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# A Gene Essential for De Novo Methylation and Development in Ascobolus Reveals a Novel Type of Eukaryotic DNA Methyltransferase Structure

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## Summary

**Molecular mechanisms determining methylation patterns in eukaryotic genomes still remain unresolved. We have characterized, in Ascobolus, a gene for de novo methylation. This novel eukaryotic gene, *masc1*, encodes a protein that has all motifs of the catalytic domain of eukaryotic C5-DNA-methyltransferases but is unique in that it lacks a regulatory N-terminal domain. The disruption of *masc1* has no effect on viability or methylation maintenance but prevents the de novo methylation of DNA repeats, which takes place after fertilization, through the methylation induced premeiotically (MIP) process. Crosses between parents harboring the *masc1* disruption are arrested at an early stage of sexual reproduction, indicating that the activity of Masc1, the product of the gene, is crucial in this developmental process.**

## Introduction

The regulation of programmed changes in methylation patterns during development and differentiation involves highly ordered processes of de novo methylation, maintenance methylation, and demethylation. The key enzymes involved in the first two processes, the DNA methyltransferases (MTases), catalyze the transfer of the methyl group from the donor S-adenosyl-methionine to the C5 position of cytosine residues within specific DNA sequence contexts. Several eukaryotic MTase genes have been cloned so far from mouse (Bestor et al., 1988), human (Yen et al., 1992), Arabidopsis (Finnegan and Dennis, 1993), chicken (Tajima et al., 1995), and sea urchin (Aniello et al., 1996). The corresponding enzymes contain the ten conserved amino acid motifs characteristic of the catalytic domain of MTases (Lauster et al., 1989; Kumar et al., 1994). They differ from prokaryotic MTases, however, by the presence of a large N-terminal domain. In contrast to prokaryotic type II MTases, which do not distinguish between unmethylated and hemimethylated substrates (Kelleher et al., 1991), the mouse MTase Dnmt (Bestor and Ingram, 1983) and the chicken MTase (Tajima et al., 1995) strongly prefer hemimethylated DNA as a substrate. Interestingly, the removal of the approximately 1000 amino acid–long N-terminal domain by proteolytic cleavage stimulates the de novo activity of the murine MTase in vitro (Bestor, 1992), suggesting that, besides a maintenance methylation activity, this enzyme also might be responsible for some in vivo de novo methylation (Leonhardt and Bestor, 1993; Bestor and Verdine, 1994; Smith, 1994). At least part of the de novo methylation activity in mammalian cells, however, should be catalyzed by a different MTase encoded by a second gene. Evidence for this comes from the analysis of mouse embryonic stem cells homozygous for the disruption of the identified *Dnmt* gene. Despite the lack of Dnmt activity, these cells still show a low amount of de novo methylation upon differentiation (Lei et al., 1996). Such a second type of MTase, responsible for de novo methylation, has not yet been identified in higher eukaryotes.

Insight into the role and the control of maintenance and de novo methylation in eukaryotes can be gained using filamentous ascomycetes as model organisms (Goyon et al., 1996a; Hagemann and Selker, 1996). In Ascobolus, DNA repeats are heavily methylated at cytosines (Goyon et al., 1996b). This de novo methylation takes place during sexual reproduction, between fertilization and karyogamy and requires pairing between the homologous repeated DNA sequences (Faugeron et al., 1990). This process has been termed "methylation induced premeiotically" (MIP; Rhounim et al., 1992). Once established, the methylation patterns are perpetuated in dividing cells by a maintenance process (Rhounim et al., 1992). Both elements of a duplication are methylated by MIP, and as a result, the genes carried by the duplication are silenced (Goyon and Faugeron, 1989). The methylation resulting from MIP of short (300–400 bp long) duplicated DNA segments is principally at CpG dinucleotides (Goyon et al., 1996a). In longer duplications, however, methylation affects all cytosines (Goyon et al., 1994), in contrast to mammals.

Toward an understanding of the genetic control and the biological function of de novo methylation processes, we report the cloning and characterization of an Ascobolus gene, *masc1*, encoding a protein with the primary structure of a MTase. The disruption of *masc1* has no effect on maintenance methylation but prevents the de novo methylation associated with MIP. Therefore, Masc1, which is involved in de novo methylation, is likely to represent a novel type of a eukaryotic MTase. Remarkably, the disruption of the *masc1* gene in the two parental strains involved in a cross invariably leads to sterility, indicating that as in the case of mouse (Li et

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al., 1992) and Arabidopsis (Finnegan et al., 1996; Kakutani et al., 1996; Ronemus et al., 1996), DNA methylation in Ascobolus is essential for the completion of the developmental processes underlying sexual reproduction.

## Results

### Isolation, Characterization, and Expression of the *masc1* Gene

All eukaryotic and prokaryotic MTases are remarkably similar in the general structure of their catalytic domain. This is manifested in the conservation of ten amino acid (aa) sequence motifs arranged in the same order and separated by nonconserved aa sequences of variable length and composition (Leonhardt and Bestor, 1993; Noyer-Weidner and Trautner, 1993). Assuming conservation of these ten motifs for MTases from Ascobolus, we performed PCR experiments using Ascobolus DNA and degenerate primers corresponding to part of the different motifs. Among several primer combinations tested, one primer pair corresponding to part of motifs IX and X (Figure 1) gave an amplification product of 78

