BamHI fragment was isolated from plasmid pot-rga2 (see below). Restriction enzymes were from New England Biolabs (Frankfurt am Main, Germany) and oligonucleotides (see supplemental Table 1) were from MWG (Ebersberg, Germany). The correctness of all plasmid constructs was verified by sequencing (Automatic DNA Isolation and Sequencing, Max-Planck-Institut, Köln, Germany). PCR products were cloned into pCR4-TOPO (Invitrogen) for sequence analysis.

**Mitotype determination:** RFLP analysis was described as described (Bortfeld et al. 2004). Total DNA from infected maize tumor tissue [9 or 10 days post inoculation (dpi) if not otherwise specified] containing thousands of fungal spor precursor cells was isolated with the DNeasy plant kit (Qiagen, Hilden, Germany); 0.2–1 μg was restricted with HindIII and separated on 1% (w/v) agarose for subsequent blotting (Hybond N+; Amersham-Pharmacica Biotech, Freiburg, Germany). For analysis of the mitotypes by PCR, the primer pairs prl01/prl02, prl03/prl04, and prl05/prl06 spanning the polymorphic region from positions 78 to 6411 were used (if not otherwise indicated all positions refer to the mitochondrial genome sequence of U. maydis strain 521; NCBI accession no. DQ157700).

**Analysis of group I and II introns:** RNA was isolated from overnight cultures (YEPSl medium) of strain FB1 (group I); from strains MF18, MF34, GF5, and GF25 (group II); and from a mixture of FB1/FB2 strains (group I) grown on solid CM charcoal medium for 2 days. RNA was treated with DNase (Roche) as described (Basse et al. 2000) and reverse transcribed for 60 min at 55°C in the presence of SuperScriptIII (200 units; Invitrogen) using gene-specific primers prl07 and prl04 for group I intron analysis, and primer prl06 for group II intron analysis. The cdNA preparations from primer prl07 were amplified with the combination prl08/prl07 (positions 431–2495), those from primer prl04 with the combination prl03/prl04 (positions 3542–6411), and those from primer prl06 with the combination prl05/prl06 (positions 2945–3667) using Taq polymerase (MBI Fermentas, St. Leon-Rot, Germany).
Generation of lga2/rga2 gene deletions: U. maydis strains deleted in lga2 and/or rga2 were generated by a PCR-based gene deletion procedure as described (Bortfeld et al. 2004, Kämper 2004). The hyg resistance cassette contained in the replacement constructs was isolated by SfiI from pBSHhn (Kämper 2004). Homologous recombination in U. maydis transformants was confirmed by diagnostic PCR to demonstrate the absence of the endogenous gene as well as integration of left and right borders as described (Kämper 2004).

Generation of U. maydis a1 strains expressing rga2: For construction of pR-rga2, the rga2 ORF—extended by 687 bp of the 5’ and 765 bp of the 3’ region—was amplified (Phusion polymerase; New England Biolabs) from genomic DNA of transformants was verified by PCR using the primer pairs insertion into the 5’ polymerase; New England Biolabs) from genomic DNA of transformants was confirmed by diagnostic PCR to demonstrate the absence of the endogenous gene as well as in- 

Netherlands) using the primer pair pril20/pril21. Products resulting plasmid pMBR2 and plasmid pMB2-2, served as vector for sequencing analysis.

Transformation into transformants was verified by PCR using the primer pairs insertion into the 5’ polymerase; New England Biolabs) from genomic DNA of transformants was confirmed by diagnostic PCR to demonstrate the absence of the endogenous gene as well as in-

mRNA Inheritance in U. maydis

RESULTS

U. maydis mitochondrial genotypes differ in the number of group I and group II introns: To address a role of lga2 and rga2 in uniparental mtDNA inheritance in U. maydis, we were interested in the identification of additional mitotypes. Previous analysis had revealed a HindIII RFLP in mitochondrial genomes of wild-type strains BUB7 (a1) and FB2 (a2), which produced diagnostic 4.5- and 2.3-kb fragments, respectively (Bortfeld et al. 2004). We termed the corresponding mitotypes B and F, respectively. To identify more mitotypes, we compared by RFLP analysis mitochondrial genomes of meiotic segregants from teliospores collected in the field (Table 1). This led to the identification of a third mitotype (termed W; Table 1) producing a diagnostic HindIII fragment of >10 kb. The identity of all new a1 and a2 strains was verified by sequence analysis of the mfa1 and lga2 ORF regions, respectively, which showed 100% identity to the known sequences. Furthermore, all new isolates carried known b alleles except for strain MF14, which carried a b allele, with 95% identity to the bW4a sequence (Table 1).
Sequence analysis of the polymorphic regions from a variety of U. maydis strains revealed that RFLPs detected among F, B, and W mitotypes were caused by substantial insertions and deletions in the region of the large subunit (LSU) rRNA gene (Table 2; Figure 1). In particular, insertions and deletions in the region of the large subunit (LSU) rRNA gene (Table 2; Figure 1). The three intronic regions of the F type were from positions 48 to 149 and 199 to 308, respectively (see discussion). The F-type 521 sequence further differs from W-type sequences by three comprehensive insertions from positions 774–2107, 4034–5160, and 5216–6333 (Figure 1). Sequence alignments with the program Rfam assigned the two regions between 4034 and 6333 to group I introns (see Supplementary Figure 1B; Saldanha et al. 1993; Haugen et al. 2005). The predicted border sequences were explicitly verified by sequencing of corresponding cDNA preparations from FB1 and FB2 strains (see materials and methods). This further confirmed the small region from 5161 to 5215 as an exon sequence and showed that the mature F-type sequence exactly corresponds to the mature W-type sequence within the LSU rRNA region (Figure 1).

