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A STE12 homologue of the homothallic ascomycete *Sordaria macrospora* interacts with the MADS box protein MCM1 and is required for ascosporogenesis

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Summary

The MADS box protein MCM1 controls diverse developmental processes and is essential for fruiting body formation in the homothallic ascomycete *Sordaria macrospora*. MADS box proteins derive their regulatory specificity from a wide range of different protein interactions. We have recently shown that the *S. macrospora* MCM1 is able to interact with the alpha-domain mating-type protein SMTA-1. To further evaluate the functional roles of MCM1, we used the yeast two-hybrid approach to identify MCM1-interacting proteins. From this screen, we isolated a protein with a putative N-terminal homeodomain and C-terminal C_2/H_2-Zn^{2+} finger domains. The protein is a member of the highly conserved fungal STE12 transcription factor family of proteins and was therefore termed STE12. Furthermore, we demonstrate by means of two-hybrid and far western analysis that in addition to MCM1, the *S. macrospora* STE12 protein is able to interact with the mating-type protein SMTA-1. Unlike the situation in the closely related heterothallic ascomycete *Neurospora crassa*, deletion (Δ) of the *ste12* gene in *S. macrospora* neither affects vegetative growth nor fruiting body formation. However, ascus and ascospore development are highly impaired by the Δ*ste12* mutation. Our data provide another example of the functional divergence within the fungal STE12 transcription factor family.

Introduction

The differentiation of a diploid cell into a haploid cell via meiotic cell cycle is a hallmark of eukaryotic organisms. In fungi, the meiotic regulatory cascade relies on transcription factors encoded at the mating-type locus. The allele type at the fungal mating-type locus determines sexual compatibility between haploid individuals in fungi (Fraser and Heitman, 2004; 2005). The genetic breeding mechanism of fungi is called heterothallism when sexual reproduction occurs only between cells of the opposite mating type. Strains of heterothallic ascomycetes exist in two mating types (MAT); these are termed MATα and MAT***a*** in the budding yeast *Saccharomyces cerevisiae* as well as MAT***A*** and MAT***a*** in the filamentous ascomycete *Neurospora crassa*. The alternative versions of the mating-type loci on homologous chromosomes are termed idiomorphs because they are completely dissimilar in the genes they encode (Metzenberg and Glass, 1990; Coppin *et al*., 1997; Shiu and Glass, 2000).

The mating system has been well studied in the budding yeast *S. cerevisiae*. The MAT-encoded transcription factors are responsible for cell type-specific gene expression. Each mating-type locus carries two genes. The MATα1 protein of the MATα locus has been shown to be a transcriptional activator of α-specific genes and carries the α-domain as a DNA-binding motif (Bender and Sprague, 1987). The gene product of *mat**a**2* is a homeodomain (HD) protein and acts as a negative regulator of **a**-specific genes. In the MAT***a*** locus, *mat**a**1* is the only gene to encode a functional protein which is also a HD transcription factor (Dolan and Fields, 1991).

The MAT loci of the filamentous ascomycete *N. crassa* share some features with those of *S. cerevisiae*; however, the encoded proteins are different. The MAT***A*** idiomorph contains three genes: *mat A-1*, *mat A-2* and *mat A-3* (Glass *et al*., 1990; Ferreira *et al*., 1998). The MAT***a*** idiomorph harbours two genes, *mat a-1* and *mat a-2*. Only *mat A-1*, *mat A-3* and *mat a-1* encode proteins with DNA-binding motifs (Staben and Yanofsky, 1990; Pöggeler and Kück, 2000).

Besides heterothallism, homothallism as a second mating system can be observed in ascomycetes. Homothallic species have no genetically definable mating type and are self-fertile. The mycelium derived from a uninucleate ascospore or a vegetative spore of a homothallic fungus is able to complete the sexual cycle without interacting with a mating partner (Pöggeler, 2001). *Sordaria macrospora* is a homothallic ascomycete that is closely related to the heterothallic *N. crassa*. Under laboratory conditions, the life cycle of *S. macrospora* is completed within 7 days. Two days after inoculation and

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without any stimulation or further treatment, sexual development starts from the vegetative mycelium with the formation of female gametangia, called ascogonia. At day four these are enwrapped by sterile hyphae to make closed spherical fruiting body precursors, the protoperithecia. Pairs of nuclei synchronously and repeatedly divide inside the ascogonium, thereby producing a great number of nuclei. The nuclei then migrate in pairs to the developing dikaryotic ascogenous hyphae emerging from the ascogonium. The ascogenous hyphae tips develop a U-shaped hook (croizer) containing two nuclei. Five days after inoculation, the two nuclei in the apical cell of each croizer fuse. Immediately after karyogamy within the young ascus, the diploid nucleus undergoes meiosis, which is followed by a post-meiotic mitosis, resulting in the formation of eight nuclei. Each of the eight nuclei is incorporated into its own ascospore. The development is finished when the fruiting bodies of *S. macrospora*, called perithecia, harbour between 50 and 150 asci. After 7 days, mature black ascospores are forcibly discharged through an apical pore at the neck of the fruiting body.

Analyses of the mating-type locus of *S. macrospora* revealed that this locus contains sequences homologous to both the MAT***a*** and MAT***A*** idiomorphs of *N. crassa* (Pöggeler *et al*., 1997a). In the mating-type locus of *S. macrospora*, four different open reading frames (ORFs) were identified, *Smta-1*, *SmtA-3*, *SmtA-2* and *SmtA-1*, and all of them are transcribed (Pöggeler and Kück, 2000). The proteins encoded by two of these genes (*Smta*-1 and *SmtA*-1) contain domains typical for eukaryotic transcription factors.

In the budding yeast, both MATα1 and MATα2 need to cooperate with the minichromosome maintenance protein 1 (MCM1) to act as transcription factors (Bender and Sprague, 1987; Keleher *et al*., 1988). In **a**-cells, MCM1 activates **a**-specific genes together with the HD transcription factor STE12 (Johnson, 1995). MCM1 is an essential sequence-specific homodimeric DNA-binding protein and a member of the MADS box transcription factor family. Members of the MADS box family of transcription factors play pivotal roles in regulating key biological processes in a diverse range of eukaryotic organisms including yeast, plants, lower vertebrates and mammals (Shore and Sharroks, 1995). The MADS box is a highly conserved sequence motif characteristic of this family of transcriptional regulators. This motif was identified after sequence comparison of MCM1 of *S. cerevisiae*, the thale cress AGAMOUS protein, DEFICIENS of *Antirrhinum majus*, and the human SRF (serum response factor), and the name MADS was derived from the 'initials' of these four 'founders' (Sommer *et al*., 1990). Beyond its function in cell type-specific gene expression and minichromosome maintenance in *S. cerevisiae*, the MADS box protein MCM1 is involved in controlling several different processes, including arginine metabolism, cell cycle progression and cell wall maintenance (Keleher *et al*., 1988; Passmore *et al*., 1988; Lydall *et al*., 1991; Messenguy and Dubois, 1993; McInerny *et al*., 1997; Kumar *et al*., 2000; Abraham and Vershon, 2005). To achieve these diverse functions, MCM1 interacts with a diverse range of transcription factors to repress or activate different sets of genes (reviewed in Messenguy and Dubois, 2003).

In contrast to *S. cerevisiae*, little is known about the role of MCM1-like MADS box proteins in other fungi. The *Schizosaccaromyces pombe* Map1p is related to the *S. cerevisiae* MCM1 protein and is also required for cell type-specific gene expression (Nielsen *et al*., 1996; Yabana and Yamamoto, 1996). In *Ustilago maydis*, an MCM1 MADS box homologue regulates the expression of pheromone-inducible genes (Krüger *et al*., 1997), whereas the MADS box transcription factor SRFA from *Dictyostelium discoideum* plays an essential role in sporulation (Escalante and Sastre, 1998). We recently isolated and characterized a *S. cerevisiae* MCM1 homologue from the homothallic ascomycete *S. macrospora*. The *S. macrospora* MCM1 protein is able to form a homodimer and to interact with the mating-type protein SMTA-1, which is the homologue to the *S. cerevisiae* MATα1 mating-type protein. Deletion (Δ) of the *S. macrospora mcm1* gene resulted in reduced biomass, increased hyphal branching, and reduced hyphal compartment length during vegetative growth. Furthermore, the *S. macrospora* Δ*mcm1* strain is unable to produce fruiting bodies or ascospores during sexual development (Nolting and Pöggeler, 2006). The pleiotropic phenotype of the *S. macrospora* Δ*mcm1* led us to suppose that the *S. macrospora* MCM1 protein might be involved in a wide range of functions via interaction with diverse transcriptional regulators. To further evaluate the physiological roles of MCM1, we performed a yeast two-hybrid screen to identify MCM1-interacting proteins. From this screen, we isolated, among several other proteins, a putative homologue of the *S. cerevisiae* HD protein STE12. The *S. macrospora* protein was therefore termed STE12 in *S. macrospora*. In *S. cerevisiae*, STE12 is known to be a transcriptional factor active downstream of FUS3/KSS1 MAP kinases where it plays critical roles in the regulation of mating, pseudohyphal growth, and cell wall biosynthesis. For example, in haploid yeast cells, STE12 is required for the response to mating pheromone produced by the opposite mating type and for invasive growth in response to limited nutrients. In diploids, STE12 regulates pseudohyphal development in response to nitrogen starvation (Madhani and Fink, 1997; Gustin *et al*., 1998; Roberts *et al*., 2000). In each case, STE12 induces transcription of genes necessary to produce the appropriate cell cycle progression and morphological alterations. Transcriptional regulation of different classes of genes is