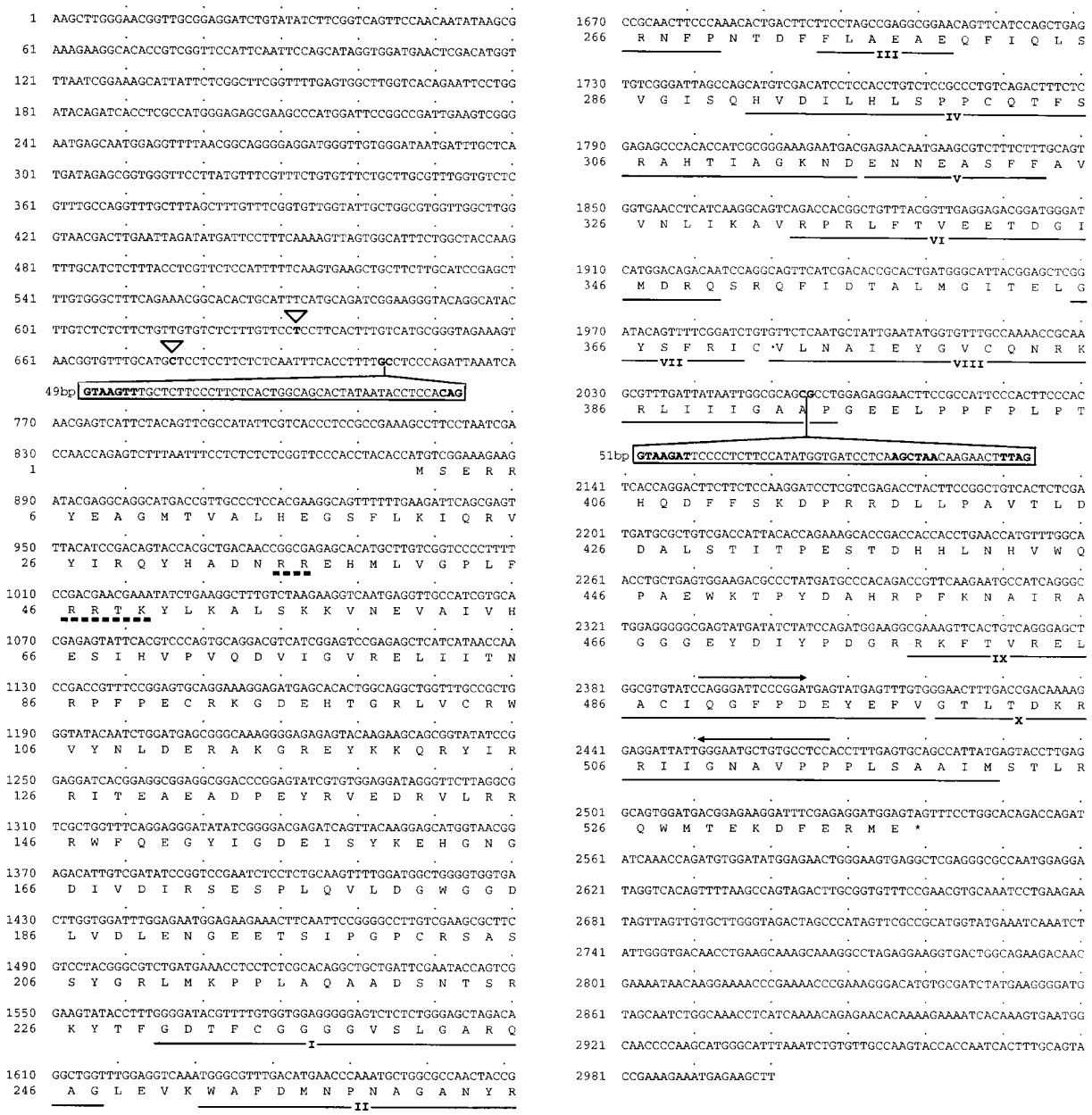

Figure 1. Nucleotide and Derived Amino Acid Sequence of *masc1*

The entire 3 kb HindIII fragment of plasmid pMI is shown. The two introns are boxed, and the consensus splice and recognition sites are printed in bold letters. Transcriptional start sites are marked by triangles. Conserved aa sequence motifs corresponding to regions I through X described in Pósfai et al. (1989) are underlined. The PCR primers used to amplify the 78 bp *masc1* segment overlapping motifs IX and X are indicated by arrows. The two motifs of a potential nuclear localization sequence (NLS) are indicated by stripple underlining.

bp. The aa sequence deduced from the primary structure of this fragment showed similarity to the open reading frame (ORF) of the MTase genes from Arabidopsis and mouse, suggesting that it represented a segment of an Ascobolus MTase gene. Using this fragment as a probe, a 3 kb HindIII genomic DNA fragment was identified by Southern hybridization and isolated in the recombinant plasmid pMI. The sequence of the HindIII fragment revealed the presence of a discontinuous ORF encoding a protein that contained all ten MTase motifs arranged in the canonical order (Figure 1). This gene was termed *masc1* (MTase from Ascobolus 1). Its ORF was split in two putative exons, which were separated by an intron of 51 bp (positions 2054–2104) exhibiting all consensus sequences of a typical intron of ascomycetes (Gurr et al., 1987). *Masc1* transcripts are apparently rare since they were not detected in Northern hybridization experiments with RNA extracted from mycelium or fruiting bodies, which contain cells engaged in sexual reproduction (data not shown). Transcripts, however, could be amplified by reverse transcription and amplification via 5′ RACE and RT-PCR experiments (data not shown). Sequencing of the PCR products allowed us to verify the proposed structure of the *masc1* gene, to determine the transcriptional start points of the gene, to confirm the location of the proposed 51 bp intron, and to identify another 49 bp intron (positions 704–752) in the untranslated part of the transcript (Figure 1). Translation is most probably initiated at the first ATG (position 876–879) of the ORF, whose sequence context shows highest similarity to that of fungal translation initiation sites (Gurr et al., 1987). The size of the putative protein is 537 aa, with a calculated $M_r$ of 61.5 kDa.

The primary structure of the Masc1 protein suggests that it is a MTase. All aa sequence motifs characteristic for the catalytic domains of MTases (Noyer-Weidner and Trautner, 1993; Kumar et al., 1994) are present and arranged in the standard order. However, Masc1 is only roughly one-third the size of the known eukaryotic MTases. This size difference mainly results from the absence of the large N-terminal domain characteristic of the other eukaryotic MTases and from the presence of a very short variable region between motifs VIII and IX (Figure 2A). In the case of the mouse enzyme, the large N-terminal domain has been shown to fulfill regulatory functions such as nuclear targeting, association to DNA replication foci, and preference of the enzyme for hemimethylated substrates (Leonhardt and Bestor, 1993).

Amino acid comparison of Masc1 with other MTases reveals that it is constituted of: (1) an N-terminal region of about 200 aa, which aside from a potential nuclear localization sequence (NLS; see Figures 1 and 2A), does not exhibit any significant similarities to corresponding regions of other MTases or other protein sequences currently in public databases; (2) a C-terminal region of about 330 aa, which includes the catalytic domain. Within the ten conserved motifs of the putative catalytic domain, Masc1 exhibits slightly greater similarity to eukaryotic (42%–45% identity) than to bacterial MTases (35% or less) (Figure 2B). However, the similarity of Masc1 to the five other known eukaryotic MTases is considerably lower than the homology they share (62%–90% identity). The differences are most apparent in motifs II–VII and in the "variable region" harboring the target recognition domain(s) (TRD). In particular, the variable region of Masc1 is unique both with respect to its length and its aa composition, although it contains TRD-like motifs (Lauster et al., 1989; Kumar et al., 1994).