| Strains | Genotypes | Mitotypes | References or source |
|---------|------------|-----------|---------------------|
| 521     | a1 b1      | F         | Kämper et al. (2006) |
| FB1     | a1 b1      | F         | Banuett and Herskowitz (1989) |
| FB2     | a2 b2      | F         | Banuett and Herskowitz (1989) |
| FB6a    | a2 b1      | F         | Banuett and Herskowitz (1989) |
| FB6b    | a1 b2      | F         | Banuett and Herskowitz (1989) |
| FB1/pR-rga2#1,2 | a1 b1 pR-rga2-nat | F | This study |
| FB2lga2 | a2 b2 lga2Δ::hyg | F | Bortfeld et al. (2004) |
| FB2lga2 | a2 b2 lga2Δ::hyg | F | Bortfeld et al. (2004) |
| FB2lga2Δrga2 | a2 b2 Δrga2Δ::hyg | F | Bortfeld et al. (2004) |
| MF14+  | a2 b4+     | F         | This study |
| MF14Δlga2Δrga2 | a2 b4 Δlga2Δrga2Δ::hyg | F | This study |
| BUB7    | a1 b3      | B         | This study |
| BUB8    | a2 b4      | B         | This study |
| MF38+  | a1 b18+    | B         | This study |
| MF18+  | a1 b17+    | W         | This study |
| MF18/pot-rga2#1,2 | a1 b17 pot-rga2-chx+ | W | This study |
| MF34+  | a1 b14+    | W         | This study |
| GF5+   | a2 b13+    | W         | This study |
| GF5lga2 | a2 b13 lga2Δ::hyg | W | This study |
| GF5lga2 | a2 b13 lga2Δ::hyg | W | This study |
| GF8+   | a2 b13     | W         | This study |
| GF8lga2 | a2 b13 lga2Δ::hyg | W | This study |
| GF8lga2 | a2 b13 lga2Δ::hyg | W | This study |
| GF25+  | a1 b13     | W         | This study |
| GF63+  | a1 b13     | W         | This study |

*Isolated as meiotic segregants from teliospores collected in the Marburg area during 2004 and 2006. A 95% identity to the bW4a sequence (accession no. AJ630076; see materials and methods). Isolated as meiotic segregants from teliospores collected in the Bonn (Germany) area. B. Scholz, unpublished data. Accession numbers for b13, b14, b17, and b18 are AJ630069, AJ630070, AJ630073, and AJ630074, respectively.

The LRIII sequence furthermore contains a putative LAGLIDADG homing endonuclease gene with two predicted LAGLIDADG domains from amino acid positions 48 to 149 and 199 to 308, respectively (see discussion). The F-type 521 sequence further differs from W-type sequences by three comprehensive insertions from positions 774–2107, 4034–5160, and 5216–6333 (Figure 1). Sequence alignments with the program Rfam assigned the two regions between 4034 and 6333 to group I introns (Evalues <10<sup>-17</sup> and 10<sup>-14</sup>, respectively), while the region from 774 to 2107 was not recognized as such. Analysis of 5′ and 3′ border sequences of the three insertions confirmed the existence of consensus motifs assigned to group I introns (see supplemental Figure 1B; Saldanha et al. 1993; Haugen et al. 2005). The predicted border sequences were explicitly verified by sequencing of corresponding cDNA preparations from FB1 and FB2 strains (see materials and methods). This further confirmed the small region from 5161 to 5215 as an exon sequence and showed that the mature F-type sequence exactly corresponds to the mature W-type sequence within the LSU rRNA region (Figure 1). The three intronic regions of the F type were designated LRI1, LRI2, and LRI3, respectively. In
TABLE 2
Sequence analysis of polymorphic mtDNA regions from various *U. maydis* strains and meiotic segregants

| Strain(s) | Deletions | Insertions | Mismatches |
|-----------|-----------|------------|------------|
| **F type** |           |            |            |
| FB1*, FB2* | 2512      | 4320       |            |
| MF14      | 2512      | 4320       | 5020/03 (8) |
| **B type** |           |            |            |
| BUB7      | 1126-1132 | 3413/14 (1960) | 3076 (a-c) |
| **W type** |           |            |            |
| MF18      | 1126-1132 | 3413/14 (1932) | 3076 (a-c) |
| MF34      | 2512      | 4320       | 5020/03 (8) |
| GF5*, GF63* | 2512     | 4320       | 5020/03 (8) |