thereby triggered through interactions of STE12 with different transcriptional regulators. STE12 interacts with itself as well as with MCM1 and MATα1 to regulate pheromone-responsive genes and cell type-specific genes respectively, and interacts with TEC1 to activate genes required for filamentous growth (Dolan *et al*., 1989; Yuan *et al*., 1993; Bruhn and Sprague, 1994; Madhani and Fink, 1997). Homologues of the *S. cerevisiae* transcription factor STE12 have also been characterized in several filamentous ascomycetes (Liu *et al*., 1994; Vallim *et al*., 2000; Borneman *et al*., 2001; Park *et al*., 2002; Tsuji *et al*., 2003; Li *et al*., 2005). While in *Aspergillus nidulans* and *N. crassa*, STE12 homologues are required for sexual development (Vallim *et al*., 2000; Li *et al*., 2005), in the plant pathogens *Colletotrichum lagenarium* and *Magnaporthe grisea*, they are involved in pathogenicity (Park *et al*., 2002; Tsuji *et al*., 2003).

In this study, we identified the STE12 homologue of *S. macrospora* as an interaction partner of MCM1. Two-hybrid and biochemical studies showed that the N-terminus of STE12 associates with MCM1. In addition, we found that the *S. macrospora* STE12 protein is able to interact with the mating-type protein SMTA-1. Analysis of a Δ*ste12* knockout mutant demonstrated that STE12 of *S. macrospora* plays an essential role in ascosporogenesis but not in fruiting body formation and vegetative growth.

Results

Yeast two-hybrid screening identifies STE12 as an interaction partner of MCM1

To further evaluate the function of the MADS box transcription factor MCM1 in *S. macrospora*, a yeast two-hybrid screening was carried out. For this screening the 5′-coding region of the *mcm1* gene, encoding the first 155 amino acids (aa) of the MCM1 N-terminus, was used as bait, because the full-length MCM1 showed strong self-activation of reporter gene expression (Nolting and Pöggeler, 2006). A stringent selection procedure was performed with yeast host strain PJ69-4A that contains three easily assayed reporter genes, each under the control of a different inducible promoter. This host strain is extremely sensitive to weak interactions and eliminates nearly all false positives using both, analysis of growth on media lacking either histidine or adenine and β-galactosidase assay (James *et al*., 1996). Eleven colonies growing on selective media and having significant β-galactosidase activity were obtained from screening the *S. macrospora* cDNA library (Fig. 1, Table S1). Sequence analysis revealed that all identified inserts encode parts of proteins with known function in *N. crassa* or closely related fungi. Homology searches demonstrated that clones MGB18-1 and MGB18-4 are two independently isolated clones encoding a homologue of the *Hypocrea jecorina* glucokinase (Hartl and Seiboth, 2005), while clones MGB13-6 and MGB17-1 were identified as two independent clones that encode a homologue of the *S. cerevisiae* TVP15 a putative integral membrane protein localized to late Golgi vesicles along with the v-SNARE TLG2 (Inadome *et al*., 2005). In this study, we focused our attention on the positive clone MGB15-3 encoding a *S. macrospora* orthologue of the *N. crassa* STE12 protein (Li *et al*., 2005).

S. macrospora *possesses a STE12 homologue*

Primers S12A and S12E (Table 1) were designed according to the insert of pMGB15-3 (Table S1), encoding a part of the putative homologue of the *S. cerevisiae* STE12. This *ste12*-specific primer pair was used to perform a high throughput PCR screening of pooled cosmid DNA as described in *Experimental procedures*. The isolated cosmid was then used for subcloning and sequencing of the putative *S. macrospora ste12* coding region and its flanks. The *ste12* ORF comprised 2407 bp interrupted by three introns of 62, 71 and 93 bp with conserved 5′ donor and 3′ acceptor sequences (Pöggeler, 1997), whose presence was confirmed by sequencing the corresponding cDNAs. The *ste12* gene encodes a putative protein of 726 aa with a predicted molecular mass of 80.0 kDa and a calculated isoelectric point of 6.2 (Fig. S1). Overall, the predicted *S. macrospora* protein shares the highest homology with STE12 of *N. crassa* (95.6% identity). The homology to other members of the fungal STE12 family is mainly found in the Ste DNA-binding domain (Pfam PF02200, Smart SM00424) that includes a conserved HD, based upon the Antennapedia consensus sequence (Scott *et al*., 1989; Yuan and Fields, 1991). The region encompassing this domain of the *S. macrospora* STE12 is 66.2–100% identical to the corresponding region from STE12 homologues of other fungi (Fig. S1). In *S. cerevisiae*, STE12 binds to pheromone response elements with helix III of the HD. This helix is highly conserved among the fungal STE12 homologues. In addition, the *S. macrospora* protein contains two C_2/H_2-Zn^{2+} finger domains at the C-terminus (aa 597–619 and 627–647) which are conserved only in STE12 homologues of filamentous fungi but are lacking in the STE12 homologues of ascomycetous yeasts (Fig. S1).

Using the program ‘Promoter Predictor’, the presumed transcriptional start site of the *ste12* gene was predicted to be 116 nucleotides upstream of the putative *ste12* translation start codon. Sequences flanking the ATG start codon show a high level of similarity to translation initiation sites of other *S. macrospora* genes (Pöggeler, 1997). Within the promoter region, a putative TATA box

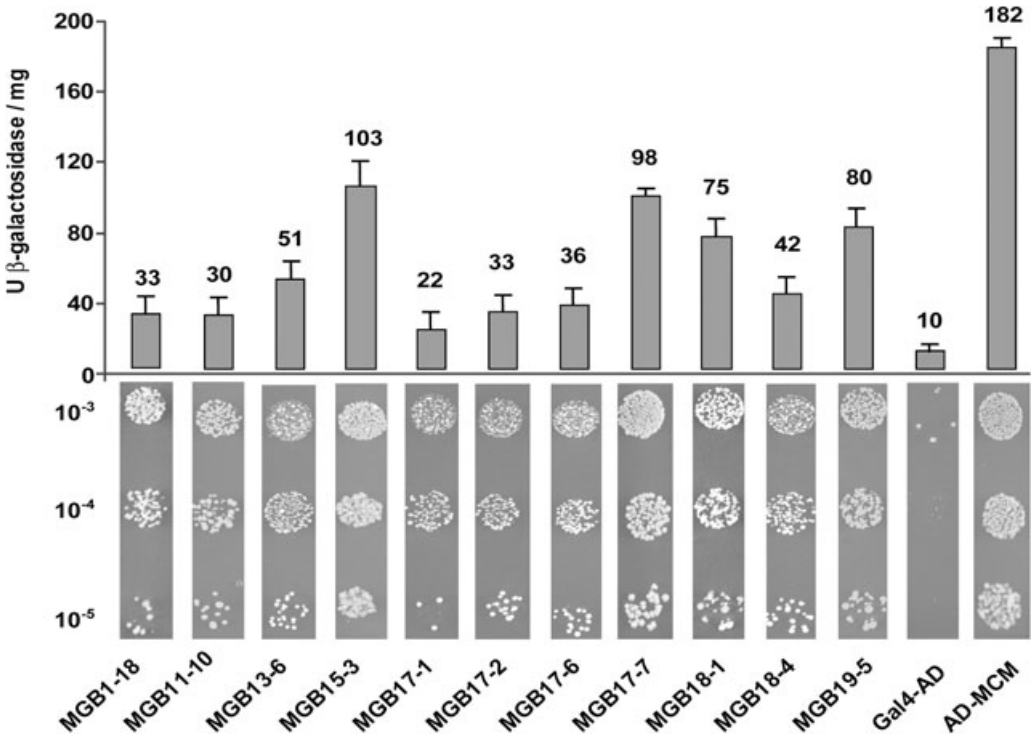

Fig. 1. Yeast two-hybrid screen between GAL4-BD-MCM1d2 and the *S. macrospora* cDNA library determined β-galactosidase activity and growth of 11 transformants expressing GAL4-BD-MCM1d2 together with GAL4-AD-fusion proteins. β-Galactosidase activity was measured in triplicate for each transformant. Error bars represent the standard deviation. For the growth determination of the cells on agar plates, 10 μl of 1:10 serial dilutions was spotted on the SD plates lacking uracil, leucine and adenine. As a negative control, PJ69-4A (+ pBMd2) was transformed with the plasmid pGAD-C1 expressing GAL4-AD, and, as a positive control, PJ69-4A (+ pBM1d2) was transformed with the plasmid pAM expressing GAL4-AD-MCM1.

was predicted by the 'Hamming-Clustering method' at position −38 relative to the transcription initiation site. Computational analysis of the *S. macrospora ste12* promoter with MatInspector (Cartharius *et al*., 2005) revealed no matches with *S. cerevisiae* pheromone or filamentation response elements (Dolan *et al*., 1989; Madhani and Fink, 1997). Possible binding sites for NIT2 (Fu and Marzluf, 1990) and the *Schizosaccaromyces pombe* HMG mating-type protein MATMc (Kjaerulff *et al*., 1997) were identified within the *S. macrospora ste12* promoter sequence.

