Gsp1 Triggers the Sexual Developmental Program Including Inheritance of Chloroplast DNA and Mitochondrial DNA in *Chlamydomonas reinhardtii*[W][OA]

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The isogamous green alga *Chlamydomonas reinhardtii* has emerged as a premier model for studying the genetic regulation of fertilization and sexual development. A key regulator is known to be a homeoprotein gene, *GAMETE-SPECIFIC PLUS1* (*GSP1*), which triggers the zygotic program. In this study, we isolated a mutant, *biparental31* (*bp31*), which lacks *GSP1*. *bp31 mt+* gametes fuse normally to form zygotes, but the sexual development of the resulting diploid cell is arrested and pellicle/zygospore/tetrad formation is abolished. The uniparental inheritance of chloroplast (cp) and mitochondrial (mt) DNA (cytoplasmic inheritance) was also impaired. *bp31* has a deletion of ~60 kb on chromosome 2, including *GSP1*. The mutant phenotype was not rescued by transformation with *GSP1* alone but could be rescued by the cotransformation with *GSP1* and another gene, *INOSITOL MONOPHOSPHATASE-LIKE1*, which is involved in various cellular processes, including the phosphatidylinositol signaling pathway. This study confirms the importance of Gsp1 in mediating the zygotic program, including the uniparental inheritance of cp/mtDNA. Moreover, the results also suggest a role for inositol metabolism in the sexual developmental program.

INTRODUCTION

In sexually reproducing eukaryotes, fertilization involves a set of orchestrated cellular and molecular events. The initial events of fertilization involve adhesive interactions between gametes of opposite mating types, cell–cell fusion, and signal transduction (Yanagimachi, 2009). The unicellular green alga *Chlamydomonas reinhardtii* has been developed as a premier model organism to dissect the complex molecular processes underlying sexual development (Goodenough et al., 2007; Harris, 2009; Nishimura, 2010) (Figure 1).

The sexual life cycle of *C. reinhardtii* includes four significant events: gametogenesis, mating (zygote formation), zygote maturation (pellicle/zygospore/tetrad formation), and meiosis. Gametogenesis is induced by the loss of a nitrogen source. After acclimation to nitrogen starvation, the gamete differentiation program is initiated in a light-dependent manner (Huang and Beck, 2003; Huang et al., 2004), which is accompanied by the upregulation of gamete-specific genes (von Gromoff and Beck,

1993; Merchán et al., 2001; Abe et al., 2004, 2005). One of the early events during gametogenesis is the synthesis of agglutinins. The agglutinins are fibrous Hyp-rich glycoproteins that are responsible for the flagellar adhesion of mating type plus (*mt+*) and mating type minus (*mt−*) gametes. The *mt+* and *mt−* agglutinins are encoded by the autosomal genes Sexual AGglutination (SAG) and Sexual Adhesion, respectively (Ferris et al., 2005). The adhesion of *mt+* and *mt−* flagella triggers a signaling pathway, which includes a protein Tyr kinase (Wang and Snell, 2003), cGMP-dependent kinase (Wang et al., 2006), and adenylyl cyclase (s) and results in an increase in intracellular cAMP (Saito et al., 1993; Zhang and Snell, 1994). In response to the cAMP signal, both gametes shed their cell walls through the action of a metalloprotease (Kubo et al., 2001), and mating structures, located on the apical cell membranes between the two flagella, are activated (Detmers et al., 1983). The *mt+* gamete-specific protein Fus1, present on the plasma membrane of the mating structure, is essential for fusion of the *mt+* and *mt−* mating structures (Misamore et al., 2003). Fusion of *mt+* and *mt−* gametes and subsequent zygote formation entail the loss of gametic properties and the activation of the zygote developmental program, including the degradation of the Fus1 protein (Liu et al., 2010) and activation of zygote-specific genes (Armbrust et al., 1993; Uchida et al., 1993; Kuriyama et al., 1999; Uchida et al., 1999; Kubo et al., 2008). Upon fusion of *mt+* and *mt−* cell nuclei and chloroplasts, the zygotes develop a thick extracellular matrix and become zygospores (Woessner and Goodenough, 1989), a dormant stage in the life
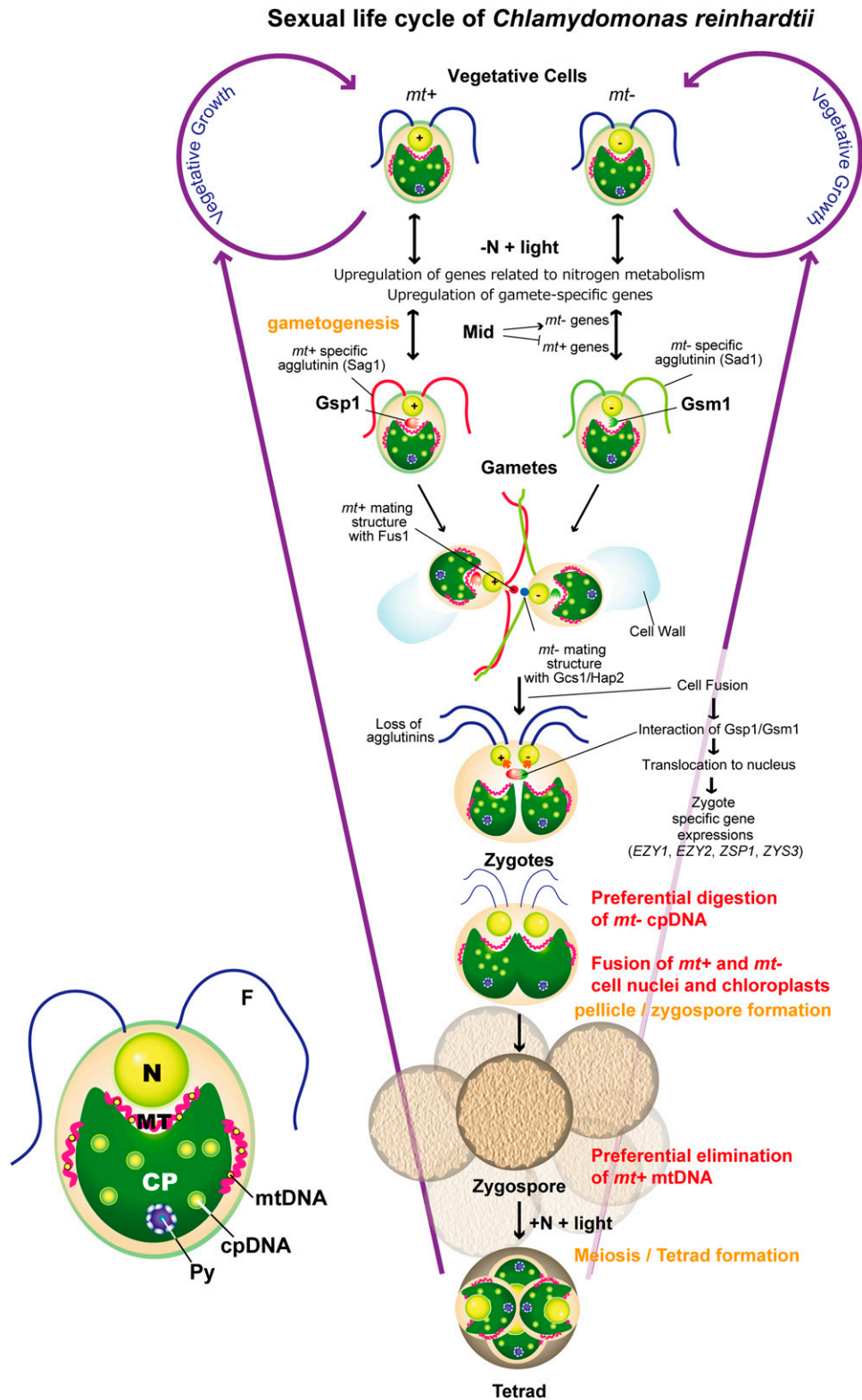
Figure 1. The Sexual Life Cycle of *C. reinhardtii*.

Bottom left panel: *C. reinhardtii* is a unicellular green alga with two flagella (F), one cell nucleus (N), and a cup-shaped chloroplast (CP) surrounded by fibrous mitochondria (MT). The cpDNA and mtDNA are complexed with proteins to form nucleoids. Center panel: The sexual life cycle of *C. reinhardtii*. Py, pyrenoid.

cycle. The cycle restarts when the appropriate environmental conditions stimulate the zygospores to undergo germination and meiosis to produce new haploid *mt+* and *mt−* vegetative cells.