Sequences of positions 1–6411 were analyzed. The numbers indicate the positions of the sequence deviations. All positions refer to the published sequence of strain 521. All sequence deviations for a given strain were confirmed by independent sequencing of different clones. SRX1, SRX2, and SRX3 were isolated from the sexual progenies of the MF34 × MF14Δlga2Δrga2, FB1/pR-rga2#1 × GF5, and MF18 × MF14Δlga2Δrga2 combinations, respectively.

The sequences of FB1 and FB2, and of GF5 and GF63, respectively, are 100% identical.

Indicates insertion of 8 bp between positions 5602 and 5603.

accordance with their identification as introns, BLASTN sequence analysis indicated the absence of LRI1, LRI2, and LRI3 from the closely related LSU rRNA gene of *Tilletia walkeri* (accession no. EF536375), while bordering sequences including the region from 5161 to 5215 were conserved. In conclusion, F-, B-, and W-type sequences differ from each other by the number of group I or group II introns.

To exclude differential fitness effects at the level of mtDNA molecules in potential mating partners that carry discernible mitotypes (*de la Bastide* and *Horjen* 2003), we explicitly compared growth, mating, and pathogenicity between W- and F-type strains. In comparison with the F-type strains FB1 and FB2, the W-type strains MF18, MF34, GF25, and GF5 were not impaired in growth in medium containing fermentable or non-fermentable carbon sources (see supplemental Figure 2, A and B). Furthermore, compatible W-type strains were able to mate and triggered disease symptoms not different from those in plants infected with the FB1/FB2 combination (see supplemental Figure 2, C and D).

**Inheritance of the mitochondrial F, B, and W types:** RFLP analysis of mtDNA inheritance in the BUB7/FB2 combination indicated a ratio close to 1 for B- to F-type signals between 1 and 2 days after host inoculation, while this ratio increased from 10- to 20-fold in the subsequent 2 days, suggesting that mtDNA elimination proceeds during fungal growth in the host (data not shown). To analyze mtDNA inheritance at the terminal stage of sexual development, we performed RFLP analysis with total DNA isolated from tumors at 6–10 dpi. At this stage, spore formation has been initiated. We first analyzed combinations between the B and F types. As in the BUB7 (a1)/FB2 (a2) combination, the B type also dominated over the F type in the BUB7/FB6a combination, whereas a biparental pattern of inheritance was detected for the BUB7/MF14 combination (Table 3). Nonetheless, heteroplasmy was only rarely detected in individual spores (Table 4; see below). Next, combinations of F and W types were tested. In the FB1/GF5 and FB1/GF8 combinations, the W type of the a2 strains GF5 and GF8 dominated over the F type (Figure 2, A and B). Conversely, the F type of the a2 strains FB2 and MF14 dominated over the W type of the a1 strains MF18 or MF34 (Table 3; Figure 2, B and C). In contrast, the GF25/FB2 and GF63/FB2 combinations produced a biparental pattern of inheritance and new mtotypes in addition (Table 3; Figure 2A; data not shown; see below).

Finally, in combinations of B and W types, the B type of the a2 strains BUB8 dominated over the F type of the a1 strains MF18 or MF34, whereas the W type of the a2 strain GF8 dominated over the B type of strain BUB7. For the BUB7/GF5 combination, biparental inheritance was detected (Table 3). This indicates that mtDNA transmission does not follow a hierarchy of individual mitotypes, and in numerous combinations tested, the a2-associated mitotype (termed m2) dominated.