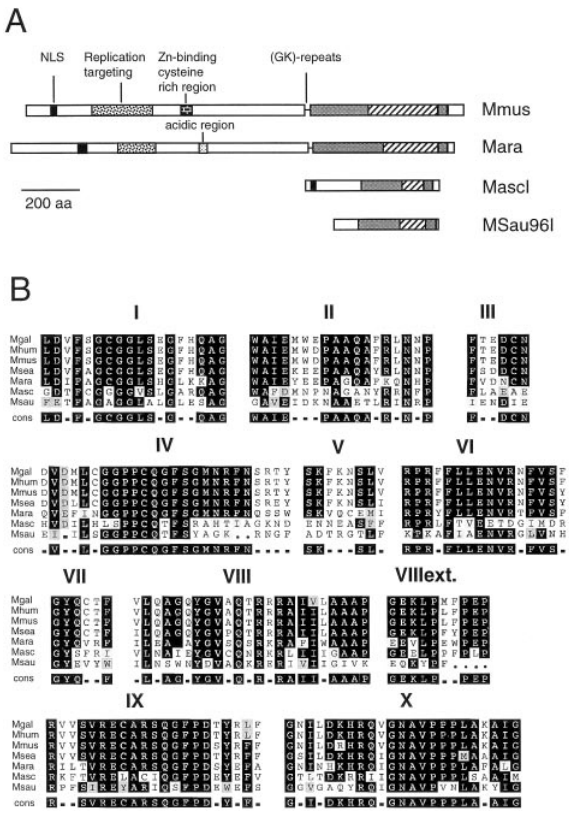


Figure 2. Amino Acid Sequence Comparison between MTases

(A) Schematic representation of different MTases. At the C terminus (right) are indicated the conserved domains I–X (solid bars) and the variable regions (hatched bars) corresponding to the catalytic parts of the enzymes. Empty bars at the N terminus with common motifs (dotted bars) represent noncatalytic parts of the enzymes (see Bestor and Verdine, 1994).

(B) Comparison of the aa sequence of Masc1 with those of five other eukaryotic MTases and the bacterial DNA-C5-methyltransferase M. Sau96I. The alignment is restricted to the aa sequences of motifs I–X, as defined by Pósfai et al. (1989). Amino acids conserved in five out of the seven sequences are highlighted as black boxes and presented as a consensus (cons) sequence below; aa with functional similarities to the consensus sequence are shown by gray boxes. Directly downstream from motif VIII, Masc1 and the other eukaryotic MTases share a high degree of homology in the block shown as VIIIext. The alignment was performed on a VAX using the programs "pileup" and "prettyplot" of the GCG8 Wisconsin Package of the Genetics Computer Group, Wisconsin, USA. Mgal, chicken (Gallus domesticus); Mhum, human; Mmus, mouse (Mus musculus); Msea, sea urchin (Paracentrotus lividus); Mara, Arabidopsis thaliana; Masc, Ascobolus immersus; Msau, the MTase M. Sau96I from bacterium Staphylococcus aureus.

When expressed in E. coli and baculovirus-infected insect cells, the Masc1-derived proteins showed no MTase activity in standard in vitro assays (see Experimental Procedures). Expression of Masc1 in these heterologous systems might produce an enzymatically inactive protein. Alternatively, the Masc1 protein might require either specific secondary modifications, cofactors, or complex DNA structures as substrates for it to

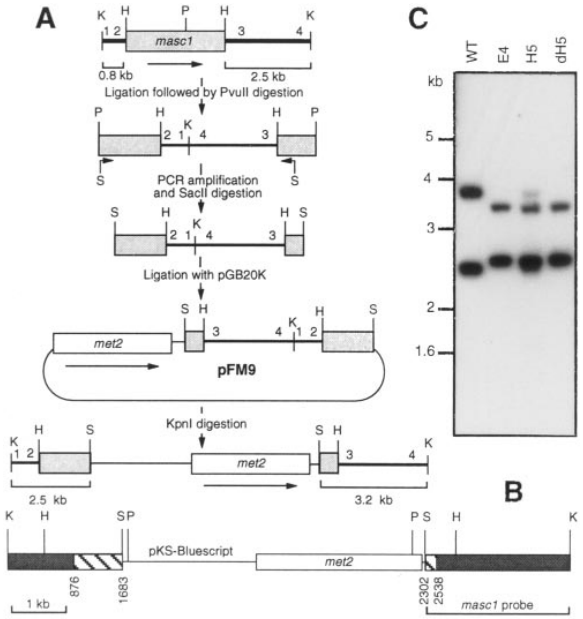


Figure 3. Disruption of the *masc1* Locus

(A) Schematic representation of the strategy used for constructing plasmid pFM9. For details, see Experimental Procedures.

(B) Map of the disrupted *masc1* locus resulting from the substitution of the native *masc1* gene by plasmid pFM9 cut at the KpnI site. Hatched and filled bars represent the *masc1* sequences (ORF and flanking sequences, respectively) left after disruption. Positions are indicated as in Figure 1. The deleted part of *masc1* corresponds to the 620 bp segment of the ORF (positions 1683–2302) encompassing motifs III–VIII. The 3.2 kb SmaI–KpnI *masc1* segment used as a probe in Southern hybridization is indicated.

(C) Analysis of the *masc1* disruption by Southern hybridization. In this example, PvuII + KpnI DNA digests from the recipient strain FB22 (wt), the primary transformants H5 and E4, and a progeny from transformant H5 (dH5) were probed with the *masc1* HindIII fragment. Transformant H5 was clearly heterokaryotic, containing a mixture of transformed and untransformed nuclei, as indicated by the presence of the 3780 and 2520 bp fragments diagnostic of the wild-type gene, in addition to the 3380 and 2636 bp fragments resulting from the disruption. A, AccI; H, HindIII; K, KpnI; P, PvuII; S, SacII.