Table 1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′–3′)
S12A	AGA TCT TAT CCT TCG CAG CAT GCC
S12B	GGA TCC TGA GAC ATG CTG CTA
S12C	GGA TCC TTC GAG AGG GTA CTC
S12D	AGA TCT CTA GAA CTC TAG TTC GTT TCG
S12E	GGAAGTCCAAGAAAGGGCTC
S12G	ATGTGCTCCCCTGAAATGTGTGCT
D1	TGCTCGATCACCCGGCCATCC
D2	GAATTCGGCATGCTGCGAAGGATACAT
D3	GGGCCCGAAACGAACTAGAGTTCTAG
D4	GGGCCCACCTTCCAAGATCCTGGTTTG
D5	CACCACCACACAGAGGAAAC
D6	GAGTGTCCGGCGGCAGTTTG
GAL4-AD	AAAGAGATCGAATTAGGATCCTCT
GAL4-T7	AATACGACTCACTATAGGGCTCTA
h3	ACTCGTCCGAGGGCAAAGGAATAG
tC1	GATCCGCCTGGACGACTAAACC

Expression of the S. macrospora ste12 *gene is developmentally regulated*

To determine the size of the *ste12* transcript and to examine whether the *ste12* expression depends on the developmental stage of *S. macrospora*, Northern blot analyses were performed. Total RNA from mycelium was isolated from *S. macrospora* wild-type strain daily from days 3 to 7 of sexual development. Under our experimental conditions, sexual development started 3 days after inoculation with the appearance of ascogonia and ended with the discharge of mature ascospores from perithecia 7 days after inoculation. The *S. macrospora ste12* transcript is 3.4 kb long, which is similar to the size of the *A. nidulans steA* transcripts (3.1 and 3.3 kb) (Vallim *et al*., 2000) (Fig. 2). Densitometrical quantification of the Northern hybridization signals revealed that the *ste12* transcript level increased until day 6, but drastically decreased at day 7 of sexual development (data not shown).

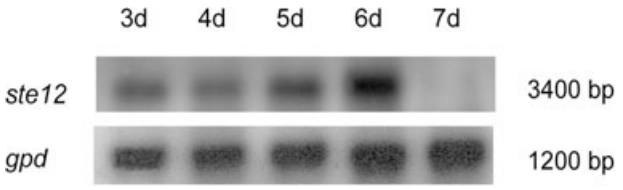

Fig. 2. Transcriptional expression of the *S. macrospora ste12* gene in the wild-type strain. Twenty micrograms of total RNA, isolated from mycelium at different time points during the life cycle of *S. macrospora* (3, 4, 5, 6 and 7 days), was loaded per lane. Northern blot was probed using a *ste12* gene-specific probe. As a control, the blot was stripped and re-probed with a *gpd* gene-specific probe. The Northern blot shown is a representative of Northern blots of five independent samples.

The N-terminus of the S. macrospora *STE12 protein interacts with MCM1 and the mating-type protein SMTA-1*

In *S. cerevisiae*, the C-terminus of STE12 is required for association with MCM1 (Errede and Ammerer, 1989; Kirkman-Correia *et al*., 1993). In contrast, the two-hybrid analysis presented revealed that the first 136 aa at the N-terminus of the *S. macrospora* STE12 protein encoded by plasmid pMGB15-3 (Fig. 1, Table 2) were able to interact with the MCM1 protein. To verify this result, the 5′ and 3′ regions of the *S. macrospora ste12* cDNA coding for either the N-terminal HD-domain (aa 1–428) or the C-terminal zinc-finger domain (aa 428–726) were fused to the GAL4-AD and GAL4-BD domain respectively. First, yeast cells transformed with the recombinant pGBDU-derivatives pBS12n and pBS12c carrying the N- and the C-terminal part of the *ste12* gene respectively, and the empty pGAD-C1 vector, were tested for self-activation of reporter gene expression. None of the transformants exhibited expression of the reporter genes (data not shown). Subsequently, both domains were separately analysed for homodimerization as well as for interaction with MCM1 in the yeast two-hybrid system. Neither the N-terminal nor the C-terminal nor the N-terminal and C-terminal parts of the STE12 protein were able to form a homodimer (Fig. 3). However, concordant with the results obtained in the two-hybrid screen, the N-terminus of STE12 was able to interact with the MCM1 protein (Fig. 3), while the C-terminus did not interact with MCM1 (data not shown).

In the yeast *S. cerevisiae*, the *ste12* gene encodes an essential DNA-binding protein that in cooperation with either MATα1 or MCM1 induces transcription of MAT***a***-specific genes (Yuan *et al*., 1993). During previous analyses, we were able to demonstrate the ability of SMTA-1 to interact with the mating-type protein SMTa-1 and the MADS box protein MCM1 (Jacobsen *et al*., 2002; Nolting and Pöggeler, 2006). To screen for the ability of *S. macrospora* STE12 to interact with mating-type proteins of this fungus, we tested the pGBDU- and pGAD-derivatives of the *ste12* gene in combination with pGBDU- and pGAD-derivatives carrying *S. macrospora* mating-type genes. In this assay, the interaction between SMTA-1 and SMTa-1 was used as a positive control. Analyses of reporter gene activity of yeast transformants carrying different combinations of pGBDU- and pGAD-derivatives revealed strong positive interactions of the N-terminal STE12 part with the mating-type protein SMTA-1 (Fig. 3A). The C-terminus of STE12 did not interact with any mating-type protein (data not shown). Similar to the positive controls [pAA1 + pBa1 (SMTA-1 + SMTa-1)], colonies carrying the N-terminus of STE12 (pBS12n) in combination with SMTA-1 (pBAA1) were able to grow on

Table 2. Plasmids used in this study.

Plasmid	Vector	Insert	Reference
pAS12n	pGAD-C1	1286 bp BglII/BamHI cDNA fragment of *ste12* (Position 4–1284)	This study
pAS12c	pGAD-C1	897 bp BglII/BamHI cDNA fragment of *ste12* (Position 1,284–2181)	This study
pBS12n	pGBDU-C1	1286 bp BglII/BamHI cDNA fragment of *ste12* (Position 4–1284)	This study
pBS12c	pGBDU-C1	897 bp BglII/BamHI cDNA fragment of *ste12* (Position 1,284–2181)	This study
pSte12-n2	pDrive	459 bp *ste12* cDNA of *S. macrospora wt*, RT-PCR with S12A/S12E (aa 1–153)	This study
pAM1	pGAD-C1	976 bp BamHI full-length *mcm1* cDNA	Nolting and Pöggeler (2006)
pBM1d2	GBDU-C1	*S. macrospora mcm1* cDNA (Position 1–465)	Nolting and Pöggeler (2006)
pAA1	pGAD-C1	Full-length *SmtA-1* cDNA (Position 1–921)	Jacobsen *et al*. (2002)
pAA2	pGAD-C1	Full-length *SmtA-2* cDNA (Position 1–1080)	Jacobsen *et al*. (2002)
pAA3	pGAD-C1	Full-length *SmtA-3* cDNA (Position 1–351)	Jacobsen *et al*. (2002)
pAa1	pGAD-C1	Full-length *Smta-1* cDNA (Position 1–864)	Jacobsen *et al*. (2002)
pBa1	pGBDU-C1	Full-length *Smta-1* cDNA (Position 1–864)	Jacobsen *et al*. (2002)
pH-SMTA1	pQE31	921 bp BamHI/BglII fragment of pB-A1	This study
pH-STE12	pQE31	468 bp BamHI *ste12* cDNA fragment (Position 4–459), sense	This study
pH-STE12i	pQE31	468 bp BamHI *ste12* cDNA fragment, inverse	This study
pG-MCM1	pGEX-4T-1	976 bp BamHI *mcm1* fragment of pAM1	This study
pSTE12-KO	pPTSTE12	930 bp upstream and 1175 bp downstream region for homologous recombination at the *ste12* locus separated by 1400 bp EcoRI *hph*-cassette of pCB1003	This study
pD-NAT1		*nat1* expression cassette	Kück and Hoff (2006)