Key regulatory factors for zygote development are Gsp1 (for Gamete-specific plus1) and Gsm1 (for Gamete-specific minus1), which are contributed by the *mt+* and *mt−* gametes, respectively. *GSP1* was identified as a gene expressed specifically in *mt+* gametes (Kurvari et al., 1998). When *GSP1* is ectopically expressed in *mt−* gametes, the cells transcribe genes that are normally transcribed only after zygote formation (Zhao et al., 2001). Lee et al. (2008) identified the *mt−* gamete–specific homeodomain gene *GSM1*. The ectopic expression of *GSM1* in *mt+* gametes also enhances the transcription of zygote-specific genes, and it has recently been shown that Gsp1 and Gsm1 physically interact upon the formation of zygotes and that the Gsp1/Gsm1 dyads translocate from the cytosol to the nucleus, initiating the zygote development program.

One notable event in the young zygote is uniparental inheritance of chloroplast (cp) and mitochondrial (mt) DNA. In *C. reinhardtii*, although the *mt+* and *mt−* gametes contribute an equal amount of cytoplasm to the zygote, only the cpDNA from the *mt+* parent is retained (Sager, 1954), due to the active degradation of *mt−* cpDNA within 60 min after mating (Sager and Lane, 1972; Kuroiwa et al., 1982; Nishimura et al., 1998, 1999). Conversely, the *mt+* mtDNA is eliminated although this elimination occurs more than 6 h after mating (Nakamura et al., 2003). As a result of the selective destruction of the organellar genomes, *C. reinhardtii* shows the uniparental inheritance of the cpDNA and the mtDNA from the *mt+* and mt− parents, respectively (Boynton et al., 1987).

To date, considerable efforts have been made to isolate and characterize mutants with defective uniparental inheritance (Sager and Ramanis, 1974; Sager et al., 1981). Gillham et al. (1987) isolated three mutations, *mat-3-1*, *3-2*, and *3-3*, that are linked to the *mt+* locus, all showing increased transmission of the *mt−* cpDNA. However, the *mat-3* cells are unusually small and contain significantly reduced amounts of cpDNA, indicating that the increased biparental transmission of cpDNA might be an indirect effect of the reduced amount of *mt+* cpDNA (Armbrust et al., 1995; Umen and Goodenough, 2001).

In the related species *Chlamydomonas monoica*, two mutants have been obtained that alter uniparental inheritance. The *mtl-1* mutant fails to protect *mt+*-derived cpDNA in zygotes and has a mating type–specific zygotic lethal effect (VanWinkle-Swift and Salinger, 1988). The *sup-1* mutant is defective for cpDNA degradation, suppressing the lethal effect of *mtl-1*, and leads to biparental inheritance of cpDNA in zygotes (VanWinkle-Swift et al., 1994). However, these genes have yet to be cloned, and their protein products and precise functions remain unknown.

In this report, we describe a screen to isolate mutants defective in uniparental inheritance. We identified the *biparental 31* (*bp31*) mutant, which shows normal agglutination of gametes and cell fusion but, when present in *mt+* cells that are mated with wild-type *mt−* gametes, shows significantly increased biparental transmission of cp/mtDNA. Furthermore, *bp31* vegetative zygotes cannot mature into sexual zygospores or tetrads, and the dynamic alteration of the transcriptome upon zygote formation is lost. Detailed analysis revealed that *bp31* is a mutant lacking the key regulator for zygote development, *GSP1*.

RESULTS

The *bp31* Mutant Is Defective in the Uniparental Inheritance of cpDNA

In this report, insertional mutagenesis was performed in *mt+* cells (CC-125 *mt+*) using the paromomycin resistance marker in an *AphVIII* gene cassette (Figure 2). The *mt+* cells were chosen because a brief UV irradiation of the *mt+* gametes prior to mating prevents the selective elimination of the *mt−* cpDNA; therefore, it is likely that *mt+*-specific genes play a more important role than *mt−*-specific genes in uniparental inheritance (Sager and Ramanis, 1967).

Transformants tagged with the *AphVIII* gene cassette were crossed individually with the *CL218 mt−* tester strain, which carries the spectinomycin resistance marker *aadA* in its cpDNA (Figure 2). The mated cells were incubated for ~90 min under the light, during which the active digestion of *mt−* cpDNA was completed in the wild-type cells. After normalizing the concentration of the cell suspensions, cells were spotted onto an agar plate containing paromomycin and spectinomycin to eliminate unmated *mt+* and *mt−* gametes and zygotes without *mt−* cpDNA. The plates were incubated in the dark for a week and subsequently exposed to the light. After performing ~3000 crosses, *bp31* was identified as a mutant with a greatly increased rate of survival (Figure 2).
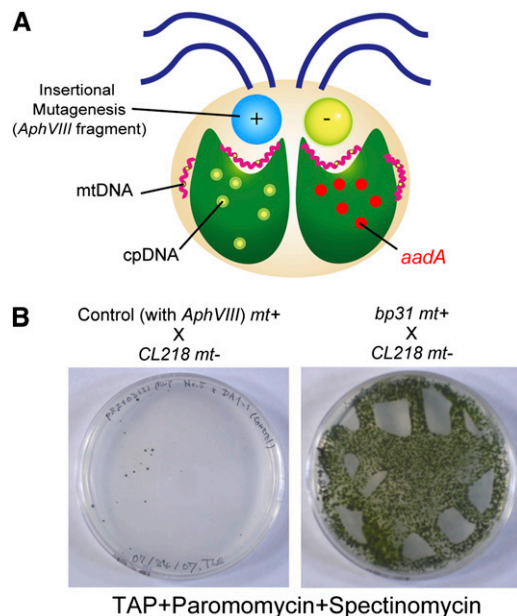
Figure 2. The Genetic Strategy to Isolate Mutants Defective in the Uniparental Inheritance of the cpDNA.

(A) Mutants defective in the uniparental inheritance of the cpDNA were obtained through insertional mutagenesis of *mt+* cells, followed by crossing the transformants with a tester strain carrying a spectinomycin resistance marker gene (*aadA*) in its cpDNA (*CL218 mt−*).
(B) The *bp31* mutant showed a higher rate of transmission of *mt−* DNA (*aadA*) than the control.

We examined *bp31* under the fluorescence microscope. In wild-type zygotes, the active digestion of the *mt−* cp nucleoid (cpDNA-protein complex) is observed within 60 min after mating (Figure 3). By contrast, the active digestion of the *mt−* cpDNA could not be observed in the *bp31 mt+* × wild-type *mt−* zygotes.

The *bp31* Mutant Produces Vegetative Zygotes Rather Than Zygospores

The absence of the active degradation of the *mt−* cpDNA was not the only phenotype of *bp31*. In wild-type cells, many events occur as the zygotes mature; for example, the zygotes stick together to form a structure called a pellicle. Four flagella are positioned closely between the two cell nuclei in wild-type zygotes, and these flagella are gradually retracted (Harris, 2009). However, these characteristic events of zygote maturation were not observed in *bp31*, and the pellicle/zygospores/tetrads were not formed (Figure 4).

These characteristics are reminiscent of vegetative diploids (Ebersold, 1967; VanWinkle-Swift, 1978; Boynton et al., 1987) or polyethylene glycol–induced diploids (Matagne and Hermesse, 1980; Matagne and Mathieu, 1983), which are defective in both sexual development and uniparental inheritance of cp/mtDNAs. In a typical wild-type cross, only 1 to ~5% of mated pairs fail to form sexual zygotes and seem instead to have a vegetative diploid identity (Ebersold, 1967; Harris, 2009), but in a cross of *bp31 mt+* × wild-type (or *CL218*) *mt−*, a majority of the mated cells fail to form sexual zygotes and grow without forming a pellicle or zygospores. PCR analysis detected both *FUS1* and *MID* sequences (markers for *mt+* and *mt−* cells, respectively; Werner and Mergenhagen, 1998) in individually isolated colonies from a *bp31 mt+* × *CL218 mt−* cross, indicating that almost all of the mated cells in this cross are vegetative diploids (Figure 4).

Inheritance of the mtDNA Was Also Disturbed in *bp31*

To analyze the inheritance of the mtDNA in *bp31*, the wild type, or *bp31 mt+*, cells were crossed to a mutant (*myx*) strain carrying a point mutation in the mitochondrial *cytochrome b* (*cytb*) gene (Bennoun et al., 1991), which creates a *Mnl*I site. The *mt+* and *mt−* mtDNAs could be distinguished by restriction digestion with *Mnl*I (Figure 4).