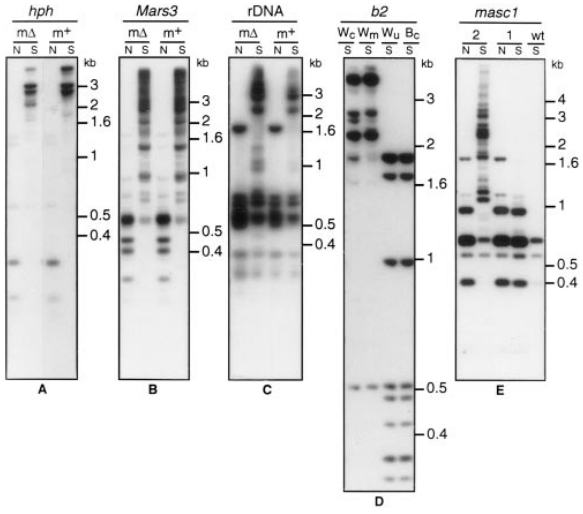


Figure 4. Southern Hybridization Analysis of Methylation Using the C-Methylation–Sensitive Enzyme Sau3AI (S) and Its Insensitive Isoschizomer NdeII (N)

(A–C) show examples of the methylation patterns obtained with two strains derived from transformant H5, one harboring the disrupted allele *masc1.*Δ*::met2* (mΔ) and the other harboring the wild-type allele of *masc1* ($m^+$); DNA digests were probed with the *hph* gene (A), plasmid pCG57 carrying *Mars3* (Goyon et al., 1996b) (B), and plasmid pCG32 carrying the rDNA unit (Goyon et al., 1996b) (C).

(D) Examples of the hybridization patterns obtained with two W strains, $W_u$ and $W_m$, (in which *b2* is nonfunctional, giving white ascospores) issued from the cross II × III (Table 1). DNA digests were probed with the *b2* gene. $B_c$ and $W_c$ were used as controls. $B_c$, in which *b2* is not methylated, issued from a brown ascospore of a 8B:0W ascus from the same cross. $W_c$, in which *b2* is methylated as a result of MIP, issued from a white ascospore of a 4B:4W ascus from the cross $IV^R$ × III (Table 1).

(E) Comparison of the hybridization patterns of the *masc1* region in the wild-type strain RN42 (wt), in the primary transformant 6–11.4 (1) and in one of its derivatives (2), which has undergone MIP. DNA digests were probed with *masc1* (Figure 3B).

be enzymatically active. We also cannot exclude the possibility that Masc1 lacks intrinsic MTase activity.

### Mutant Strains Carrying the Disrupted *masc1* Gene Are Viable and Display Normal Methylation Maintenance

To investigate its function in vivo, the native *masc1* gene was disrupted by substituting it with a recombinant gene in which the central part of the coding region encompassing motifs III–VIII was replaced by the Ascobolus *met2* gene (Figures 3A–3C). We first checked whether homokaryotic mutant strains harboring the disrupted *masc1.*Δ*::met2* allele were viable and, if so, whether they were able to maintain methylation through mitotic divisions. For this, we used strains harboring an *hph* transgenic copy previously methylated and silenced by MIP. When active, the *hph* transgene from E. coli (encoding hygromycin B phosphotransferase) confers hygromycin resistance to Ascobolus (Rhounim et al., 1994). When *hph* is silenced, the transgenic strains harboring it exhibit the same sensitivity to hygromycin as untransformed strains. Fourteen strains carrying both the disrupted *masc1* allele and the silenced *hph* transgene were isolated after appropriate crosses involving either of two primary heterokaryotic transformants that harbored the *masc1.*Δ*::met2* disruption (Figure 3C). All 14 knockout strains exhibited a wild-type growth phenotype, indicating that *masc1* is not essential for vegetative growth. They were all sensitive to hygromycin, indicating that *hph* was still silenced. The methylation pattern of *hph* investigated in four disruptants (two from each transformant) was identical to that observed with four control strains carrying the wild-type allele of *masc1* (Figure 4A). In the same way, we showed that methylation of the native DNA repeats (Goyon et al., 1996b) was not impaired by the *masc1* disruption (Figures 4B and 4C).

### Mutants Carrying the Disrupted *masc1* Gene Are MIP Deficient

Since Masc1 activity was not required for the maintenance of methylation, we asked whether the *masc1* disruption would affect de novo methylation in the sexual phase when duplicated sequences are silenced by MIP

(Rhounim et al., 1992). We examined whether an ectopic duplication of the ascospore pigmentation gene *b2*, which is required for the generation of brown ascospore color, would undergo MIP in *masc1.*Δ*::met2* mutant strains. A *b2* allele silenced by MIP gives white (W) instead of brown (B) ascospores (Colot and Rossignol, 1995). In the progeny of a cross between two strains carrying the wild-type allele of *masc1*, one harboring two wild-type copies of *b2* and the other harboring a single copy of this gene, a fraction of the asci shows 4B:4W segregation of ascospore pigmentation. The white ascospores (W) in these asci result from the silencing by MIP of the duplicated *b2* copies (Colot and Rossignol, 1995). In order to perform test crosses with either one or both parental strains carrying the *masc1.*Δ*::met2* mutation, four types of strains were constructed (Table 1). This was achieved by crossing one primary disruptant with a strain that carried two copies of the *b2* gene. The type I and type II strains harbored the *masc1.*Δ*::met2* mutation, together with either a single copy of *b2* (type I), or the duplication of *b2* (type II). The type III and type IV strains harbored the wild-type allele of *masc1*, together with either a single copy of *b2* (type III) or the duplication of *b2* (type IV). Test and control crosses were performed in multiple pairwise combinations (Table 1). In crosses between type III and type IV strains, the *b2* duplication was silenced by MIP in 30.7% of the progeny, on average.

All crosses between type I and type II strains, thus homozygous for the *masc1* disruption, were sterile, indicating that Masc1 is essential for sexual reproduction (see below).

In crosses heterozygous for the *masc1* disruption, fertility was not affected. Progeny from these crosses, however, displayed a decreased frequency of MIP. The decrease was most pronounced in the crosses between type II and type III strains, in which the *b2* duplication was present in the same nucleus as the *masc1* disruption. On average, only 0.25% of the asci progeny showed the 4B:4W segregation. By Southern hybridization, we analyzed methylation of *b2* in 18 strains derived from white ascopores from 18 different asci issued from seven distinct crosses (Figure 4D). Six strains exhibited a *b2* methylation pattern typical of MIP (one example, $W_m$, is shown in Figure 4D) whereas the other 12 strains were unmethylated (one example, $W_u$, is shown in Figure 4D). By genetical analysis, we showed that for these 12 strains the lack of pigmentation was not caused by silencing of *b2* but was due to mutations that occurred in other spore color genes. We do not know the reason for this abnormally high mutation rate, but it suggests that the percentage of asci in which *b2* was silenced by MIP is close to 0.1% after correcting for this mutation frequency. We showed, in addition, that in 64 strains derived from brown ascospores from 64 8B:0W asci, *b2* was not methylated, indicating that the absence of silencing reflected an absence of methylation.