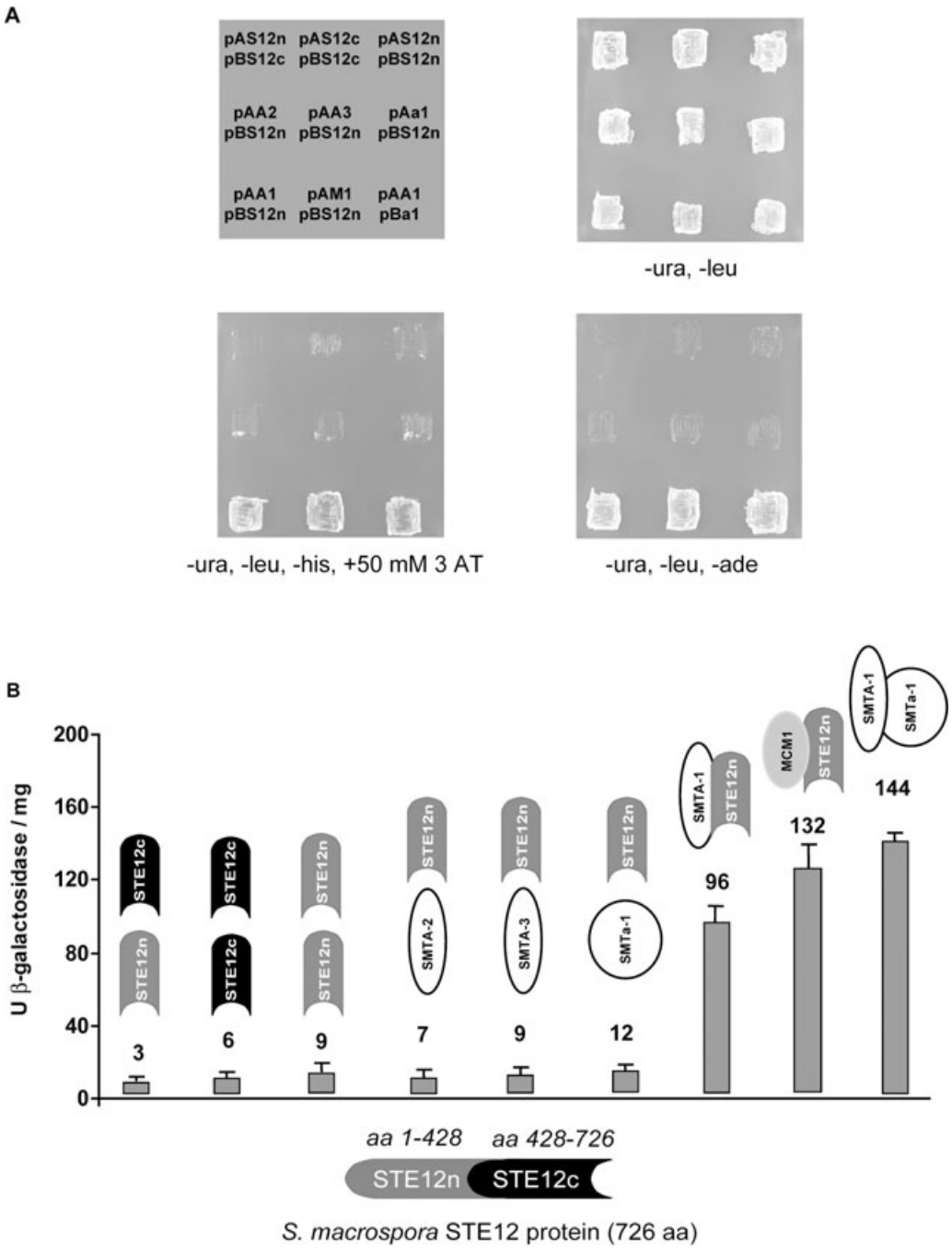

Fig. 3. Two-hybrid analyses of STE12 with MCM1 and mating-type proteins.
A. Yeast transformants carrying different combinations of pB- and pA-derivatives (Table 2) were examined for growth on –Ura, –Leu (top, right); –Ura, –Leu, –His + 50 mM 3-AT medium (bottom, left) or –Ura, –Leu, –Ade medium (bottom, right).
B. The β-galactosidase activity of the transformants carrying the GAL4-AD and BD-fusion proteins is given in units per milligram and represents the average of three independent measurements.

media lacking either histidine or adenine. The corresponding yeast transformants displayed a β-galactosidase activity comparable with the positive control, in contrast to yeast cells without associated protein combinations. From our two-hybrid analysis, there was no indication of interaction of the *S. macrospora* STE12 protein with the mating-type proteins SMTA-2, SMTA-3 or SMTa-1 (Fig. 3). To confirm our results from the two-hybrid assay, we examined the interactions of STE12 with SMTA-1 and MCM1 by far western blot analyses. For this purpose, we His-tagged the first 153 N-terminal aa of the STE12 protein containing the HD-domain (Table 2). After

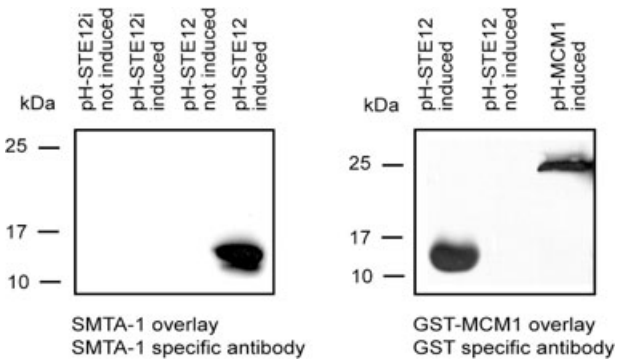

Fig. 4. Far western analyses of STE12 interacting with MCM1 and SMTA-1. His-tagged STE12 (pH-STE12) and MCM1 (pH-MCM1) fusion proteins and the corresponding inverse fusion construct (pH-STE12i) were purified and separated on a SDS-PAGE, blotted and overlaid with either a His-SMTA-1 fusion protein or a GST-MCM1 fusion protein. Bonded His-SMTA-1 and GST-MCM1 was detected by an anti-SMTA-1 and anti-GST antibody respectively.

induction of gene expression with isopropyl-β-D-galactopyranoside (IPTG), His-tagged fusion proteins were isolated, separated on gels, and analysed by far western blot. Filters were incubated with a purified His-tagged SMTA-1 or a GST-tagged MCM1 fusion protein which were detected by a polyclonal anti-SMTA-1 or anti-GST antibody respectively (Fig. 4). Interaction of His-MCM1 and GST-MCM1 was used as a positive control. As shown in Fig. 4, the His-STE12 N-terminal fragment of 15 kDa was detected with both antibodies. Thus, indicating specific positive interactions between His-SMTA-1 and His-STE12 as well as His-STE12 and GST-MCM1. To confirm that the observed interactions were not artefacts from non-specific cross reactions, we separated extracts of *Escherichia coli* cultures carrying the anti-sense construct pH-STE12i and additionally used non-induced *E. coli* cultures as negative controls. Taken together, the far western analyses corroborate the yeast two-hybrid data and confirm that the *S. macrospora* STE12 protein is able to interact with the α1-domain mating-type protein SMTA-1 and the MADS box transcription factor MCM1.

Deletion of ste12 *impairs ascus and ascospore development*

To assess the role of STE12 in *S. macrospora*, we constructed a Δ*ste12* deletion strain by gene replacement (Fig. 5). For this purpose, we constructed plasmid pSTE12-KO in which the *ste12* gene was deleted and replaced with a hygromycin-resistance cassette. The 3505 bp amplification product obtained was transformed into the *S. macrospora* Δ*ku70* strain. The *S. macrospora* Δ*ku70* strain is defected in the repair of DNA double-strand breaks by non-homologous end joining and has recently been demonstrated to be an ideal recipient for gene targeting of developmental genes in *S. macrospora* (Pöggeler and Kück, 2006). Five hygromycin-resistant colonies were isolated. Among these, two strains appeared to be heterokaryotic and contained wild-type nuclei (corresponding to a 2.1 kb hybridizing fragment) as well as Δ*ste12* nuclei (3.3 kb hybridizing fragment); whereas, three transformants contained only the Δ*ste12* 3.3 kb hybridizing fragment (Fig. 5B). To segregate the *hph* and *nat1* markers of one of the homokaryotic primary transformants (T1Δ*ste12*) and to obtain a Δ*ste12* knockout mutant without the Δ*ku70::nat1* background, we performed conventional genetic analyses. Primary transformant T1Δ*ste12* was crossed with spore colour mutant *fus1-1*. The vast majority of hygromycin-resistant ascospores isolated from the cross did not germinate. The poor recovery of hygromycin-resistant progeny, only three hygromycin-resistant ascospores out of 1000 isolated spores geminated, indicates that Δ*ste12::hph* might cause ascospore lethality. Only in one out of 110 analysed asci, Southern blot analyses and PCR amplification revealed a 1:1:1:1 segregation of hygromycin-resistant, nourseothricin-resistant, nourseothricin- and hygromycin-resistant, and non-resistant progeny; thereby, illustrating that the *ste12* gene is a nonessential gene. From these crosses, the hygromycin-resistant/nourseothricin-sensitive single-spore isolate S68567 (Δ*ste12*) was selected for further phenotypic analyses. PCR amplification confirmed that this Δ*ste12* single-spore isolate shows the genetic features of the original mutant strain T1Δ*ste12* without any wild-type background (Fig. 5C).