However, one problem was that the *bp31* zygotes do not form pellicles/zygospores /tetrads, and the cell suspension after the mating reaction remains heterogeneous, containing unmated gametes and zygotes that cannot be analyzed by either tetrad dissection or DNA gel blot/PCR analyses. To circumvent this difficulty, a single zygote was isolated using optical tweezers. The optical tweezer is a fluorescence microscope equipped with a strongly focused infrared laser beam, with which one can observe, trap, and harvest single cells (Nishimura et al., 1999, 2006). The single mated cells were collected and analyzed using nested PCR followed by *Mnl*I digestion. These analyses revealed that both *mt+* and *mt−* mtDNA were present in *bp31* × *myx mt−* diploids, even after 1 week of dark treatment, whereas only *mt+* mtDNA was detected in the wild-type *mt+* × *myx mt−* zygotes (Figure 4).

These results indicated that the *bp31* mutant is defective in the zygotic developmental program, including the uniparental inheritance of the cpDNA and mtDNA.

The *bp31* Mutant Has a ~60-kb Deletion

To identify the genes responsible for the pleiotropic phenotypes, we used thermal asymmetric interlaced (TAIL)-PCR to identify the 3′ flanking region and the genome database to search for
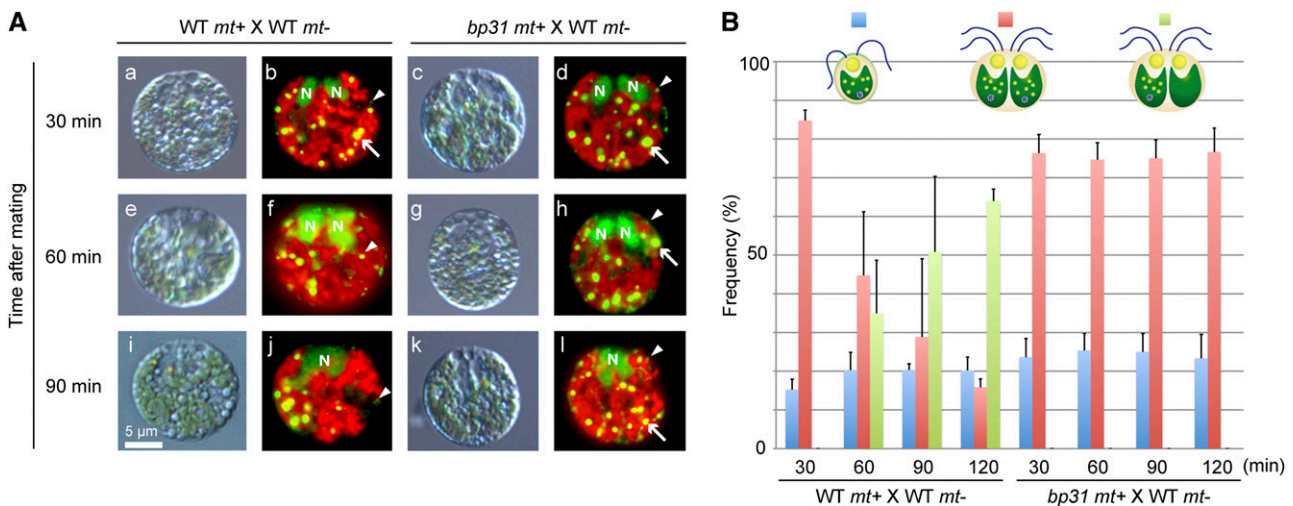
Figure 3. The Inhibition of the Preferential Degradation of the *mt−* cpDNA in *bp31*.

(A) DIC (**[a]**, **[c]**, **[e]**, **[g]**, **[i]**, and **[k]**) and fluorescent (**[b]**, **[d]**, **[f]**, **[h]**, **[j]**, and **[l]**) images of SYBR Green I–stained young zygotes. N, nucleus; arrow, cp nucleoid; arrowhead, mt nucleoid; red autofluorescence indicates chlorophyll. In wild-type (WT) *mt+* × wild-type *mt−* (left panel), the *mt−* cp nucleoids were digested for ~60 min after mating. By contrast, the *mt−* cp nucleoids remained even after the fusion of the cell nuclei in *bp31 mt+* × wild-type *mt−* (left panel). The preferential degradation of *mt−* cpDNA was not observed in *bp31*.

(B) The frequencies of unmated gametes (blue), zygotes with *mt−* cp nucleoids (red), and zygotes lacking *mt−* cp nucleoids (green) at 30, 60, 90, and 120 min after mating.
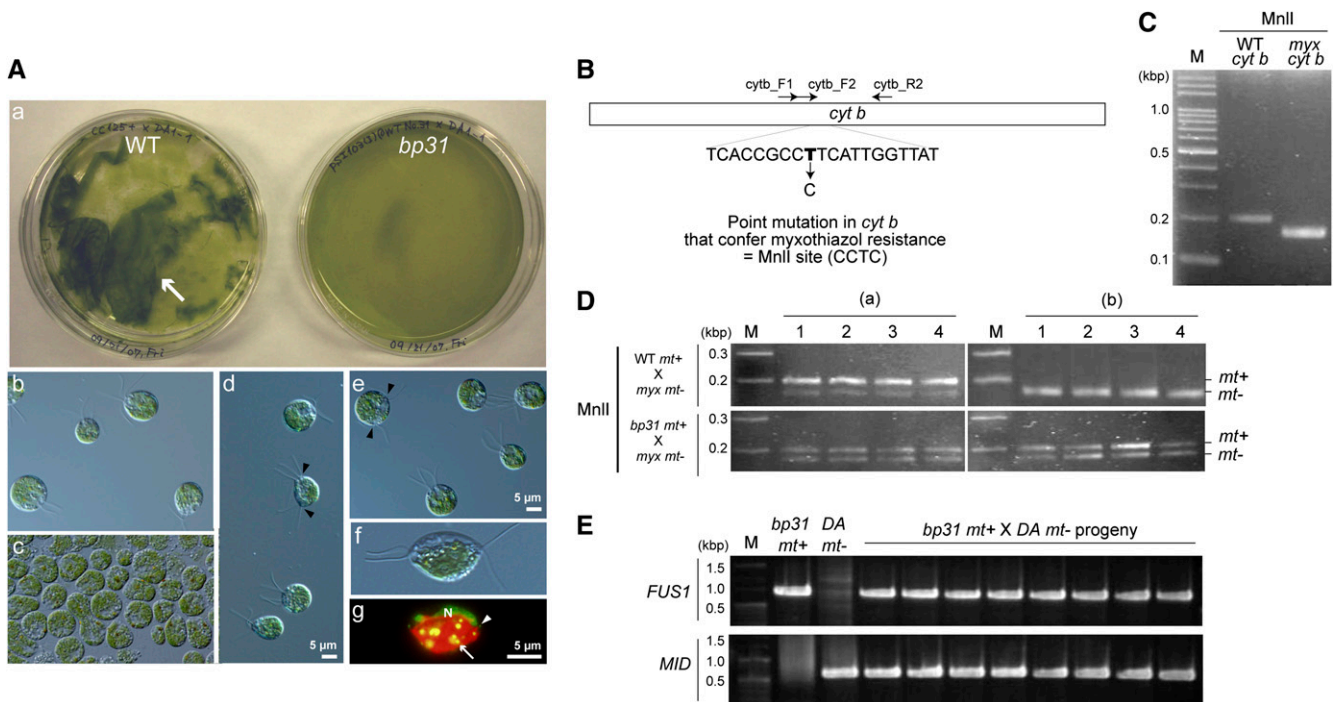
Figure 4. The *bp31* Mutant Is Defective in Zygote Maturation and the Paternal Inheritance of mtDNA.

(A) In the wild type (WT), zygotes stick together to form a film-like structure called a pellicle (**[a]**; arrow), and the pellicle is not observed until ~90 min after mating **(b)**. A DIC image of a wild-type pellicle 1 d after mating **(c)**. In bp31, however, pellicle formation is not observed (**[a]** and **[d]** to **[g]**). Certain cells (~1 to 2%) show abnormal positioning and numbers of flagella (black arrowheads). DIC (**[d]** to **[f]**) and SYBR Green I–stained fluorescent images **(g)** of a bp31 zygote at 1 d after mating, which does not show the active digestion of the mt− cpDNA, retraction of the flagella or pellicle formation, despite the fusion of the cell nuclei (N) and chloroplasts (red) (**[f]** and **[g]**). Arrow, cp nucleoids; white arrowhead, mt nucleoids.
(B) The mitochondrial *cytb* gene of the myxothiazol resistant (myx) strain has a fortuitous *Mnl*I site.
(C) The PCR product (primer set cytb_F2+cytb_R2) of mitochondrial *cytb* from wild-type and myxothiazol-resistant (myx) strains digested using *Mnl*I. M, marker.
(D) Wild-type mt+ and bp31 mt+ gametes were crossed to myxothiazol-resistant mt− gametes. Mated cells at 30 min after mating **(a)** and mated cells incubated in the dark for 1 week and then in the light for 8 h **(b)** were individually isolated using optical tweezers (approximately one to four) and analyzed using nested PCR.
(E) Single-colony PCR was used to investigate the presence or absence of the FUS and MID sequences in the progeny of *bp31* mt+ × *CL218 mt−*.

the 5′ end of the tag as determined by the presence or absence of upstream genes using genomic PCR (see Supplemental Figure 1 and Supplemental Table 1 online). Unexpectedly, this search showed that *bp31* harbors an extensive deletion of ~60 kb in chromosome 2. Twelve candidate genes were mapped to this deleted region. Based on their expression profiles (see Supplemental Figure 2 online), the 12 genes could be classified into four groups: (a) constitutive, (b) mating type–specific, (c) mating type/gamete-specific, and (d) zygote-specific. Intriguingly, seven of the 12 genes in this region showed mating type–, gamete-, or zygote-specific expression profiles.