In the crosses between type I and type IV strains, in which the duplication was present in the nucleus that harbored the wild-type allele of *masc1*, the frequency of MIP was intermediate between the latter crosses and the control crosses. In this case, a wide range of variation of the frequencies of asci reflecting MIP was observed between individual crosses (Table 1). Altogether these results indicate that Masc1 plays an essential role in the de novo methylation associated with MIP.

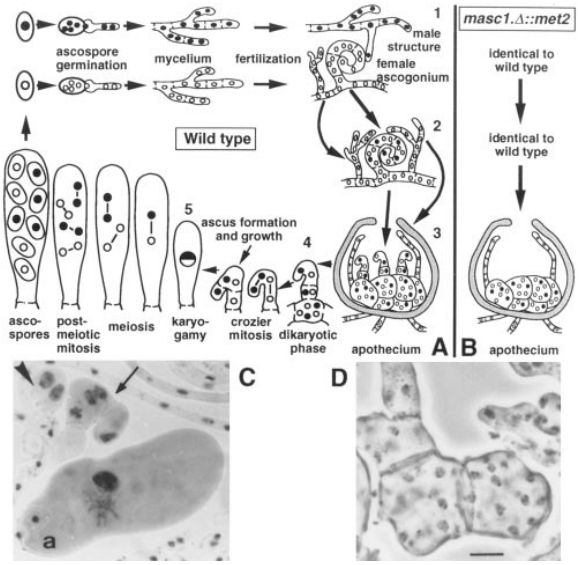


Figure 5. Comparison between Wild-Type and Mutant Sexual Development

(A) Life cycle of wild-type Ascobolus. The sexual development can be divided into four main steps. (1) Differentiation of reproductive structures, only induced when two strains of opposite mating type meet, and fertilization by transportation of the male nucleus into the plurinucleated female structure (called ascogonium). (2) Formation of the apothecium while male and female nuclei divide within the coenocytic female structure. (3) Individualization of dikaryons into specialized structures called croziers (4). Karyogamy occurs in the upper crozier cell (5), followed immediately by two meiotic divisions and a single mitotic division to form the characteristic 8-spored ascus.

(B) The right part of the drawing shows the step where *masc-1.*Δ*::met2* homozygous crosses are blocked: all steps from 1 to 3 are normal, but apothecia remain empty because only rare dikaryotic cells and croziers are formed.

(C) Light microscopy picture of a wild-type apothecium with one ascus (a) at meiotic prophase I (the dark spot corresponds to the nucleolus) and two croziers, one with four nuclei (arrow) and one with two nuclei (arrowhead).

(D) Large plurinucleate ascogonial cells seen in all *masc1.*Δ*::met2* apothecia. Scale bar, 5 μm.

### Sexual Development in Crosses Homozygous for the *masc1.*Δ*::met2* Mutation Is Blocked before the Individualization of Dikaryotic Cells

In Ascobolus, an ordered series of differentiation steps leads to the formation of sexual organs, fertilization, formation of the fruiting bodies (apothecia), and compartmentalization of dikaryotic cells that will undergo karyogamy, leading to diploid cells that immediately enter meiosis to give ascospores (Figure 5A).

In the homozygous *masc1.*Δ*::met2* crosses, the first three steps of sexual reproduction were normal both in timing and number of induced reproductive structures, as compared to the homozygous wild-type and heterozygous crosses made in parallel. Apothecia were normally formed, indicating that fertilization had indeed occurred (Figure 5B). Dissection of their contents showed

Table 1. Effects of the *masc1* Disruption on MIP and Fertility

| Crosses[a] | Genotypes[a] | No. of Crosses Screened[b] | No. of Asci Analyzed | No. of Asci Showing | | % of MIP[d] |
|---|---|---|---|---|---|---|
| | | | | 8B:0W | 4B:4W[c] | |
| $IV^R \times III$ | $(m^+ b2–b2) \times (m^+ b2)$ | 22 | 11,000 | 7,623 | 3,377 | 30.7 (24.2–39.2) |
| II × I | $(m\Delta b2–b2) \times (m\Delta b2)$ | 21 | (STERILE) | | | |
| II × III | $(m\Delta b2–b2) \times (m^+ b2)$ | 45 | 22,500 | 22,442 | 58 | 0.25 (0–1.6) |
| I × IV | $(m\Delta b2) \times (m^+ b2–b2)$ | 31 | 15,500 | 14,286 | 1,214 | 7.8 (2–25) |

[a] All strains (type I to type IV and type $IV^R$) carry the *met2*.Δ::*amdS* mutation (Goyon et al., 1996) and the resident wild-type $b2^+$ allele. Genotypes are the following, $m^+$: $masc1^+$; mΔ: *masc1*.Δ::*met2*; b2: single resident $b2^+$ copy; b2–b2: duplication of *b2* consisting of the resident gene and the ectopic copy carried by the transgenic construct "$met2^m$ $b2^+$ $hph^+$" in type II and IV strains, or "$met2^+$ $b2^+$ $hph^+$" in type $IV^R$ strains. Since crosses of two Ascobolus $Met^-$ strains could not mature ascospores (unpublished data), type IV strains were reverted to prototrophy (Rhounim et al., 1992) before being crossed with type III strains, resulting in type $IV^R$ strains. Phenotypes: type I, $Met^+$ $Hyg^S$; type II, $Met^+$ $Hyg^R$; type III, $Met^-$ $Hyg^S$; type IV, $Met^-$ $Hyg^R$; type $IV^R$, $Met^+$ $Hyg^R$.

[b] Five hundred asci were screened from each cross.

[c] Besides 4B:4W asci, some asci showing the segregation 2B:6W or 0B:8W were found; they also resulted from a MIP event, but this was followed by meiotic transfer of methylation (Colot et al., 1996); they were thus counted as 4B:4W asci.

[d] Ranges are indicated in parentheses.

a normal number of plurinucleate ascogonial cells but a near total absence of dikaryotic cells, indicating that the blockage occurred before their individualization (compare panels 5A and 5B). In the wild-type and heterozygous crosses, approximately ten dikaryotic cells were formed in every apothecium, while in the three homozygous *masc1*.Δ::*met2* crosses analyzed only two dikaryons could be observed among the whole of 50 apothecia submitted to cytological analysis. These rare dikaryotic cells evolved into croziers similar in shape and division pattern to wild-type croziers (step 4 in Figure 5), but they did not form asci, which implies that karyogamy never occurred. Observation of 200 apothecia from three different crosses showed that, even after 20 days following fertilization, mutant apothecia remained blocked while wild-type and heterozygous crosses had shot their ripe ascospores.