The Δ*ste12* mutant displayed nearly no alterations in the vegetative phenotype when compared with the wild type (data not shown). It showed only a slightly decreased growth of 18.1 (±1.4) mm day^{-1}, compared with 20.0 (±0.3) mm day^{-1} of the wild-type strain. Moreover, the mutant produced about 10% less mass of mycelium than wild type after 7 days of growth. Compared with the *S. macrospora* wild-type strain, there were no differences in branching frequency and hyphal compartment length (data not shown). Thus, deletion of the *ste12* sequence seems not to have, if any only minor, effects on the vegetative growth of *S. macrospora*.

To prove the effects of the *ste12* deletion on the sexual development, formation of sexual reproductive structures was analysed on fructification medium. Similar to the wild type, the mutant strain formed ascogonia, protoperithecia, and fruiting bodies (data not shown). The Δ*ste12* mutant strain produced the same amount of protoperithecia and perithecia per cm^2 when compared with wild type and the size of these fruiting bodies is also comparable. However, even after extended incubation time, the Δ*ste12* mutant was not able to discharge ascospores. After opening the fruiting bodies, we observed that the number of asci in the mutant strain was reduced to about 75% of wild-type level

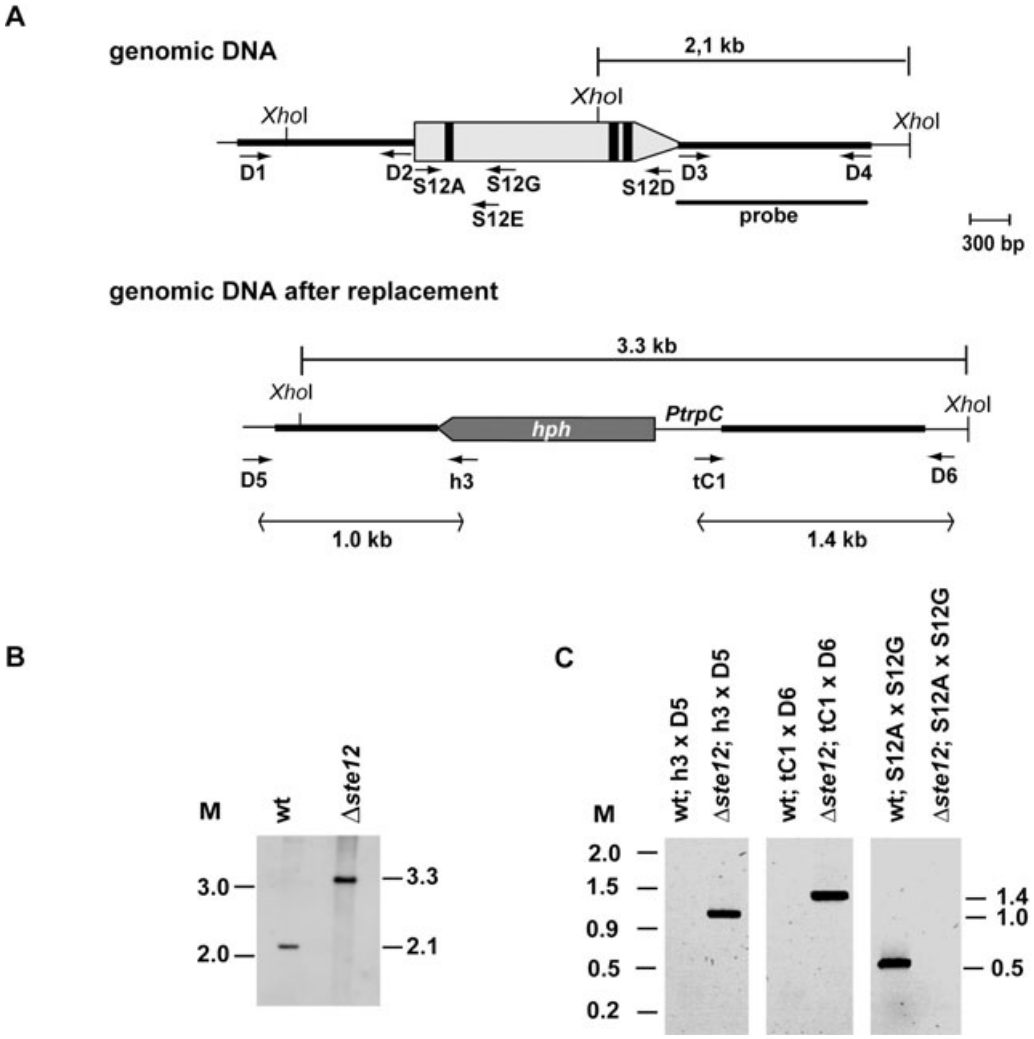

Fig. 5. Construction of a Δ*ste12* strain.
A. Schematic illustration of the genomic region of the *S. macrospora ste12* gene and its flanking sequences and generation of the Δ*ste12* replacement vector. The arrow represents the *ste12* coding region, interrupted by three introns (black boxes). Positions of primers used to amplify the disruption construct from plasmid pSTE12-KO and to verify the homologous recombination at the *ste12* locus are indicated. PtrpC, *A. nidulans trpC* promoter; *hph*, *E. coli* hygromycin B-resistance gene.
B. Southern analysis of *S. macrospora* wild-type (wt) strain and single-spore isolate S68567 (Δ*ste12*) were hybridized with the probe indicated in (A). Sizes of hybridized fragments in wt and knockout transformants are given in (A).
C. PCR analyses of homologous recombination from wt and single-spore isolate S68567 (Δ*ste12*). Positions of primers are indicated in (A).

and many asci seem to be burst (Fig. 6A). On average, the wild-type ascus rosettes were determined to be comprised of 64 asci, whereas only 18 asci were found in the ascus rosettes of the Δ*ste12* mutant (Fig. 6A).

To further analyse the defect within the reproductive structures of the Δ*ste12* mutant, DAPI staining was performed. This staining of nuclei revealed that hook-cell formation, karyogamy, and meiosis proceeds as in the wild type, but that the number of these reproductive structures was drastically reduced (data not shown).

In addition to the reduced number of asci, the development of asci and ascospores seemed to be affected in the Δ*ste12* mutant. Cell walls of asci and ascospores appeared to be very fragile and often bursted during the microscopic investigation. Calcofluor staining of the spore walls demonstrated that fluorescence was not restricted to the spore wall as in the wild type, but appeared to be uniformly distributed throughout the spore. In the mutant strain, the Calcofluor stain seemed to infiltrate into the cytoplasm due to the thinner ascospore wall (Fig. 6B). All morphological changes of the phenotype co-segregated with the knockout *hph* marker in crosses between Δ*ste12* and wild-type strains, indicating that deletion of the *ste12* gene is responsible for the mutant phenotype defective in ascosporogenesis. To further demonstrate that the phenotype was due to deletion of the *ste12* gene, we comple-