Complementation of *bp31*

Considering the observed defects in the zygotic developmental program, it appeared likely that the causative gene(s) would be expressed preferentially in the *mt+* gametes. There were two genes that showed such an expression profile: *GSP1* and *INO-SITOL MONOPHOSPHATASE-LIKE1* (*INM1*) (accession number

JN412560). We constructed various expression vectors for *GSP1* and *INM1* (Figure 5) and conducted multiple rounds of transformations. Eventually, we determined that *bp31* could be complemented through the simultaneous introduction of *GSP1* and *INM1* (Figure 5). As discussed in the next section, neither gene alone was sufficient to rescue the *bp31* mutation.

When *bp31* mutants were cotransformed with *GSP1* and *INM1*, the active digestion of the *mt−* cpDNA was restored. The timing and efficiency of the *mt−* cpDNA degradation were almost fully restored to the wild type (Figure 5). Moreover, because pellicle/zygote/tetrad formation was restored, we were able to perform a tetrad analysis (see Supplemental Tables 2 and 3 online). The inheritance of cpDNA was assessed using tetrad dissection in the complemented *bp31 mt+ × CL218 mt−* (see Supplemental Table 2 online), in which 125 tetrads were analyzed. The segregation of the paromomycin cassette (paromomycin-resistant:-sensitive progeny = 60:65) and mating type (*mt+:mt−* = 63:62) was consistent with Mendel's law, but none of the progeny showed spectinomycin
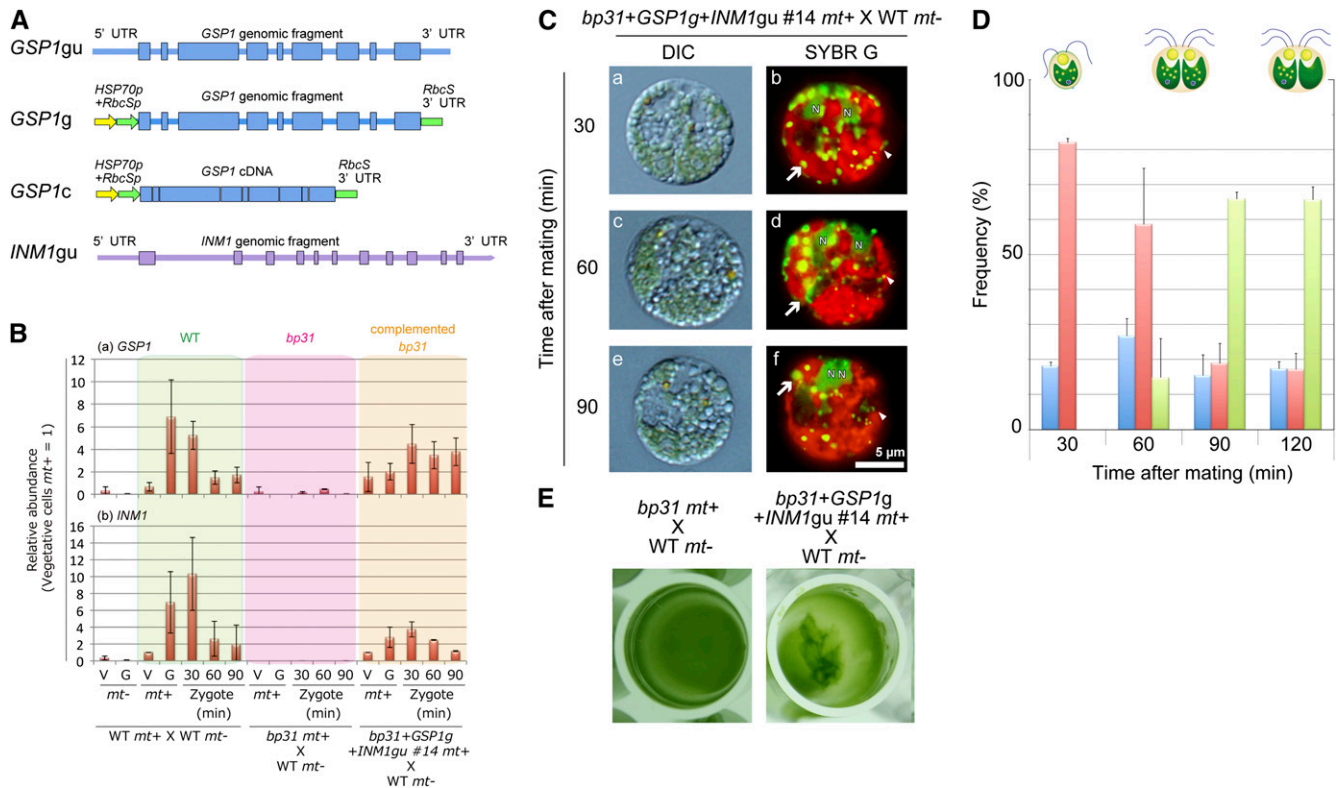
Figure 5. The *bp31* Mutation Was Complemented by Cotransformation with *GSP1* and *INM1*.

(A) Schematic drawings of the expression constructs for *GSP1* and *INM1*. A *GSP1/INM1* genomic fragment including the 5′ and 3′ untranslated regions (UTRs) (*GSP1*gu/*INM1*gu); a *GSP1* genomic fragment from the initiation codon to the termination codon ligated to the *HSP70+RbcS* promoter and *RbcS* 3′ untranslated region (*GSP1*g), and a *GSP1/INM1* cDNA ligated to the *HSP70+RbcS* promoter and *RbcS* 3′ untranslated region (*GSP1*c/*INM1*c) were prepared.

(B) Comparison of the expression profiles of *GSP1* and *INM1*. The cDNAs were prepared from the vegetative cells and gametes of *mt−* and *mt+* cells (V−, G−, V+, and G+), and zygotes (30, 60, and 90 min after mating) and analyzed using qRT-PCR. The relative levels were normalized to the *mt+* wild-type (WT) vegetative cells (normalized to 1).

(C) DIC (**[a]**, **[c]**, and **[e]**) and fluorescence (**[b]**, **[d]**, and **[f]**) images of SYBR Green I–stained young zygotes of *bp31+GSP1*g+*INM1*gu #14 *mt+* × wild-type *mt−*.

(D) The frequencies of gametes (blue), zygotes with *mt−* cp nucleoids (red), and zygotes lacking *mt−* cp nucleoids (green) at 30, 60, 90, and 120 min after mating in *bp31+GSP1*g+*INM1*gu #14 *mt+* × wild-type *mt−*.

(E) Pellicle formation in *bp31 mt+* × wild-type *mt−* and *bp31+GSP1*g+*INM1*gu #14 *mt+* × wild-type *mt−*.

resistance, indicating that cpDNA was strictly inherited from the *mt+* parents. To examine the inheritance of mtDNA, the complemented *bp31* lines were crossed with the *myx mt−* strain (see Supplemental Table 3 online). A total of 53 progeny were analyzed, all showing inheritance of mtDNA from the *mt−* parent. In summary, the phenotype of the *bp31* mutant, including the defects in pellicle, zygospore, and tetrad formation and the uniparental inheritance of the cpDNA and mtDNA, was fully restored through the introduction of two genes, *GSP1* and *INM1*.

The Transcription Profiles of Gamete- and Zygote-Specific Genes in *bp31*

To compare the expression profiles of the wild type, *bp31*, and the complemented *bp31*, quantitative RT-PCR (qRT-PCR) was used to analyze the transcription profiles of gamete- and zygote-specific genes (Figure 6).