### Complementation of the *masc1*-Disrupted Strain by the Wild-Type Gene

To verify that the disruption was responsible for the lack of fertility and the MIP deficiency, the HindIII fragment containing *masc1* was introduced into the knockout *masc1*.Δ::*met2* mutant by transformation using plasmid pFM12. From the transformants obtained, appropriate crosses allowed us to isolate three strains harboring simultaneously the *masc1*.Δ::*met2* mutation, a single *masc1* transgenic copy, and the *b2* duplication. We also isolated as a control one strain that harbored a methylated and hence silenced *masc1* transgene, together with the *masc1*.Δ::*met2* mutation and the *b2* duplication (Experimental Procedures). Each of these strains was crossed with type I strains that harbored the *masc1*.Δ::*met2* mutation (Table 1).

Fertility was fully restored in the crosses involving each of the three strains carrying the *masc1*.Δ::*met2* mutation associated with the single-copy *masc1* transgene. In contrast, sterility persisted in the crosses involving the strain carrying the methylated and silenced *masc1* transgene. This confirmed that Masc1 is involved in fertility.

The effect of the *masc1* transgene upon MIP was checked by crossing the same four strains with type III strains (Table 1). The strain carrying the methylated *masc1* transgene gave a very low frequency of asci indicative of MIP (0.35%), close to the values observed in the crosses between type II and type III strains (see Table 1). Among the three other strains carrying the *masc1* transgene, one exhibited full restoration of MIP (35.4%). The two other strains exhibited lower MIP frequencies (5% and 1%). This may reflect incomplete expression of the transgene due to an undetected alteration or to a position effect on its expression. Taken together, these results further confirm that Masc1 is involved in MIP and indicate that the 3 kb HindIII fragment of pMI contains all the sequence information necessary for the expression of the *masc1* gene.

### The *masc1* Duplication Is Prone to MIP

In previous experiments aimed at disrupting *masc1*, we generated a transformant harboring a tandem duplication of the whole *masc1* region. In this transformant, a transgenic copy of *masc1* lacking an internal 463 bp segment (this segment, positions 1750–2214 in Figure 1, was replaced by the *met2* gene) had integrated upstream from the resident gene. The two *masc1* copies were methylated in the sexual progeny of this transformant, attesting that they had been subject to MIP (Figure 3E). By appropriate crosses, we constructed strains carrying the methylated tandem duplication of *masc1* together with the *b2* duplication. When intercrossed, these strains exhibited the same sterility as that observed in crosses homozygous for the *masc1* disruption. In some matings, however, ascospores were formed in tiny sectors of the plates, indicating local restoration of sexual reproduction, which we attributed to reversion of *masc1* silencing in at least one of the two parental nuclei. In crosses between the same strains and strains carrying the *masc1* wild-type allele, no asci indicative of MIP was observed, except in rare tiny sectors of some plates. The presence of sectors indicating the occurrence of sexual reproduction and MIP was expected if *masc1* silencing is not faithfully maintained as observed also for other silenced genes in Ascobolus (Rhounim et al., 1992). This demonstrates that sterility and absence of MIP do result from silencing of the *masc1* resident gene rather than from its eventual inactivation due to the plasmid integration. The fact that methylation is maintained at the *masc1* locus despite the

silencing of the *masc1* gene constitutes further evidence that the product of *masc1* is not a maintenance MTase.

## Discussion

### Masc1 Displays the In Vivo Features of a De Novo DNA Methyltransferase

The structural features of Masc1 suggest that it is a novel type of a eukaryotic MTase. The differences in its primary structure with other known eukaryotic MTases are presumably related to differences in specific enzyme functions. Masc1 is required during the differentiation processes of sexual reproduction and for the targeted methylation of duplicated DNA sequences that occurs during this phase of the life cycle. The structural and genetical data strongly suggest that it exercises its primary function as an enzyme performing de novo methylation, in contrast to the known MTases of mouse, human, and Arabidopsis, which have been shown to function mainly as maintenance MTases. Nevertheless, as long as in vitro activity has not been shown, we cannot formally exclude that Masc1 is not itself the MTase. Maintenance methylation is apparently not affected in the absence of Masc1 activity, indicating that at least one more MTase is expressed in Ascobolus. At least one other MTase is thought to exhibit a developmentally controlled de novo methylation activity in mouse (Lei et al., 1996). Possibly, this proposed mammalian de novo MTase might have structural and also functional similarities to Masc1, and reciprocally, the yet unidentified maintenance MTase of Ascobolus may belong to the family of already identified eukaryotic MTases.

MIP occurs in the dikaryotic tissue formed after fertilization. Nevertheless, the disruption of the *masc1* gene in only one parent is sufficient to reduce the frequency of MIP. This cannot be simply explained by a negative dominant effect of the *masc1* disruption, since a transgenic *masc1* copy can fully restore the wild-type MIP frequency despite the presence of the disruption. It may reflect the fact that MIP efficiency strongly depends on the concentration of the Masc1 product. The most drastic reduction of MIP frequency is seen when the gene duplication used to test MIP and the active *masc1* allele are in separate nuclei. This suggests that the product of *masc1* tends to be relocalized into the nucleus from which it originated. Such nuclear restriction was evidenced by Griffiths (Griffiths, 1976) for tRNA supersuppressor gene products in Neurospora heterokaryons. Finally, it should be noted that, although MIP events are extremely rare in nuclei harboring the *masc1* disruption, they lead to full methylation of the MIP products. This suggests that, once the duplication has been targeted, Masc1-induced methylation occurs in a highly processive manner.