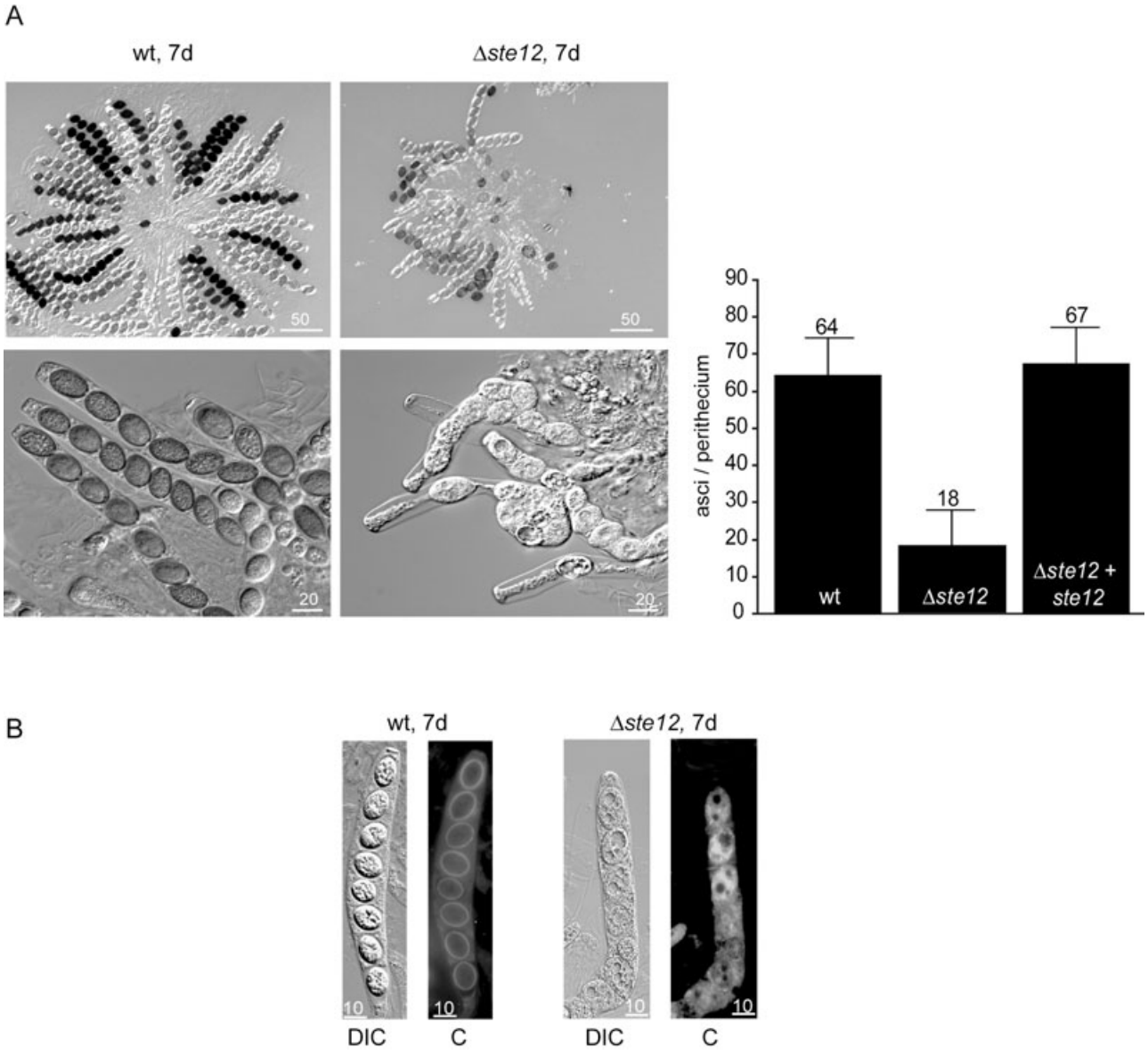

Fig. 6. Phenotype of *S. macrospora* Δ*ste12* strain.
A. For examination of ascospore development, the content of wild-type (wt) and Δ*ste12* perithecia was dissected. The average number of asci per perithecium was determined. Fifty perithecia of the wt strain, the Δ*ste12* mutant strain and complemented mutant strain were squeezed and after that the contents were analysed under the microscope.
B. Comparison of wt and Δ*ste12* ascus stained with calcofluor. DIC, differential interference contrast. C, Calcofluor staining. Bars represent sizes (μm) as indicated.

mented the mutant phenotype by transformation with the wild-type *ste12* gene. Like the wild type, complemented mutants were able to produce mature asci and ascospores (Fig. 6A).

Discussion

Recently, we have demonstrated that deletion of the *S. macrospora mcm1* gene resulted in a pleiotropic phenotype including reduced biomass, increased hyphal branching during vegetative growth, and impairment in sexual development. This phenotype suggested that the *S. macrospora* MCM1 protein might be involved in the transcriptional regulation of many different genes by means of interaction with different cofactors (Nolting and Pöggeler, 2006). Using a yeast two-hybrid approach, we have now identified a set of putative MCM1-interacting proteins of *S. macrospora*. For most of these proteins, further work is needed to confirm whether or not the two-hybrid association is meaningful in terms of a biologi-

cally relevant MCM1 complex. In this study, we focused on the analysis of the interaction of MCM1 with the HD protein STE12. The *S. macrospora ste12* gene encodes a 726 aa protein with an N-terminal HD and two C-terminal C_2/H_2-Zn^{2+} finger DNA-binding domains. The C_2/H_2-Zn^{2+} finger domains are present in STE12 homologues of filamentous ascomycetes and the basidiomycete *Cryptococcus neoformans*, but are lacking in the STE12 proteins of ascomycetous yeasts (Yuan and Fields, 1991; Liu *et al*., 1994; Wickes *et al*., 1997; Vallim *et al*., 2000; Borneman *et al*., 2001; Park *et al*., 2002; Calcagno *et al*., 2003; Tsuji *et al*., 2003; Li *et al*., 2005). Similar to STE12 homologues of *N. crassa*, *A. nidulans*, *M. grisea*, *C. lagenarium* and *Penicillium marneffei*, the *S. macrospora* STE12 protein shared little homology with the *S. cerevisiae* STE12 within the region downstream from the HD. In the *S. cerevisiae* STE12, this region is known to interact with MAP kinase KSS1, or the repressor DIG1, or the co-activators MCM1 and TEC1, or the karyopherin KAP121 (Kirkman-Correia *et al*., 1993; Bruhn and Sprague, 1994; Madhani and Fink, 1997; Bardwell *et al*., 1998; Olson *et al*., 2000; Leslie *et al*., 2002). Like STE12 homologues of other filamentous ascomycetes, the *S. macrospora* STE12 completely lacked the minimal pheromone induction domain (aa 301–335 in the *S. cerevisiae* STE12) which is necessary for response to pheromone stimulation (Pi *et al*., 1997).

However, stretches of amino acid residues EPAYIANEETGLYTAIP (residues 375–391) and VIEGSP TYKQGRRRSSIPP (residues 440–458), which were previously identified to be identical in the STE12 homologous of filamentous ascomycetes (Park *et al*., 2002), are conserved in the *S. macrospora* STE12. Moreover, one putative cAMP- and cGMP-dependent phosphorylation site (RRRS aa 451–454), six putative protein kinase C phosphorylation sites, and 10 putative casein kinase II phosphorylation sites are predicted for *S. macrospora* STE12 by the computer program PROSITE (Falquet *et al*., 2002).

Interestingly, our two-hybrid and *in vitro* studies revealed that the *S. macrospora* STE12 specifically interacted with MCM1 through its N-terminal half encompassing the conserved HD. In contrast, the HD in *S. cerevisiae* is required for DNA-binding (Yuan and Fields, 1991), while the C-terminal region enables association with MCM1 (Errede and Ammerer, 1989; Kirkman-Correia *et al*., 1993). Thus, it is therefore possible that in contrast to yeast, the C-terminal C_2/H_2-Zn^{2+}-finger region of *S. macrospora* STE12 is responsible for DNA binding and that the HD might modulate transcription by protein–protein interactions as observed in *Drosophila* and other organisms (Ikeda and Kawakami, 1995; Darling *et al*., 1998; Bondos and Tan, 2001). However, as for the *C. neoformans* STE12α, the HD region and not the C_2/H_2-Zn^{2+}-finger region is required for DNA binding, we cannot exclude that the HD region of *S. macrospora* STE12 confers DNA-binding ability (Chang *et al*., 2004). It is also possible that protein–protein interaction mediated through the HD region impedes DNA binding as demonstrated for mammalian HD proteins (Zhang *et al*., 1997; Bendall *et al*., 1998). Similar to our results, yeast two-hybrid experiments indicated that the STE12α of *C. neoformans* does not form homodimers in *S. cerevisiae* (Chang *et al*., 2004). However, in contrast to our results, Chang *et al*. (2004) failed to identify any *C. neoformans* protein that interacts with STE12α in a two-hybrid screen.

We previously have shown that the MADS box protein MCM1 interacts with the *S. macrospora* mating-type protein SMTA-1 (Nolting and Pöggeler, 2006). In this study, we found that STE12 is also able to interact with the α-domain mating-type protein SMTA-1. These results are in agreement with data from the yeast *S. cerevisiae*. In MATα cells of the budding yeast, STE12 activates the expression of the α-specific *ste3* pheromone receptor gene and α-factor precursor genes in a complex with MCM1 and the mating-type protein MATα1, the homologue of the *S. macrospora* SMTA-1 protein (Johnson, 1995). This indicates a role for *S. macrospora* STE12 in a regulatory network for the control of mating and sexual development.

To determine the roles of STE12 in *S. macrospora*, we generated a Δ*ste12* gene disruption mutant by replacing the entire coding region of the *ste12* gene with the bacterial *hph* gene. Interestingly, vegetative growth rates were only slightly decreased; this finding strongly differs from what was observed in the closely related heterothallic ascomycete *N. crassa*. In this case, mycelial elongation rates of *N. crassa* Δ*pp-1* strains were only 25% of the wild-type controls (Li *et al*., 2005), while the vegetative growth rate of the *S. macrospora* Δ*ste12* mutant was 90% of wild-type controls. Like the *S. macrospora ste12*, homologues in other filamentous ascomycetes, such as *mst12* in *M. grisea*, *steA* in *A. nidulans*, *cst1* in *C. lagenarium*, and *stlA* in *P. marneffei* are also not significantly relevant for vegetative growth. However, all of them are found to be associated either with mating, reproduction or pathogenicity (Vallim *et al*., 2000; Borneman *et al*., 2001; Park *et al*., 2002; Tsuji *et al*., 2003). The *S. macrospora* Δ*ste12* mutant is able to form protoperithecia and perithecia; the latter contain a drastically reduced number of asci with predominantly inviable ascospores (Fig. 6). In contrast, Δ*pp-1* strains of the closely related heterothallic *N. crassa* are female sterile and show a complete absence of protoperithecia, but similar to *S. macrospora* they cause ascospore lethality when used as male partner (Li *et al*., 2005). Homothallic *A. nidulans* Δ*steA* strains are sterile and do not differentiate tissue or fruiting bodies, whereas the *mst12* gene was shown to be dispensable for mating and sexual

reproduction in the heterothallic *M. grisea* but essential for plant infection (Vallim *et al*., 2000; Park *et al*., 2002).