EARLY ZYGOTE1 (EZY1) is a tandemly repeated sequence (approximately seven to eight repeats) at both the mt+ and mt− mating loci, and the repeats are transcribed immediately upon zygote formation. The Ezy1 protein localizes to both mt+ and mt− cp nucleoids, suggesting that Ezy1 is likely involved in the uniparental inheritance of cpDNA (Armbrust et al., 1993). EZY2 is a tandemly reiterated gene in the mt+ locus, alternating with OTU2 (Ferris et al., 2002). ZSP1 is a zygote-specific gene that encodes a Hyp-rich glycoprotein, which is a part of the cell wall (Woessner and Goodenough, 1989). ZYS3 is also zygote specific, and its product has two types of protein–protein interaction motifs: ankyrin repeats and a WW domain. ZYS3 localizes to the endoplasmic reticulum (Kuriyama et al., 1999).

In wild-type samples, the expression of EZY1, EZY2, ZSP1, and ZYS3 is rapidly upregulated upon the formation of the zygote, but in bp31, these genes remain undetected by quantitative PCR. The upregulation of these zygote-specific genes was
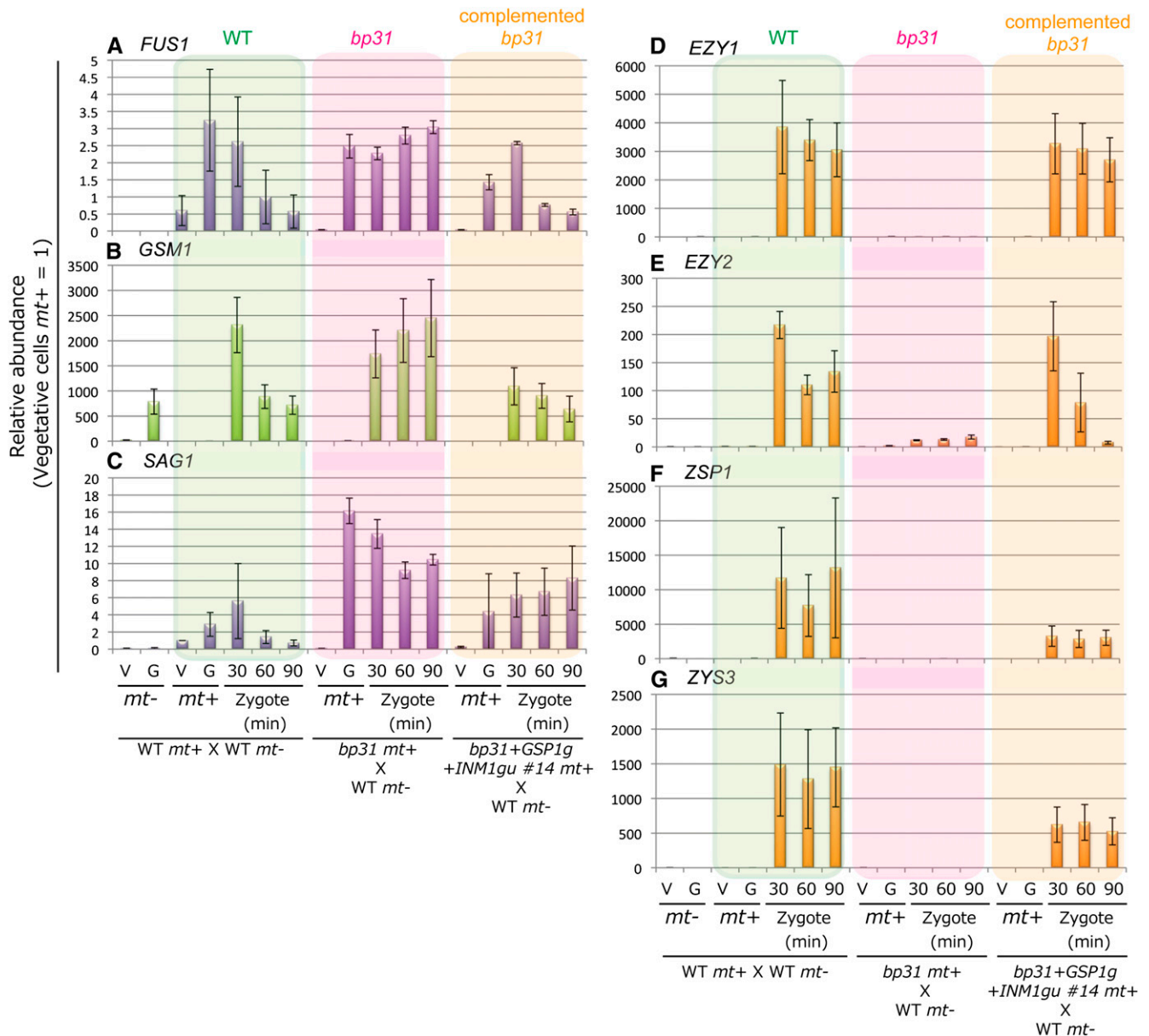
Figure 6. The Dynamic Changes in Gene Expression upon Zygote Formation Are Severely Disturbed in *bp31* and Are Restored in *bp31* Transformed with *GSP1* and *INM1*.

Comparison of the expression profiles of gamete- and zygote-specific genes in wild-type (WT) *mt+* × wild-type *mt−* (green), *bp31 mt+* × wild-type *mt−* (pink), and *bp31+GSP1*g+*INM1*gu #14 (complemented *bp31*) *mt+* × wild-type *mt−* (orange). The cDNAs were prepared from the vegetative cells and gametes of *mt−* and *mt+* cells (V−, G−, V+, and G+) and zygotes (30, 60, and 90 min after mating) and analyzed using qRT-PCR. *mt+* gamete-specific genes (*FUS1* **[a]** and *SAG1* **[c]**), *mt−* gamete-specific gene (*GSM1* **[b]**), and zygote-specific genes (*EZY1* **[d]**, *EZY2* **[e]**, *ZSP1* **[f]**, and *ZYS3* **[g]**) were investigated. The relative levels were normalized to the *mt+* wild-type vegetative cells (normalized to 1).

successfully restored in the complemented bp31 strain (Figure 6). The gamete-specific genes *FUS1*, *GSM1*, and *SAG1* were also analyzed. In the wild type, these genes are transiently up-regulated during gametogenesis in mt+ or mt− cells, reaching a maximum at 30 min after mating, and then decreasing rapidly. This rapid silencing of the gamete-specific genes in the zygotes was not observed in bp31 mt+ × wild-type mt−; rather, these

genes continued to be expressed at high levels even at 90 min after the mixing of the mt+ and mt− gametes. These results indicate that the dynamic alterations in the transcriptome upon zygote formation, including the rapid silencing of the gamete-specific genes and the upregulation of the zygote-specific genes, are lost in bp31. However, these transcriptional profiles are restored through the introduction of GSP1 and INM1. One

exception was SAG1, which was not silenced in the zygotes of the rescued bp31, suggesting that the expression of SAG1 might be controlled through a different pathway from that of FUS1 and GSM1.

Introduction of a Single Gene Was Not Sufficient to Complement *bp31*

When the *bp31* cells were transformed only with *INM1*, neither active degradation of the *mt−* cpDNA nor formation of the pellicle/zygospore/tetrad was observed (Figure 7). When the *bp31* cells were transformed with *GSP1*, an unexpected defect was observed. The cells showed normal flagellar adhesion but were unable to fuse with *mt−* gametes (Figure 7). We attempted the introduction of *GSP1* into *bp31* with different expression vectors (Figure 5) and tested 60 transformants for *GSP1*c, 56 for *GSP1*g, and 22 for *GSP1*gu. None of these 138 transformants showed either mating competency or pellicle formation, indicating the necessity of both *GSP1* and *INM1* to regulate the sexual developmental program and the uniparental inheritance of the cpDNA and mtDNA.

Treatment with cAMP did not rescue this nonfusing phenotype (Goodenough, 1989), suggesting that the signal transduction pathway for activating the fertilization tubule is blocked downstream of cAMP or that the ability to generate a fine-tuned cAMP pulse or oscillation (Valeyev et al., 2009) to achieve the successful mating reaction has been disrupted and cannot be rescued through supplementation with high levels of cAMP. To discover the basis for the nonfusing phenotype, the expression levels of *GSP1*, *FUS1*, and *SAG1* were compared among wild-type, *bp31*, and *bp31+GSP1*g gametes incubated with or without cAMP (Figure 7). This analysis showed that the expression of *FUS1* was almost completely absent in the *bp31+GSP1*g gametes, whereas the *FUS1* genomic sequence was intact. The silencing of *FUS1* would be a plausible explanation for the unexpected nonmating phenotype because *inm1*, an *mt+* mutant, also has reduced expression of *FUS1* and displays a similar phenotype (Ferris et al., 1996).