### A Dual Role of Masc1 in Defense Mechanisms and Development?

Two roles for genomic methylation in mammals have been proposed (reviewed by Bestor and Tycko, 1996). According to the first hypothesis, cytosine methylation is part of a genome defense system, which inactivates parasitic sequences such as transposable elements and proviral DNA (Bestor, 1990; Doerfler, 1991) and makes them inaccessible to recombination (Faugeron et al., 1990; L. Maloisel and J.-L. R., unpublished data). The second hypothesis suggests that programmed demethylation and de novo methylation play a direct role in gene control during development. The essential role of MTase in development (Li et al., 1992) and the role of methylation in gametic imprinting (Barlow, 1993) support this hypothesis. Barlow (1993) suggested that imprinting in mammals may have evolved as an extension of the host defense role that methylation plays against invading DNA. In her proposal, foreign DNA becomes de novo methylated as part of the host defense function of MTase because it contains sequences that are recognized as foreign DNA, and imprinted genes are methylated by the same system because they contain an imprinting box that looks foreign to the organism. A common feature shared by imprinted genes is that they contain, or are closely associated with, a region rich in direct DNA repeats (Barlow, 1995). The consequences of *masc1* disruption on sexual development and MIP, and the previous finding that repeated retroelements contain the hallmarks of MIP (Goyon et al., 1996b), suggest that in Ascobolus, a lower eukaryote, methylation may play a dual role: it is involved in the control of development and it acts as a defense mechanism. On an evolutionary point of view, and in keeping with Barlow's hypothesis (Barlow, 1993), this suggests that the extension of the host defense role of methylation to a role in developmental control may have arisen early in evolution or may have happened several times in the course of evolution. On the other hand, a striking parallel can be put forward between the presence of direct DNA repeats in genes submitted to gametic imprinting and the fact that the targets for MIP are DNA repeats. Hence, it is tempting to hypothesize that the target of Masc1 involved in sexual development is constituted by one or several clusters of DNA repeats. Methylation of these repeats would control expression of genes whose activity, or inactivity, is required for the proper completion of the sexual cycle.

## Experimental Procedures

### Genetic Procedures and Media

Standard Ascobolus genetic techniques, transformation procedures, and media were used (Rhounim et al., 1994; references therein).

### Isolation and Manipulation of DNA

Most experimental procedures were as previously described (Goyon and Faugeron, 1989). Other standard techniques were as described in Sambrook et al. (1989). For PCR amplifications, unless otherwise noticed, standard conditions were used (Rhounim et al., 1992).

### PCR Amplification, Cloning, Sequencing of the *masc1* Gene and RT-PCR Experiments

The *masc1*-specific 78 bp fragment was PCR amplified from genomic DNA of the wild-type Ascobolus strain RN42 (Goyon et al., 1994) using the pair of primers 5′-CCGAATTCCARGGNTTYCCNGA-3′ and 5′-CGGGATCCGGNGGNACNGCRTTNCC-3′, whose 3′ parts correspond to the sequence encoding the most conserved part of motifs IX and X, located at positions 2391–2404 (sense strand) and 2467–2451 (antisense strand), respectively (Figure 1). Each primer was designed such that the third position of every codon was constituted of degenerated bases and was tailed at its 5′ end with eight nucleotides generating an EcoRI or a BamHI site, respectively (underlined).

Amplification was performed in 40 μl reaction volumes containing 200 ng of genomic DNA, 2 nmol of each primer, 200 μM (each) dNTPs, and one unit of Ampli-Taq polymerase (Perkin Elmer) and the buffer supplied by the manufacturer, under the following cycling conditions: an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 46°C, and 30 s at 65°C. The last cycle was followed by a 2 min extension at 65°C. PCR fragments in the range of 70–100 bp were gel purified, cleaved with BamHI and EcoRI, ligated into pBluescript KS$^-$, and transformed into E. coli XL-1 Blue cells (Stratagene). Plasmid pK11 containing the 78 bp insert was used in Southern hybridization to probe Ascobolus genomic DNA digests. To clone the 3 kb HindIII hybridizing fragment, a size-fractionated (2.5–4.7 kb) HindIII digest of strain RN42 DNA was subcloned into the HindIII-digested vector pBluescript KS$^-$. Clones containing the correct insert were identified by colony hybridization using the 78 bp fragment as a probe. The insert of one recombinant plasmid, pMI, was entirely sequenced by using the ABI PRISM Ready Reaction DyeDeoxyterminator Cycle sequencing kit. *Masc1* transcripts were reverse transcribed and amplified via 5′ RACE (using a reverse primer, positions 2302–2272, Figure 1) and conventional RT-PCR experiments (using forward and reverse primers, positions 1507–1531 and 2540–2518, Figure 1, respectively). Total RNA and poly(A)$^+$ RNA extracted from vegetatively grown mycelia were used as templates for RT-PCR and 5′ RACE experiments, respectively. Kits from BRL were used.

**Construction of *masc1* Expression Plasmids**

To obtain a continuous *masc1* ORF for cloning into expression vectors, the 51 bp intron (positions 1891–2274, Figure 1) was deleted in plasmid pMI. Two PCR fragments A and B flanking the 51 bp intron were first generated. Fragment A was obtained with primers located at positions 1645–1661 and 2054–2032 (Figure 1), blunt ended, and digested with BsmBI. Fragment B was obtained with primers at positions 1645–1661 and 2540–2520 (Figure 1) and digested with StuI and BspMI. The A and B fragments were jointly ligated into plasmid pMI cut at the unique BsmBI and BspMI sites, to form plasmid pMasc1. The *masc1* continuous ORF present in pMasc1 was verified by sequencing.

Using pMasc1 as a template, the *masc1* ORFs starting at each of the three in-frame ATG codons respectively located at positions 876–878, 990–992, and 1506–1508 (Figure 1) were amplified by PCR using forward primers located at positions 876–897, 990–1011, and 1506–1527, respectively, and a reverse primer located at positions 2540–2520 (Figure 1). Forward primers were synthesized with 5′ linkers generating an EcoRI site (CGGAATTCCGG); the reverse primer was synthesized with an XbaI linker (GCTCTAGAG). The restriction sites were used to insert the PCR fragments in the expression vectors pMal-c2 (New England Biolabs) and pAcHLT-A (Stratagene). All constructs were sequenced. Overexpression and purification of fusion proteins were performed according to the manufacturer protocols. In case of the pMal-c2 constructs, fusion proteins were cleaved with factor Xa. To verify correctness of cleavage products, Masc1 polypeptides were subjected to N-terminal microsequencing (on an ABI 494 PROCISE sequencer) after SDS gel separation and blotting on a PVDF membrane (Schleicher and Schuell). In vitro methylation by the expressed proteins was measured as described in (Günthert et al., 1981).