The *U. maydis* *lga2* and *rga2* genes affect mtDNA inheritance during sexual development: On the basis of dominant inheritance of the m2 mitotype in numerous combinations, we addressed whether *lga2* and *rga2* influenced mtDNA inheritance during sexual development. Furthermore, it was interesting to examine mtDNA
Strikingly, in the FB1/GF8 combination, inheritance was strongly biased toward the m1 mitotype W, instead of prevalent inheritance of the m2 mitotype F, accompanied with loss of the parental F type. Next, we analyzed combinations with the W type with either the F or B types allowed detection of new alleles by RFLP analysis (see Figure 1). Hence, we examined mtDNA transmissions in combinations involving the W type a1 strains GF25, GF63, MF18, and MF34 and a2 strains GF5 and GF8 (Table 1). To discriminate between the effects of lga2 and rga2, these genes were individually deleted in the a2 mating partner. Interestingly, in contrast to exclusive inheritance of the W type in the FB1/GF8 combination, in the FB1/GF8Δlga2 combination, three additional nonparental bands were detected, indicative of new mitotypes. These were named X1, X2, and X3 (Figure 2A). These mitotypes, along with both parental mitotypes, were also detected in the GF25/FB2 wild-type combination, while in the GF25/FB2Δrga2 combination, the parental F type was no longer detected (Figure 2A). A similar influence of the lga2 deletion was seen for the MF34/FB2 combination in which the m2 mitotype F dominated, while in the absence of lga2, the F type was lost and formation of the W type was strongly increased (Figure 2B). In contrast to exclusive inheritance of the W type in favor of the m2 mitotype F (Figure 2B and data not shown). Together, this indicates that the rga2 deletion strongly compromises inheritance of the m2 mitotype in favor of the m1 mitotype in both F/W and W/F combinations. The influence of lga2 and rga2 on mtDNA inheritance was further analyzed in the BUB7 (B)/GF5 (W) combination. While the wild-type combination produced biparental inheritance in favor of the m2 mitotype W, in the absence of lga2, inheritance of the m1 mitotype B, along with the X2 type, dominated. Further, in the absence of rga2, the m1 mitotype was exclusively inherited (see supplemental Figure 3 and discussion). The critical requirement for rga2 for m2 inheritance in all combinations tested raised the question of whether rga2 influenced mtDNA inheritance in dependence on lga2. We therefore generated a2 strains deleted in both lga2 and rga2. Analysis of mtDNA inheritance in the MF34/FB2Δlga2Δrga2, MF18/FB2Δlga2Δrga2, and GF25/FB2Δlga2Δrga2 strain combinations revealed that the Δlga2Δrga2 double deletion led to a very similar outcome of inheritance as the single lga2 deletion in the respective combinations (Figure 2C and data not shown). Taken together, these results indicate that Lga2 and Rga2 play distinct roles in mtDNA inheritance and that rga2 is critical for inheritance of the m2 mitotype only in conjunction with lga2.

Expression of rga2 in both mating partners eliminates the influence of lga2: The critical requirement for rga2 in inheritance of the m2 mitotype implied a func-
tion in protecting mtDNA from elimination. In such a scenario, expression of \textit{rga2} in both mating partners was expected to enable inheritance of both parental mitotypes. To test this assumption, plasmid \textit{pR-rga2}, which provides for \textit{rga2} expression under native regulatory sequences, was inserted ectopically into the \textit{a1} strain \textit{FB1}. Expression of \textit{rga2} in \textit{FB1/pR-rga2} strains under pheromone-stimulating conditions was verified by RNA gel blot analysis. This showed that \textit{rga2} transcript levels were elevated ~13-fold more in strain \textit{FB1/pR-rga2}\#1