We attempted to rescue *bp31* through cotransformation with *GSP1* and *FUS1* driven by an artificial chimeric promoter (*HSP70+RbcS:FUS1c*). The competence for cell fusion, the active digestion of the *mt−* cpDNA, and the formation of the
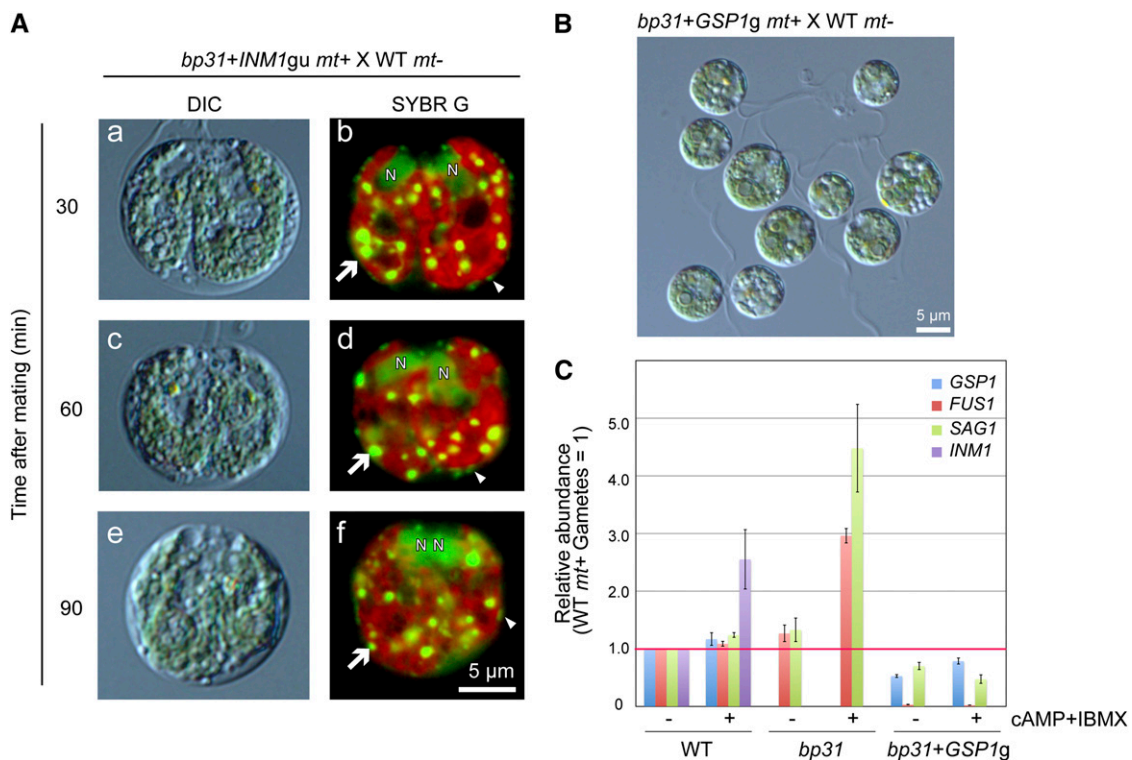

Figure 7. The *bp31* Mutant Could Not Be Complemented Solely by *INM1* or *GSP1*.

(A) DIC (**[a]**, **[c]**, and **[e]**) and fluorescent (**[b]**, **[d]**, and **[f]**) images of SYBR Green I–stained young zygotes of *bp31+INM1*gu *mt+* × wild-type (WT) *mt−*. The phenotype was identical to *bp31*, and neither the active digestion of the *mt−* cpDNA nor pellicle formation was observed.
(B) A DIC image of gametes of *bp31 mt+* transformed with *GSP1*gu mixed with wild-type *mt−* gametes. These gametes showed agglutination but did not form zygotes.
(C) The expression levels of *GSP1*, *FUS1*, *SAG1*, and *INM1* in wild-type, *bp31*, and *bp31+GSP1*g gametes incubated with or without 10 mM N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate and 1 mM 3-isobutyl-1-methylxanthine, as analyzed by qRT-PCR. The gametic expression of *FUS1* was suppressed almost completely in the *bp31+GSP1*g gametes.
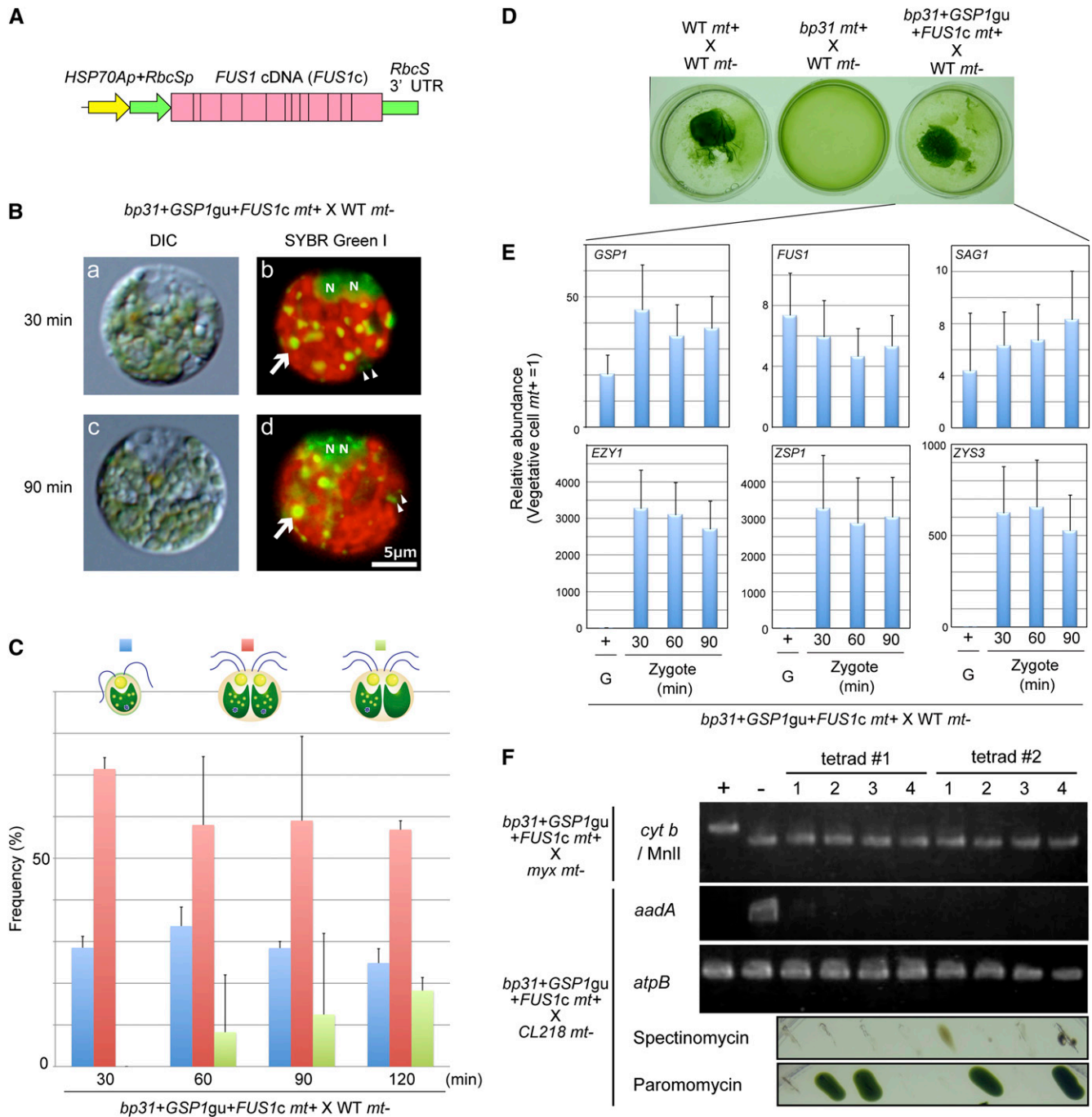
Figure 8. Cotransformation with *GSP1* and *FUS1* Rescues the *bp31* Phenotype.

(A) A schematic drawing of the construct used to express *FUS1* mRNA. The *HSP70+RbcS* promoter is connected to the *FUS1* cDNA (*FUS1c*). UTR, untranslated region.