Despite being self-fertile, *S. macrospora* carries two pheromone precursor genes and two pheromone receptor genes. Theses genes encode two pheromone receptor pairs (PPG1/PRE2 and PPG2/PRE1) which upon interaction are supposed to trigger pheromone-induced responses (Pöggeler, 2000; Pöggeler and Kück, 2001; Mayrhofer and Pöggeler, 2005). Recently, pheromones and pheromone receptors were shown to be required for optimal sexual reproduction in *S. macrospora* (Mayrhofer *et al*., 2006). The phenotype of double-knockout strains lacking both compatible pheromone receptor pairs (Δ*pre1*/Δ*ppg1*, Δ*pre2*/Δ*ppg2*) displayed a drastically reduced number of perithecia and sexual spores, and thus resembles the phenotype of the Δ*ste12* mutant. These common phenotypes suggest that STE12 might be a pheromone response regulator. However, fragile ascus and ascospore cell walls, as in the Δ*ste12* mutant strain, have never been observed in Δ*pre1*/Δ*ppg1* and Δ*pre2*/Δ*ppg2* mutant strains. This implies that STE12, in addition to its role in pheromone signal transduction, might be involved in cell wall integrity of asci and ascospores. In *S. cerevisiae*, STE12 plays a major role in maintaining cell wall integrity as it can be targeted to the promoters of a set of genes involved in cell wall biology by interaction with MCM1 (Kuo *et al*., 1997). Moreover, in *Candida glabrata*, the absence *ste12* is known to results in derepression of genes involved in cell wall biology (Calcagno *et al*., 2003). In *N. crassa*, a putative polyketide synthase gene (*pks/NCU02918*) that might be involved in melanin biosynthesis within ascospores was downregulated in the Δ*pp-1* mutant (Li *et al*., 2005).

However, microarray analysis revealed that a gene encoding mannitol-1-phosphate dehydrogenase (NCU07318), the first enzyme in the mannitol biosynthesis pathway, displayed an elevated expression in the absence of STE12 in *N. crassa* (Li *et al*., 2005). Mannitol was identified to be the major sugar component within asci to build up turgor pressure for ascospore discharge in filamentous ascomycetes (Trail *et al*., 2002; 2005). Thus, it might also be that in the *S. macrospora* Δ*ste12* mutant an increased accumulation of mannitol leads to premature bursting of the asci. Alternatively, there might be a defect in microtubule reorganization during ascus formation, because in *M. grisea*, the STE12 homologue MST12 controls microtubule rearrangements at late stages of appressorium formation (Park *et al*., 2004).

Taken together, the role of *ste12* homologues in vegetative growth and sexual development appears to be different and species-specific and also does not reflect phylogenetic relationships or mating behaviour.

Our study revealed that some aspects of the *S. cerevisiae* complex formation to determine cell type specificity seem to be conserved in *S. macrospora*. However, there are also obvious differences. In *S. cerevisiae*, the HD protein MATα2 interacts with MCM1 to repress cell type-specific gene expression. In *N. crassa*, no homologue to this transcription factor could be identified. Instead, the *S. macrospora* SMTA-1 mating-type protein is able to interact with the HMG-domain mating-type protein SMTa-1 (Jacobsen *et al*., 2002).

In conclusion, regulatory interactions between STE12, MCM1, SMTA-1 and SMTa-1 may represent a mechanism to control fertilization, fruiting body development, and ascosporogenesis in the homothallic ascomycete *S. macrospora*. Interactions between mating-type proteins and other proteins have not yet been described in filamentous ascomycetes and the ones described here may help to better understand regulatory networks underlying sexual development in this fungal species. However, it is also apparent from our study that our ability to unravel the complexitiy of sexual development in fungi requires further studies to identify upstream components regulating these transcription factors and downstream targets dependent on their protein associations.

Experimental procedures

Strains and growth conditions

Cloning and propagation of recombinant plasmids was done in *E. coli* strain SURE under standard culture conditions (Greener, 1990; Sambrook *et al*., 2001). *S. cerevisiae* strain PJ69-4A was used as host strain for the two-hybrid experiments and was cultivated as described by James *et al*. (1996). All *S. macrospora* strains used in this work are summarized in Table 3. *S. macrospora* strains were cultivated on corn-meal medium or CM complete medium (Esser, 1982; Nowrousian *et al*., 1999). The *S. macrospora* strain K used for the isolation of RNA was grown in Westergaard's synthetic medium (Westergaard and Mitchell, 1947). *S. macrospora* wild-type and Δ*ste12* growth velocity was determined according to Nowrousian and Cebula (2005). Growth of mycelia was monitored as dry cell weight (DCW), described by Nolting and Pöggeler (2006). Transformation of *S. macrospora* was performed after Nowrousian *et al*. (1999). Transformants were selected either on nourseothricin- (50 μg ml^{-1}) or on hygromycin B-containing medium (110 U ml^{-1}).

Yeast two-hybrid library construction and screening

An λ-phage cDNA library was constructed by Stratagene (La Jolla, CA, USA) based on *S. macrospora* poly(A)-RNA. Excision and construction of the two-hybrid vector library was done according to the HybriZAP®-2.1 Two-Hybrid Predigested vector Kit. The activation-domain constructs (pAD-4-2.1, Stratagene) containing the different *S. macrospora* cDNAs, were isolated from *E. coli* using the Hi Speed® Plasmid Maxi Kit (Qiagen, Hilden, Germany). The primary library size was estimated to contain 2×10^6 independent

Table 3. *Sordaria macrospora* strains used in this study.

Strain	Relevant genotype	Reference
S48977	Wild type, homothallic	[a]
S23442	*fus1-1*, brown coloured spores	[a]
S66001	Δ*ku70*::*nat*^{R}, single-spore isolate	Pöggeler and Kück (2006)
T1Δ*ste12*	Primary transformant Δ*ste12*::*hph*^{R}, Δ*ku70*::*nat*^{R}, homokaryotic	This study
S68567	Single-spore isolate of cross T1Δ*ste12* × S23442, Δ*ste12*::*hph*^{R}, *ku70*$^+$*nat*^{S}, *fus*$^+$	This study
TSte12-nat	Δ*ste12*::*hph*^{R}, *nat*^{R}*ste12*$^+$	This study

a. Culture collection, Department for General and Molecular Botany, Ruhr-University Bochum, Germany.
nat^R, nourseothricin-resistance; *nat*^{s}, nourseothricin-sensitive; *hph*^{R}, hygromycin-resistance.

clones. In each case, 1 μg of the cDNA-library (containing a Leu selectable marker) was co-transformed with 100 ng of the bait-plasmid pBM1d2, carrying a truncated version of *mcm1* (selectable marker Ura, Table 2) (Nolting and Pöggeler, 2006). Transformation of yeast cells was done by electroporation according to Becker and Lundblad (1994) in a Multiporator (Eppendorf, Hamburg, Germany) at 1.5 kV. *S. cerevisiae* strain PJ69-4A was used for the two-hybrid experiments. To reduce the incidence of false positives, this strain contains three easily assayed reporter genes under the control of different GAL4-inducible promoters (*gal2-ade2*, *gal1-his3*, *gal7-lacZ*) (James *et al*., 1996). Transformants were initially selected by plating on dropout agar medium (DO) lacking leucine, uracil and histidine and supplemented with 50 mM 3-amino-1,2,4-triazole (3-AT) and on DO minus leucine, uracil and adenine.

To rescue the prey plasmid from yeast cells, positive colonies were subsequently plated on –Leu media containing 1 μg ml^{-1} Fluorooroticacid (FOA). Plasmid DNA of growing colonies was then isolated with the NucleoSpin® Plasmid Kit (Macherey-Nagel), including an initial step of disrupting the yeast cells with glass beads (0.5 mm diameter). To isolate prey plasmids, DNA was subsequently transformed into *E. coli* strain SURE after standard protocols (Greener, 1990; Sambrook *et al*., 2001). Plasmid DNA isolation from *E. coli* transformants was carried out according to Birnboim and Doly (1979) and plasmids were retransformed into PJ69-4A cells carrying the bait plasmid pBM1d2. Interaction of the bait and prey fusion constructs was then again confirmed by selection on selective media (DO, –Leu, –Ura, –His + 50 mM 3-AT or –Ade). To assess the strength of interactions we performed a growth assay. Equal numbers of cells were collected after the strains were grown to early log phase ($OD_{600} = 1$) in liquid DO media –Leu, –Ura, –His + 50 mM 3-AT. Ten microlitres of 1:10 serial dilutions of the cells were spotted on SD plates –Leu, –Ura, –His + 50 mM 3-AT and incubated at 30°C for 2 days. Measurement of β-galactosidase activity was done as described by Nolting and Pöggeler (2006). Inserts of plasmids encoding putative MCM1 interaction partners were sequenced (MWG Biotech Customer Service, Ebersberg, Germany).