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Influence of the \textit{a2} locus genes \textit{lga2} and \textit{rga2} on mtDNA inheritance. (A) Combinations with \textit{\Delta lga2} strains (left: \textit{FB1} with \textit{GF8} or \textit{GF8}\textit{\Delta lga2}; right: \textit{GF25} with \textit{FB2} or \textit{FB2}\textit{\Delta lga2}). (B) Combinations with \textit{\Delta rga2} strains (left: \textit{FB1} with \textit{GF8}\textit{\Delta rga2}, \textit{FB1} with \textit{GF5} or \textit{GF5}\textit{\Delta rga2}; right: \textit{GF25} with \textit{FB2}\textit{\Delta rga2}, \textit{MF34} with \textit{FB2} or \textit{FB2}\textit{\Delta rga2}). (A and B) Maize plants were inoculated with the combinations indicated above the panels for RFLP analysis. The \textit{a1} partner (\textit{m1}) is on the left, and the \textit{a2} partner (\textit{m2}) is on the right. Each lane represents the total DNA isolated from one tumor and hybridized with the mtDNA-specific probe (see Figure 1). Each tumor was isolated from a different plant. Lanes \textit{m1} and \textit{m2} are controls loaded with DNA from the corresponding parental strains. All lanes in each panel are from the same blot. Mitochondrial bands indicative for the \textit{W}, \textit{X1}, \textit{X2}, and \textit{F} types are marked (arrowheads). Lanes clearly showing \textit{X2} are denoted by open triangles. (C) Quantitative analysis of the influence of the \textit{\Delta lga2}, \textit{\Delta rga2}, and \textit{\Delta lga2}\textit{\Delta rga2} deletions on uniparental mtDNA inheritance in combinations with three different \textit{a1} partners. Bands corresponding to the \textit{W}, \textit{X1}, and \textit{F} types were quantified from Southern blot analysis. For each strain combination (written as \textit{a1/a2} below the graph; \textit{\Delta l} = \textit{\Delta lga2}, \textit{\Delta r} = \textit{\Delta rga2}), average values and standard deviations of the \textit{W} (solid), \textit{X1} (shaded), and \textit{F} types (open bars) are indicated. Values of each bar express relative percentages, with a sum of 100% for all bars within a bracket. Quantification of the \textit{W} band may include signals of the \textit{X2} band due to close proximity of the \textit{W} and \textit{X2} bands. The number of individual tumors evaluated for each combination is indicated in parentheses.}
\end{figure}
than in strain FB1/pR-rga2#2, presumably due to position effects (Figure 3A). To assess an influence of ectopic rga2 expression on mtDNA inheritance, these strains were mated with strain GF5. In the FB1/GF5 wild-type combination, the W type of strain GF5 was exclusively inherited as expected (Table 3). Interestingly, in the FB1/pR-rga2#1/GF5 combination, the RFLP pattern showed the X1 and X2 types in addition to the W type, whereas the X1 type was hardly detected in the FB1/pR-rga2#2/GF5 combination in which rga2 is only weakly expressed (Figure 3B). To substantiate the effect of rga2 expression in the a1 partner, plasmid pot-rga2 was constructed, providing for rga2 expression under the constitutive otef promoter (Spellig et al. 1996), and inserted ectopically into the MF18 and MF34 strains. As judged from RNA gel blot analysis, the resulting MF18/pot-rga2 and MF34/pot-rga2 strains displayed rga2 transcript levels comparable to those of strain FB1/pR-rga2#1 (Figure 3A). Transcripts resulting from expression under the native and otef promoters varied widely in size, likely due to the long 5′ untranslated sequence of the native rga2 transcript (accession no. U37796; Urban et al. 1996a). As expected, in the wild-type MF18/FB2 and MF34/FB2 combinations, inheritance of the m2 mitotype F clearly dominated. However, in the MF18/pot-rga2/FB2 combination, both parental mitotypes, the constitutive otef promoter (Spellig et al. 1996), and inserted ectopically into the MF18 and MF34 strains. As judged from RNA gel blot analysis, the resulting MF18/pot-rga2 and MF34/pot-rga2 strains displayed rga2 transcript levels comparable to those of strain FB1/pR-rga2#1 (Figure 3A). Transcripts resulting from expression under the native and otef promoters varied widely in size, likely due to the long 5′ untranslated sequence of the native rga2 transcript (accession no. U37796; Urban et al. 1996a). As expected, in the wild-type MF18/FB2 and MF34/FB2 combinations, inheritance of the m2 mitotype F clearly dominated. However, in the MF18/pot-rga2/FB2 combination, both parental mitotypes, the constitutive otef promoter (Spellig et al. 1996), and inserted ectopically into the MF18 and MF34 strains. As judged from RNA gel blot analysis, the resulting MF18/pot-rga2 and MF34/pot-rga2 strains displayed rga2 transcript levels comparable to those of strain FB1/pR-rga2#1 (Figure 3A). Transcripts resulting from expression under the native and otef promoters varied widely in size, likely due to the long 5′ untranslated sequence of the native rga2 transcript (accession no. U37796; Urban et al. 1996a). As expected, in the wild-type MF18/FB2 and MF34/FB2 combinations, inheritance of the m2 mitotype F clearly dominated. However, in the MF18/pot-rga2/FB2 combination, both parental mitotypes,
along with new mitotypes, were inherited. In the MF34/pot-rga2/FB2 combination, inheritance of the F type was lost in favor of the W and new mitotypes (Figure 3C). Together, this demonstrated that rga2 expression in both mating partners led to a very similar mtDNA inheritance pattern than that received in the corresponding a1/a2Δlga2 combinations (see supplemental Figure 3). This implies that the m1 mitotype is protected in response to rga2 expression in the a1 partner, suggesting that Rga2 counteracts the elimination mechanism.

X1, X2, and X3 are new mitotypes that originate from parental mitotypes: To obtain clues on the generation of the new mitotypes X1, X2, and X3, we identified by RFLP analysis descendant spores carrying these mitotypes. As expected, the vast majority of single spores isolated from one tumor displayed only one mitotype whose identity was additionally confirmed by PCR (Table 4). Sequence analysis comprised the LSU rRNA region from positions 1 to 6411 (see Table 2). This revealed that the analyzed X1 type resulting from the MF34/MF14Δlga2Δrga2 combination matched the parental F-type sequence, but additionally contained the LRII1 intron of the parental W-type strain and thus corresponds to the B type (Figure 1). The finding of a W-type-specific nucleotide variation upstream of the LRII1 5' splice site in the X1 sequence (position 2882 in Table 2) implies that flanking regions were transferred. The analyzed X2 type sequence resulted from the FB1/pR-rga2#1/GF5 strain combination and matched the parental W-type sequence downstream of position 2882 in