(B) DIC (**[a]** and **[c]**) and fluorescence (**[b]** and **[d]**) images of SYBR Green I–stained young zygotes. N, cell nucleus; WT, the wild type; arrows, cp nucleoids; arrowheads, mt nucleoids. The preferential digestion of the *mt−* cp nucleoids was observed in *bp31* transformed with the *GSP1*gu and *FUS1c*.

(C) The frequencies of gametes (blue), zygotes with *mt−* cp nucleoids (red), and zygotes lacking *mt−* cp nucleoids (green) at 30, 60, 90, and 120 min after mating (*bp31+GSP1*gu+*FUS1c mt+* × wild-type *mt−*).

(D) Pellicle formation in wild-type *mt+* × wild-type *mt−*, *bp31 mt+* × wild-type *mt−*, and *bp31+GSP1*gu+*FUS1c* × wild-type *mt−*.

pellicle/zygospore/tetrad were all restored (Figure 8). The restoration of the strict uniparental inheritance of the cpDNA and mtDNA was also confirmed using tetrad dissection (see Supplemental Table 4 online).

Taken together, these results indicate the importance of Gsp1 in regulating the sexual developmental program and the uniparental inheritance of cpDNA and mtDNA, while also showing an essential role for *INM1* in mating.

DISCUSSION

In this study, we identified a mutant (*bp31*) lacking the BELL-related homeoprotein Gsp1. Our analyses of *bp31* confirmed that Gsp1 is a major regulatory factor that controls the sexual developmental program; these findings validate a previous study (Lee et al., 2008). Moreover, the fact that *bp31* was defective in the uniparental inheritance of cp/mtDNA (Figures 3 and 4) indicated that the Gsp1-Gsm1 system mediates the uniparental inheritance of cpDNA and mtDNA.

This study also indicated the importance of inositol metabolism in the mating reaction. Inositol monophosphatase, which is the other gene required for the complementation of *bp31* (Figure 5), dephosphorylates inositol monophosphate to *myo*-inositol, thereby regulating the accumulation of *myo*-inositol as part of the phosphatidylinositol (PtdIns) signaling pathway (Reynolds, 2009).

Although the actual function of *INM1* is still unclear, Musgrave et al. (1992) showed that the activation of the mating structure induces a rapid turnover of phospholipids PtdIns(4)P and PtdIns(4,5)P_2 in *Chlamydomonas eugametos* gametes (Brederoo et al., 1991). These minor phospholipids are important precursors for inositol 1,4,5-triphosphate and diacylglycerol, which could mediate the formation of the Ca^{2+}/cAMP signal transduction system needed for successful mating and zygote development (Schuring et al., 1990; Goodenough et al., 1993; Musgrave et al., 1993; Kuin et al., 2000). Notably, PtdIns(4,5)P_2 becomes enriched at the shmoo tip in the mating reaction of yeast. The preferential enrichment of PtdIns(4,5)P_2 in yeast is required for the specific localization and membrane anchoring of a mitogen-activated protein kinase signaling complex in the mating pathway (Garrenton et al., 2010).

An unexpected finding was the interaction of *GSP1*, *INM1*, and *FUS1*. The introduction of *GSP1* to *bp31* inhibited gametic fusion because of the repression of *FUS1* mRNA (Figure 7). The negative effect of *GSP1* on *FUS1* expression was nullified when a *FUS1* expression vector driven by an artificial chimeric

promoter or an *INM1* genomic fragment was introduced. Recently, it was reported that gamete fusion induces the ubiquitin-mediated protein degradation of Fus1 and Hap2 to block multicell adhesion (Liu et al., 2010). Rapid suppression of *FUS1* mRNA in zygotes could be another mechanism to avoid multicell adhesion.

Lee et al. (2008) showed that Gsp1 and Gsm1 physically interact upon the formation of the zygote and that the Gsp1/Gsm1 dyads translocate from the cytosol to the nucleus, initiating the zygotic developmental program (Lee et al., 2008), suggesting that there could be a mechanism that controls the intracellular localization of Gsp1. One speculation is that Gsp1 functions as a repressor for the *FUS1* promoter when it is translocated to the nucleus. *INM1* might be required to inactivate this repression, for example, by activating a signal transduction cascade to upregulate protein factors and inhibit the nuclear entry of Gsp1 or by upregulating the synthesis of PtdIns(4,5)P_2 to scaffold Gsp1. A possible future study would be to investigate the question of the localization of PtdIns molecules during gametogenesis and determine their influence on the localization of Gsp1.

Collectively, our studies showed that regulation of the molecular mechanism of uniparental inheritance is part of the sexual developmental program that is governed by the homeodomain protein Gsp1. In addition, they demonstrated the importance of inositol monophosphatase in orchestrating the sexual developmental program. Future studies will address the details of the mechanism for the active digestion of *mt*− cpDNA and how preferential protection of *mt*+ cpDNA is accomplished. Possible approaches to elucidating these mechanisms would include further screening for other *mt*+ mutants defective in uniparental inheritance of cpDNA and transcriptomic (RNA-seq) and proteomic analysis of *bp31* zygotes. *bp31* is the first mutant that lacks *GSP1* and is defective in the uniparental inheritance of mt/cpDNA in *C. reinhardtii*. This mutant will serve as an invaluable tool for further exploration to reveal the molecular mechanisms underlying the sexual developmental program and uniparental inheritance.

METHODS

Strains, Culture Conditions, and Mating Reactions

Cells were grown on Tris-acetate-phosphate (TAP), Sager and Granick (SG) II, or minimal medium agar plates under continuous light (Harris, 1989). The light intensity was ∼6600 lux at the surface. The mating reaction was induced through the transfer of cells onto the SG II agar plates (1.5%). After 2 d of incubation, the cells were suspended in a nitrogen-free

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Figure 8. (continued).

(E) The expression profiles of gamete-specific (*GSP1*, *FUS1*, and *SAG1*) and zygote-specific (*EZY1*, *ZSP1*, and *ZYS3*) genes in *bp31*+*GSP1*gu+*FUS1*c × wild-type *mt*−. Gametes (G) and zygotes (30, 60, 90 min after mating) were analyzed.

(F) Tetrad analysis of *bp31*+*GSP1*gu+*FUS1*c × *myx mt*− to examine the mtDNA inheritance and *bp31*+*GSP1*gu+*FUS1*c × *CL218 mt*− to examine the cpDNA inheritance. The typical results of two complete tetrads are shown. The total DNA was extracted, and the *cytb*, *aadA*, and *atpB* sequences (as a control chloroplast gene) were amplified using PCR. The *cytb* PCR product was digested with *MnlI*. The bottom panel shows the result of plating tetrads on TAP agar plates containing spectinomycin and paromomycin to determine the transmission of *AphVIII* and *aadA*, respectively. The uniparental inheritance of the cp/mtDNA was restored in *bp31*+*GSP1*gu+*FUS1*c, whereas a 1:1 Mendelian transmission was present for the nuclear *AphVIII* transgene.

Tsubo mating buffer (1.2 mM HEPES, pH 6.8, and 1 mM MgSO$_4$) at a concentration of ~5 × 10^6 cells per mL. After 5 to 8 h under light, equal numbers of $mt+$ and $mt-$ gametes were mixed and allowed to mate. Gametogenesis was initiated upon transfer to SG II agar plates. For certain experiments, the cells were treated with 10 mM N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (Sigma-Aldrich) and 1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich)(Goodenough, 1989).

Fluorescence Microscopy and SYBR Green I Staining

Vital staining of the DNA was performed as previously described (Nishimura et al., 1998). SYBR Green I (Invitrogen) was used at a final dilution of 1:2000. In all experiments, the mixture was kept in the same conditions as the original culture and either illuminated or placed in the dark. The stained cells were observed under blue excitation with a fluorescence/differential interference (DIC) microscope (BX51; Olympus) connected to a charge-coupled device camera (DP71; Olympus).

Cell Counting

The mating efficiency was calculated by counting cells under a microscope, using the equation [gametes/(gametes + 2 × zygotes) × 100 (%)]. The frequency of gametes and zygotes with or without $mt-$ cp nucleoids was calculated by counting the cells stained with SYBR Green I. The cell types (gametes, zygotes with or without $mt-$ cp nucleoids) were distinguished under a fluorescence microscope during cell counting. For each time point, ~100 cells were counted, and the experiments were repeated at least three times. The means and standard deviations were calculated, and graphs were prepared using Microsoft Excel 2008 for Mac.

Insertional Mutagenesis and Mutant Screening

Nuclear transformation was performed using the glass bead method (Kindle, 1990) or through electroporation (Shimogawara et al., 1998) using the ECM399 (BTX molecular delivery systems; Harvard Apparatus). A 1-μg SacI-KpnI fragment containing the paromomycin resistance marker (AphVIII) was introduced into the $mt+$ cells. Colonies were obtained on TAP agar plates supplemented with 10 μg/mL paromomycin and then transferred individually to fresh TAP agar plates containing paromomycin and maintained separately.