**Plasmids**

The strategy used to construct plasmid pFM9 is presented in Figure 3. Two KpnI sites were shown to flank the 3 kb HindIII genomic *masc1* fragment. The fragments obtained after digestion of the Ascobolus DNA with KpnI were circularized by intramolecular ligation (performed at a concentration of 5 μg/ml). They were then digested with PvuII, which has a unique recognition site in the *masc1* KpnI fragment. Digested DNA was used as a template for *masc1*-specific PCR amplification using the pair of primers located at positions 2302–2325 and 1683–1660 (Figure 1). Each primer was tailed at its 5′ end by nine nucleotides (GTTCCGCGG and ACTCCGCGG, respectively) generating a SacII site. These primers were designed to generate the internal deletion of motifs III–VIII of the Masc1 catalytic domain. The PCR product was digested with SacII and inserted into the SacII site of plasmid pGB20K carrying *met2*, generating plasmid pFM9. pGB20K is a derivative of pGB20 (Goyon et al., 1996a) in which the KpnI site had been deleted.

Plasmid pFM12 carries the *masc1* HindIII fragment and the *hph* gene used as a selectable marker in transformation. It results from the integration of the *hph* carrying HindIII fragment from plasmid pMP6 (M. Plamann, personal communication) into the downstream HindIII site of plasmid pIM.

**Disruption of the Native *masc1* Gene**

Plasmid pFM9 (Figure 3A), cut at its unique KpnI site, was used to transform the Met$^-$ strain FB22 (*masc1*$^+$, *met2*.Δ::*amdS*, *b2*$^+$) that harbored a *met2* disrupted allele (Goyon et al., 1996a). Met$^+$ transformants (720) were screened for homologous integration of pFM9 at the *masc1* locus. This was done by probing with *masc1* (Figure 3B) Southern blots of KpnI DNA digests from pools of 15 transformants (data not shown). The two transformants E4 and H5, in which the 6.3 kb KpnI resident fragment appeared to be replaced by the expected 12.2 kb KpnI fragment, were isolated from two distinct pools. The *masc1*.Δ::*met2* disruption was further verified in these transformants using various restriction enzymes (see Figure 3C).

**Construction of the Ascobolus Strains Used to Test the Effect of the *masc1*.Δ::*met2* Mutation on Methylation Maintenance and MIP**

To construct these strains, we used a transgenic construct "*met2 b2 hph*," containing the *b2* gene bordered in its 5′ part by a copy of *met2* and in its 3′ part by a copy of *hph*. This construct was integrated at a chromosomal site unlinked to *b2* and *met2* (L. Maloisel and J. -L. R., unpublished data).

To associate the *masc1* disruption with a methylated *hph* transgenic copy, we crossed each of the two E4 and H5 primary transformants (*masc1*.Δ::*met2*, *met2*.Δ::*amdS*, *b2*$^+$, *mt*+) with a *masc1*$^+$, *met2*.Δ::*amdS*, *b2*.Δ*1230*, "*met2*$^+$*b2*$^+$*hph*$^m$," *mt*− strain of the opposite mating type (*mt*), in which the transgenic *hph* had been methylated (L. Maloisel and J.-L. R., unpublished data). The ascospore pigmentation phenotype was used to follow the segregation of the transgenic construct in the progeny of these crosses. The null *b2*.Δ*1230* allele gives white ascospores (W) (Colot and Rossignol, 1995). In the asci showing the 4B:4W segregation of the ascospore pigmentation, the brown ascospores (B) would have inherited the *b2* duplication constituted by the resident wild-type *b2* allele and the transgenic construct. Such brown ascopores were germinated and checked for the presence of the *masc1*.Δ::*met2* allele by PCR amplification using the pair of primers located at positions 1764–1782 and 2191–2172 (Figure 1).

Types I to IV strains (Table 1) were issued from a single cross between the primary transformant H5 (*masc1*.Δ::*met2*, *met2*.Δ::*amdS*, *b2*$^+$, *mt*$^+$) and a *met2*.Δ::*amdS*, *b2*$^+$, "*met2*$^m$ *b2*$^+$ *hph*$^+$," mt$^-$ strain in which the *met2* transgenic copy was silenced. Brown ascospores were harvested in 8B:0W asci, in which the *b2* duplication had not undergone MIP. Their Met$^+$ or Met$^-$ phenotype indicated the presence of *masc1*$^+$ or *masc1*Δ::*met2*, respectively. Their Hyg$^S$ or Hyg$^R$ phenotype indicated the absence or the presence of the *b2* duplication, respectively.

**Complementation of the *masc1*.Δ::*met2* Mutation by the Wild-Type *masc1* Gene**

A type I strain (*masc1*.Δ::*met2*, *met2*.Δ::*amdS*, *b2*$^+$) was transformed with plasmid pFM12, which carries the *masc1* 3 kb HindIII fragment together with the *hph* gene used as a selectable marker. To construct strains harboring simultaneously the *masc1*.Δ::*met2* mutation, a single *masc1* transgenic copy and a *b2* duplication, 53 Hyg$^R$ transformants (*masc1*.Δ::*met2*, *met2*. Δ::*amdS*, *b2*$^+$, "*masc1 hph*") were crossed with a *masc1*$^+$, *met2*.Δ::*amdS*, *b2*.Δ*1230*, "*met2*$^m$ *b2*$^+$ *hph*$^+$" strain. Brown ascospores from individual 4B:4W asci were germinated and screened for their Met$^+$ phenotype. These strains had inherited the *b2* duplication together with the *masc1*.Δ::*met2* mutated allele. Those having inherited the *masc1* transgene were identified by PCR amplification of a short internal segment of *masc1* using primers located at positions 1764–1782 and 2191–2172, respectively. Since a transforming plasmid becomes often truncated through chromosomal integration in Ascobolus (Faugeron et al.,

1989), we further selected 12 strains having integrated an entire, untruncated copy of *masc1* by PCR amplification using primers located at positions 6–24 and 2865–2845 (Figure 1). Southern hybridization indicated that *masc1* was methylated in nine of these strains (data not shown) probably because the pFM12 plasmid had integrated in multiple clustered copies, which would have inevitably suffered MIP (Rhounim et al., 1994). Three strains (20, 34, and 17) issued from two distinct primary transformants exhibited the expected genotype with a single nonsilenced transgenic *masc1* copy. For control crosses, we isolated in parallel from other transformants two strains (12 and 17), which had not integrated the *masc1* transgenic copy, and one strain (2) exhibiting a complete methylation of the *masc1* transgenic copy(ies).

### Light Microscopy

Apothecia were fixed in fresh fixative (butanol, proprionic acid, and 10% aqueous chromic acid, 9:6:2 v/v). After 10 min of hydrolysis at 70°C, asci were stained in 2 drops of 2% hematoxylin mixed on the slide with 1 drop of ferric acetate solution.

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