**Figure 3.—**Effect of rga2 expression in the a1 mating partner on uniparental mtDNA inheritance. (A) RNA gel blot analysis to verify rga2 expression in the individual strains transformed with pR-rga2 or pot-rga2 (bottom). The individual strains are marked at the top of each lane. Strains FB1, FB1/pR-rga2#1, and FB1/pR-rga2#2 (lanes 1–3) were cultivated in CM and stimulated with Mfa2 pheromone for 6 hr. The remaining strains (lanes 4–9) were cultivated in YEPSl medium for 12 hr. Two identical filters were hybridized with [32P]-labeled rga2 and ppi fragments, respectively. Radioactive signals (both for rga2 in lanes 2 and 3; bracket) were quantified and the ratios of rga2/ppi signals were calculated, with the ratio in lane 9 set to 100. Scheme of pR-rga2 and pot-rga2: pR-rga2 expresses rga2 under its own regulatory sequences. Nontranslated sequences (5' and 3') (shaded boxes) flank the rga2 ORF. pot-rga2 expresses rga2 under the constitutive otef promoter (solid arrow). The thick lines represent the plasmid backbone. (B) Combination of either FB1/pR-rga2#1 or FB1/pR-rga2#2 with GF5. (C) Combinations of MF18 or MF18/pot-rga2 with FB2 (top panels) and MF34 or MF34/pot-rga2 with FB2 (bottom panels). #1 and #2 refer to the two MF18/pot-rga2 or MF34/pot-rga2 strains (A). (B and C) Note the additional appearance of the X and/or W bands in dependence on rga2 expression in the a1 partner. Lanes clearly showing X2 are denoted by open triangles. Maize plants were inoculated with the combinations indicated at the top of the panels for RFLP analysis. See legend of Figure 2 for further details. All lanes in all panels are from the same blot.
exon II (see Table 2), while the upstream region matched the parental F type. Finally, the analyzed X3 type resulting from the MF18/MF14\textit{Δlga2Δrga2} strain combination matched the parental W-type sequence, but contained the F-type region with the LRI2 and LRI3 introns (Figure 1 and Table 2). Together, this documents that the X1, X2, and X3 types are recombinants of their parental mitotypes (see discussion).

Analysis of mitochondrial fusion between W and F types: Fusion of mitochondria is a prerequisite for the generation of recombinant genotypes. Fusion of parental mitochondria is strongly enhanced in dikaryotic cells resulting from mating of FB1/FB2 or BUB7/FB2 combinations when \textit{lga2} is deleted in the \textit{a2} partner, while \textit{rga2} did not show this effect (M. Mahlert, C. Vogler, K. Stelter, G. Hause and C. W. Basse, unpublished results). Specifically, we wondered whether mitochondrial fusion was increased in those combinations of F and W types in which efficient formation of new mitotypes was detected. To test this, compatible mating partners equipped with reporter constructs pKS1 or pKS2, which provide for arabinose-inducible expression of mitochondrial matrix-targeted green fluorescent protein (mtGFP) and red fluorescent protein (mtRFP), respectively, were generated and analyzed for mitochondrial fusion. In the FB1/GF5 combination, complete mitochondrial fusion was observed in only 1.2% of the dikaryotic hyphae. In contrast, in the FB1/GF5\textit{Δlga2} combination, mitochondrial fusion was detected in >80% of hyphal cells, while mitochondrial fusion was not promoted in the absence of \textit{rga2} as expected (Figure 4; see supplemental Table 2). The negative influence of \textit{lga2} on mitochondrial fusion was also detected for the MF34/FB2 combination. When \textit{lga2} was deleted in the \textit{a2} partner the mitochondrial fusion frequency increased from 1.8 to 87.7% (see supplemental Table 2). The finding that new mitotypes were efficiently generated in wild-type GF25/FB2 or GF63/FB2 combinations (see Figure 2A and data not shown) raised the question of whether this was independent of \textit{lga2}. Analysis of mitochondrial fusion in the GF63/FB2 strain combination confirmed low mitochondrial fusion efficiency in the wild-type combination and a clear increase (from 11 to 83.4%) in the absence of \textit{lga2}, indicating that \textit{lga2} also prevented mitochondrial fusion in this combination (see supplemental Table 2 and discussion).

DISCUSSION

On the basis of the identification of different mitochondrial genotypes, we have investigated uniparental mtDNA inheritance in \textit{U. maydis} during sexual pro-
Control of mtDNA Inheritance in *U. maydis* 