For the screening experiment, each of the tagged strains was transferred onto SG II agar plates. After 2 d, the cells were suspended in Tsubo mating buffer and incubated under the light for over 4 h. The concentrations of the cell suspensions were estimated spectrophotometrically at OD$_{595}$ using a plate reader (model 680; Bio-Rad). The chlorophyll (a+b) content was calculated using the equation chlorophyll (a+b) (μg/mL) = 17.76*$A_{646.6}$ + 7.34*$A_{663.6}$. Each of the gametes was then crossed to a tester $mt-$ strain that harbored a spectinomycin-resistant marker (the aadA cassette) in the cpDNA (CL218 $mt-$) (Hicks et al., 2002). The zygote suspensions were maintained in the light for 90 min. Using an eight-channel pipette, aliquots of the cell suspension containing ~3 μg of chlorophyll were spotted onto TAP agar plates supplemented with paromomycin and spectinomycin. The concentrations of the cell suspensions were kept low to reduce the background colonies through the natural occurrence of vegetative diploids (Harris, 2009). In the presence of paromomycin and spectinomycin, the unmated $mt+$ and $mt-$ gametes and zygotes lacking $mt-$ cpDNA were not viable, but unusual zygotes that retained $mt-$ cpDNA (aadA) survived.

The plates were cultured in the dark for 1 week and then exposed to light. Mutants defective in uniparental inheritance were obtained through screening for strains that showed a higher rate of survival than the 5% observed in the wild type (Harris, 2009). Concomitantly, the mutants

defective in pellicle formation were also screened. For this purpose, the zygote suspensions were kept in the light condition overnight after spotting, and strains that formed pellicles were identified.

The tetrad analysis was performed using an inverted microscope (BX41; Olympus) equipped with a micromanipulator (Narishige) and a glass needle (Singer Instruments). The mating type of the progeny was determined using colony PCR (Cao et al., 2009) with primers specific for $mt+$ (FUS1: FUS1_Fq1 + EcoRI_FUS1_R) and $mt-$ (MID: MID_Fq + MID_R) (Werner and Mergenhagen, 1998).

RNA Purification and cDNA Synthesis

The total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA was cleaned using a Purelink RNA mini kit (Invitrogen) with Purelink DNase set (Invitrogen). One microgram of RNA was used to synthesize single-stranded cDNA in a total volume of 20 μL using the Superscript III kit (Invitrogen) with random hexamers as primers.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed using the FastStart SYBR Green Master (ROX; Roche) and a MX3000P system following the manufacturer's instructions. The quantitative estimations were calculated with MxProTM software using the $\Delta\Delta C_t$ (cycle threshold) method (Stratagene, Agilent Technologies). The nuclear gene maa7 was used as an internal control, and the data were calibrated using wild-type vegetative cells as 1 (Nishimura and Stern, 2010). The experiments were repeated three times to calculate the means and standard deviations.

TAIL-PCR

For the TAIL-PCR analysis of the downstream flanking regions, we used the specific primers TR1, TR2, and TR3 for the primary, secondary, and tertiary reactions, respectively (see Supplemental Table 1 online for the primer sequences). We also used the degenerate primer RMD227 (Dent et al., 2005). The PCR was performed using the proofreading enzyme KOD-Plus-Neo (TOYOBO Life Science) according to the manufacturer's instructions. The following conditions for the primary PCR were used: 94°C for 1 min; 5 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 3 min; ramping over 3 min to 68°C; 15 cycles of 94°C for 30 s, 68°C for 1 min, 72°C for 3 min, 94°C for 30 s, 68°C for 1 min, 72°C for 3 min, 94°C for 30 s, 44°C for 1 min, and 72°C for 3 min; and 72°C for 7 min. The primary PCR products were diluted 50-fold, and 1-μL aliquots were used for the secondary amplification, using the same reagents but with the following conditions: 12 cycles of 94°C for 30 s, 64°C for 1 min, 72°C for 3 min, 94°C for 30 s, 64°C for 1 min, 72°C for 3 min, 94°C for 30 s, 44°C for 1 min, and 72°C for 3 min; and 72°C for 7 min. The secondary reaction products were diluted 10-fold, and 1-μL aliquots were used for the tertiary amplification with conditions identical to those used for the secondary reaction. The PCR products were separated using 1.2% agarose gel electrophoresis, and the DNA fragments were extracted from the gel and cloned into the pGemT Easy Vector (Promega) using the TArget cloning kit (TOYOBO Life Science). The cloned PCR fragments were sequenced using the T7 and SP6 primers.

Plasmid Construction

The expression vector pTT-CrGFP was a generous gift from H. Fukuzawa (Kyoto University) and contains a chimeric promoter (HSP70+RbcS). The cDNAs and genomic sequences were amplified based on the gene model in the Joint Genome Initiative version 4.0. BglII and EcoRI sites were added to the 5′ and 3′ ends with modified primer sets (see Supplemental

Table 1 online). The 5′-BglII-cDNA/genomic sequence-EcoRI-3′ fragments were ligated to pTT, which was digested with *Bgl*II and *Eco*RI. In pTT-CrGFP, the *gfp* sequence flanked by two *Eco*RI sites was cleaved to avoid interference with SYBR Green I fluorescence.

To amplify *GSP1*gu and *INM*gu, BAC libraries (CRB1-010A02 and CRB1-006O17) were used as templates (generous gifts from Hideya Fukuzawa). PCR was performed using the proofreading enzyme KOD-FX (TOYOBO Life Science) under the following conditions: 94°C for 2 min; 35 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 18 min; and 68°C for 7 min. The PCR products were separated using 1.2% agarose gel electrophoresis, extracted, and cloned into the pGemT Easy vector using the TArget cloning kit. *GSP1*gu and *INM*gu cloned into the pGemT easy vector were introduced through cotransformation with *APH7* (a hygromycin-resistant marker). The preparation of multiple numbers of plasmid constructs was facilitated with an automatic plasmid preparation system (Gene Prep Star, PI-80X).

Optical Tweezers and PCR Analysis

The main components of the optical tweezers device (LMS-M1064-2000; Sigma Koki) are a laser and a microscope system. The inverted microscope (IX51; Olympus) was connected to a fiber laser that emits continuous infrared light at 1064 nm, with a maximum power of 2000 mW (ASF1JE01; Furukawa Electric). In this study, the optical manipulations were conducted at a power of 1000 mW. The cells were suspended at a concentration of 10^{2-3} cells/mL and fixed with 4% formaldehyde. Individual cells were observed under a fluorescence microscope, trapped by the optical tweezers, and harvested into PCR tubes as described previously (Nishimura et al., 1999).

To study mtDNA inheritance, a myxothiazol-resistant strain (Bennoun et al., 1991) was used as a tester strain. The individual zygotes or diploid cells were analyzed using nested PCR: first amplification, cytb_F1 + cytb_R2 primers; and second amplification, cytb_F2 + cytb_R2 primers. The PCR products were digested with *Mnl*I and separated on a 4% NuSieve 3:1 agarose gel.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: *GSP1* (*C. reinhardtii*, XM_001699894), *INM1* (JN412560), *FUS1* (XM_001696588), *GSM1* (EU029996), *SAG1* (XM_001694232), *EZY1* (XM_001696321), *EZY2* (XM_001696583), *ZSP1* (XM_001690612), and *ZYS3* (XM_001700546).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The 60-kb Deletion in *bp31*.

Supplemental Figure 2. The Expression Profiles of Genes Deleted in *bp31*.

Supplemental Table 1. The Primer Sequences Used for PCR and qRT-PCR.

Supplemental Table 2. The Inheritance of cpDNA in Tetrad Progeny (*bp31*+*GSP1*gu+*INM1*gu strains [#4, 7, 13] *mt+* × *CL218 mt−* and *bp31*+*GSP1*g+*INM1*gu [#14] *mt+* × *CL218 mt−*).

Supplemental Table 3. The Inheritance of mtDNA in Tetrad Progeny (*bp31*+*GSP1*gu+*INM1*g strains [#4, 13] *mt+* × *myx mt−* and *bp31*+*GSP1*g+*INM1*g [#14] *mt+* × *myx mt−*).

Supplemental Table 4. The Inheritance of cpDNA and mtDNA in Tetrad Progeny (*bp31*+*GSP1*gu+*FUS1c mt+* × *CL218 mt−* and *bp31*+*GSP1*gu+*FUS1*c *mt+* × *myx mt−*).

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