The individual roles of Lga2 and Rga2 in uniparental mtDNA inheritance: Investigation of uniparental mtDNA inheritance in *U. maydis* has shown that its outcome is strongly influenced by the *a2* mating-type locus. In wild-type combinations, the m2 mitotype is frequently inherited and in the absence of *lga2*, new mitotypes are detected in F/W and B/W combinations. This implicates survival of both parental mitotypes in the absence of *lga2*, as has been explicitly detected for combinations involving the *a1* strain MF18 (see Figure 2C and supplemental Figure 3). Mitochondrial fusion is prerequisite for mtDNA exchange among mating partners and a negative effect of *lga2* on mitochondrial fusion was demonstrated for strain combinations that produced new mitotypes (see supplemental Table 2). However, Lga2 might play an additional role in mtDNA elimination. First, *rga2* expression in the *a1* partner confers the same outcome of inheritance as in the absence of *lga2*. Second, *rga2* is required only in the presence of *lga2* since the Δ*lga2Δrga2* double deletion leads to the same pattern of inheritance as the Δ*lga2* single deletion. Third, deletion of *rga2* in the *a2* partner promotes loss of the m2 mitotype. Together, this suggests that Rga2 apparently protects from Lga2-dependent mtDNA elimination. In support of such a role, conditional overexpression of *rga2* conferred selective loss of mtDNA in *U. maydis* *a1* and *a2* cells (Bortfeld et al. 2004; C. Vogler and C. W. Basse, unpublished data). Our results have also revealed that mitochondrial elimination occurs at a post-fusion step in *U. maydis*. If mitochondrial elimination of the m1 mitotype occurred prior to cell fusion, new mitotypes could not be generated in combinations in which *lga2* is deleted in the *a2* partner. These conclusions are summarized in a model (Figure 5). While in the wild-type strain combination, Rga2 protects the m2, but not the m1, mitotype from Lga2-mediated elimination, in the absence of *lga2*, loss of mtDNA is prevented and mitochondrial fusion is stimulated, thus enabling mtDNA exchange (Figure 5, A and B). However, the same outcome is also seen when *rga2* is expressed in both mating partners (Figure 5C). This implies that mitochondrial fusion also occurs in these combinations despite *lga2* expression. Previous investigation has provided evidence that *lga2* transcript levels decline during sexual progression in the host plant (Bortfeld et al. 2004), suggesting that mtDNA...
exchange occurs at a later stage of development if both parental genotypes are maintained. This likely makes an early elimination process to prevent generation of new mitotypes, and we speculate that this is facilitated by Lga2 interfering with mitochondrial fusion after mating; additionally, Lga2 could support the elimination process. How the m1 mitotype is maintained in combinations in which rga2 is deleted in the a2 partner is presently unknown (Figure 5D).

**Additional factors involved in mtDNA inheritance in U. maydis.** The m2 mitotype did not dominate over the m1 mitotype in all combinations analyzed (see Table 3). In a1 (B)/a2 (F) combinations, dominant inheritance of the B type was detected. Furthermore, new mitotypes were frequently generated in the GF25/FB2 and GF63/FB2 wild-type combinations despite the negative effect of Lga2 on mitochondrial fusion, suggesting that additional factors influence mtDNA inheritance, possibly by interfering with mtDNA elimination. In this case, dominant inheritance of the B type in B/F combinations could be equivalent with generation of the X1 type, which cannot be discriminated from the parental B type by RFLP analysis (see Figure 1). Increased inheritance of the B (X1) type has been explicitly detected for the BUB7 (B)/GF5 (W) combination when lga2 is deleted in the a2 partner (see supplemental Figure 3). The strain-specific outcomes of mtDNA inheritance detected in this study can now be used for genetic approaches to identify additional genes involved in this process.

Maintenance of more than one mitotype during sexual progression did not reflect heteroplasmacy because the vast majority of individual spores contained only one mitotype irrespective of lga2 and rga2 (see Table 4). Prior to spore formation, cellular compartments, which contain one diploid nucleus each, are formed. We therefore assume that an efficient elimination process ensures one mitotype per cell compartment. Heteroplasmacy is quickly lost in fungi (Barr et al. 2005). For example, in *Saccharomyces cerevisiae*, mating leads to a transient heteroplasmic state. However, this state rarely persisted through subsequent zygotic divisions and was strongly biased to only one parental mitochondrial population. The components governing this nonrandom segregation process are presently elusive (Birky 1994; Berger and Yaffe 2000).

**Generation of new mitotype alleles in U. maydis.** The B and W types differ from the F type by the LRII1 intron in the second exon of the LSU rRNA gene (see Figure 1). The secondary structure deduced from the LRII1 sequence matches the model of group II self-splicing introns. Both group I and II introns can act as mobile elements and proliferate through intron homing; however, the underlying mechanisms are different (Bonen and Vogel 2001; Haugen et al. 2005; Stoddard 2005). The LRII1 sequence encodes a putative homing endonuclease (HE) of the LAGLIDADG family typically associated with group I self-splicing introns (Chevalier and Stoddard 2001; Haugen and Bhattacharya 2004; Stoddard 2005). There is precedence for LAGLIDADG-encoding group II introns in basidiomycetes and the sequences of predicted dVand dVI of LRII1 are remarkably similar to the group II intron SSUI3 of *Cryphonectria parasitica* (Toor and Zimmer 2002 and references therein; see supplemental Figure 1A). Furthermore, the predicted LRII1-derived HE protein sequence exhibits strong similarities (E-values <10^{-20}) to putative mitochondrial LAGLIDADG endonucleases of numerous fungal species, with highest similarities to those of Gibberella zeae (accession no. YP_001249530), *Podospora anserina* (accession no. P15563), and the chytridiomycete *Rhizophydia* (accession no. AAK84275). Thus, the LRII1-derived HE gene (HEG) appears to be widespread in fungal genomes. Introns containing a HEG may spread by HE-mediated cleavage of their sequence recognition sites (Lambowitz and Belfort 1993; Burt and Koufopanou 2004; Stoddard 2005). In contrast to reciprocal exchange, HE-mediated intron homing provides for unidirectional exchange leaving the donor strand intact and modifying the recipient strand, which gets a copy of the HEG-containing region as a by-product of repair. This may occur with efficiencies close to 100% (Burt and Koufopanou 2004).

The recombinant X1 type frequently resulted from parental W/F (F/W) combinations and corresponds to the F type additionally carrying the LRII1 region (see Figure 1). Intragenomic rearrangements can be excluded as the underlying mechanism since the LRII1 sequence is missing in the mitochondrial genome of the F type. Hence, the X1 type may be generated by a double crossing-over event or by HE-mediated intron homing. In support of unidirectional transfer, the F type was selectively eliminated in the majority of W/F combinations under conditions of biparental mtDNA inheritance. Intriguingly, the parental F type was maintained in the MF18 (W)/FB2lga2 (F) and MF18/pot-rga2/FB2 combinations. In this context, it appears interesting that the putative HE encoded by the MF18 LRII1 sequence contains only one instead of two LAGLIDADG domains due to a frameshift mutation caused by a 7-bp deletion (ORF positions 516–522). Hence, it would be informative to compare predicted HE activities on the basis of the identification of target sites on intron-free alleles to assess whether intron homing accounts for generation of recombinant alleles.

Numerous reports describe recombination between mtDNAs of different descent in fungal species (Saville et al. 1998 and references therein; Anderson et al. 2001; Birky 2001; De la Bastide and Horgen 2003; Barr et al. 2005). This raises the question of whether mtDNA recombination also occurs distant from the LSU rRNA region and what the respective frequencies are. To address this question, it is necessary to identify corresponding sequence variations within mtDNA molecules of *U. maydis.*
In Saccharomyces yeasts, the high rate of inbreeding likely constrains mtDNA exchange, which efficiently occurs in sexual crosses (Burt and Koufopanou 2004; Johnson et al. 2004). In U. maydis, however, mating is prerequisite for the production of masses of sexual teliospores, thus requiring an alternative mechanism as provided by Lga2/Rga2 for controlling mtDNA exchange. What could be the advantage of constraining mtDNA exchange? Occasional DNA recombination may be advantageous to clear deleterious mutations from a lineage and thus in the long run contribute to the integrity of the mitochondrial genome (Barr et al. 2005). Constrained mtDNA exchange further avoids HEGs going to fixation owing to inactivation of HE recognition sites and thus maintains their capacity to cause mtDNA recombination (Bonen and Vogel 2001; Burt and Koufopanou 2004; Haugen et al. 2005).

**Effectors for Lga2 and Rga2:** Presently, the downstream effectors for Lga2 and Rga2 are unknown. In P. polycephalum, degradation of mtDNA of one parental mitochondrial genotype precedes the disappearance of mitochondria in the zygote (Moriyama and Kawano 2003). A Mn^{2+}-dependent nuclease activity was shown to be activated in mitochondria upon formation of the zygote and suggested to play a role in selective mtDNA degradation (Moriyama et al. 2005). In C. reinhardtii, a Ca^{2+}-dependent nuclease activity specifically expressed in mt{gamma} gametes was identified and proposed to digest mt-chloroplast DNA in the zygote (Nishimura et al. 2002). However, the responsible genes have not yet been identified. In this context, it appears intriguing that Rga2 strongly interacts with Mrb1 (Bortfeld et al. 2004), a member of the p32 family proteins, which, on the basis of structural features and surface distribution of acidic residues, was proposed to be involved in Ca^{2+} homeostasis of mitochondria (Jiang et al. 1999). Thus, it will be challenging to explore whether interaction between Rga2 and Mrb1 affects cellular Ca^{2+} levels and the activity of a Ca^{2+}-dependent nuclease. There are also conceivable alternative mechanisms for protection. For C. reinhardtii, evidence was provided for a methylation-dependent chloroplast DNA protection mechanism. The DMT1 gene of C. reinhardtii encodes a chloroplast resident cytosine methyltransferase suggested to methylate maternal chloroplast DNA resulting in protection from nuclease activity (Nishiyama et al. 2004). The identification of the effector proteins of Lga2 and Rga2 holds promise to reveal mechanistic insight into conserved or unique processes governing uniparental mtDNA inheritance in sexual eukaryotes.

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