For the construction of the *ste12* two-hybrid plasmids, cDNA of the N-terminal (1286 bp, Position 4–1339) and the C-terminal (897 bp) coding region of the *ste12* gene were amplified with primer pairs S12A/S12B and S12C/S12D respectively. After subcloning and sequencing, the 1284 bp BamHI/BglII *ste12* fragment was cloned into the yeast two-hybrid vectors pGBDU-C1 (James *et al*., 1996), containing the GAL4 DNA-binding domain (pBS12n, Table 2), and into pGAD-C1 (James *et al*., 1996), containing the GAL4-activation domain (pAS12n, Table 2) respectively. The C-terminal 897 bp BamHI/BglII *ste12* fragment of plasmid pSte12-c was also cloned into the yeast two-hybrid vectors pGBDU-C1 and pGAD-C1, resulting in plasmids pBS12c and pAS12c (Table 2). Assays for determining the transactivation activity of pBS12n and pBS12c were performed as described by Jacobsen *et al*. (2002). Two-hybrid plasmids encoding mating-type proteins and the *S. macrospora* MCM1 protein are shown in Table 2.

Cloning of the ste12 *gene*

An indexed *S. macrospora* cosmid library was screened by high throughput PCR (Pöggeler *et al*., 1997b) with oligonucleotide primers S12A and S12B (Table 1), derived from the sequence of two-hybrid plasmid pMGB15-3 (Table S1). This led to the isolation of cosmid H5 from Pool VI 1346–1442 containing the *ste12* gene of *S. macrospora*. Subsequently, overlapping fragments from cosmid H5 were subcloned into vector pBCKS(+) (Stratagene). The sequences of the subcloned fragments were determined by DNA sequencing (MWG Biotech Customer Service). The nucleotide sequence of the *S. macrospora ste12* gene has been deposited in the EMBL database under accession number AJ879472.

Sequence analyses

Protein sequence data were obtained from the public database NCBI Entrez (http://www.ncbi.nlm.nih.gov/entrez/), or by BLASTP searches of the fully sequenced *N. crassa* genomes at the BROAD INSTITUTE (http://www.broad.mit.edu/annotation/fungi/fgi/). Sequence similarity searches of the inserts of yeast two-hybrid vectors were done by BLASTX at http://www.ch.embnet.org Protein sequence alignments were performed using the CLUSTALX program (Thompson *et al*., 1997). The prediction of promoter elements was done by using different 'Hamming-Clustering' methods (http://www.itb.cnr.it/sun/webgene//), the 'Promoter Predictor' (http://www.fruitfly.org/seq_tools/promoter.html) and the MatInspector (http://www.genomatix.de).

Preparation of nucleic acids, hybridization protocols and PCR

Isolation of *S. macrospora* genomic DNA was carried out as described by Pöggeler *et al*. (1997a). Southern blotting and

hybridization was performed according to standard techniques (Sambrook *et al*., 2001), using ^{32}P-labelled DNA probes. PCR amplification of *S. macrospora* genomic DNA or cosmid pools was performed with the HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) following the manufacturer's protocol. Primers used for PCR experiments were synthesized by MWG Biotech (Table 1). Total RNA of *S. macrospora* was isolated from *S. macrospora* at 3–7 days of growth using the method of Hoge *et al*. (1982). RT-PCR was performed with the specific oligonucleotide primer pairs S12A/S12B, S12C/S12D, S12A/S12E (Table 1) and was accomplished after Mayrhofer *et al*. (2006). The mRNA for cDNA library construction was isolated from 1 mg of total RNA by using the PolyATract mRNA isolation system IV (Promega, Mannheim, Germany). Northern blotting was carried out according to Sambrook *et al*. (2001).

Generation of a S. macrospora Δste12 *strain*

To create a *ste12* knockout construct for homologous recombination in *S. macrospora*, flanking regions of the *ste12* ORF were amplified by PCR from *S. macrospora* genomic DNA using primer pairs D1/D2 for the upstream region (930 bp) and D3/D4 for the downstream region (1175 bp) respectively (Table 1). The primer pair D3/D4 generated ApaI ends. The PCR fragments were subcloned into vector pGEM-T (Promega), so that the 1175 bp ApaI fragment was fused with the 930 bp upstream fragment. The two sequences were separated by a single EcoRI restriction site which was used to introduce the 1.4 kb EcoRI *hph* cassette of pCB1003 (Carroll *et al*., 1994). The resulting plasmid pSTE12-KO was used as a template to amplify the *ste12-hph* cassette with oligonucleotides D1/D4 as primers (Table 1). The 3505 bp PCR fragment obtained was transformed into the Δ*ku70* strain (S66001) of *S. macrospora* to facilitate the knockout of the *ste12* gene by homologous recombination (Pöggeler and Kück, 2006). Primary transformants were screened for homologous recombination by Southern blot analyses. Successful homologous recombination was confirmed by PCR amplification. The 5′-flanking region of the *ste12* gene was amplified with primers D5 and h3, whereas D6 and tC1 verified homologous recombination at the 3′-flanking region of *ste12* (Fig. 5).

Conventional genetic analyses of *S. macrospora* was performed as described by Esser (Esser and Straub, 1958; Esser, 1982). For segregation of the nourseothricin and hygromycin marker, the homokaryotic Δ*ku70* primary transformant T1Δ*ste12* (*ku70*::*nat1*) carrying a deletion of the *ste12* gene (*ste12*::*hph*) was crossed with mutant *fus1-1* (S23442), producing brown ascospores (Table 3). Subsequently, we isolated from this cross the hygromycin-resistant, nourseothricin-sensitive single-spore isolate S68567 (Table 3).

To rescue the phenotype of the Δ*ste12* mutant S68567, cosmid H5 was co-transformed with plasmid pD-NAT1 (Kück and Hoff, 2006), resulting in transformant TSte12-nat (Table 3). In this experiment, the nourseothricin-resistance gene *nat1* of plasmid pD-NAT1 was used as a selectable marker for co-transformation (Krugel *et al*., 1993). In plasmid pD-NAT1, the *nat1* gene is under the control of the *gpd* promoter and *trpC* terminator of *A. nidulans* (Kück and Hoff, 2006).

Protein synthesis and far western experiments

A His-tag fusion of the N-terminal STE12 domain (aa 1–159) was synthesized in *E. coli* strain M15 [pREP4] (Qiagen). For this purpose, plasmids pH-STE12 (fragment in sense orientation) and pH-STE12i (fragment in inverse orientation) were created (Table 2). Both plasmids were obtained after RT-PCR with primer pair S12A and S12E, subcloning into vector pDrive (pSte12-n2) and sequencing. The 456 bp *ste12* fragment of pSte12-n2 was isolated using the BglII site from primer S12A and the BamHI site of plasmid pDrive and inserted into the single BamHI site of plasmid pQE31 (Table 2). A His-tagged fusion of SMTA-1 was generated by cloning the 921 bp BglII/BamHI fragment of pB-A1 (Jacobsen *et al*., 2002) into the BamHI site of pQE31 resulting in plasmid pH-SMTA1 (Table 2).

The His-tagged STE12 and SMTA-1 fusion constructs were purified on nickel nitrilotriacetic acid agarose columns (Qiagen) according to the supplier's instruction. To obtain a GST-MCM1 fusion, a 976 bp BamHI *mcm1* fragment of plasmid pAM1 was cloned in frame into the single BamHI site of the GST vector pGEX-4T-1 (Amersham Biosciences, Europe GmbH, Freiburg, Germany). The GST-MCM1 fusion protein of the resulting plasmid pG-MCM1 was synthesized in *E. coli* strain BL21(DE3) (Stratagene) and purified by affinity chromatography using 50% slurry on glutathione-agarose beads according to the instructor's manual (Amersham Biosciences). Expression of the genes was induced by adding 2 mM IPTG and the cells were harvested after incubation for 3 h at 37°C or overnight at 30°C respectively. For antiserum production, purified eluates of *E. coli* strains expressing *SmtA-1* were dialysed against 50 mM ammonium carbonate and evaporated. The production of polyclonal antiserum against SMTA-1 in rabbits was performed by Eurogentec (Belgium).

Far western experiments were carried out as described previously by Nolting and Pöggeler (2006) with the following modifications: The SMTA-1 overlay was performed using 5 μg of purified His-SMTA-1 fusion protein, detected by a specific polyclonal SMTA-1 antibody (1:3000). For the GST-MCM1 overlay, the blot was blocked overnight in blocking buffer (Roche Diagnostics) and then incubated with 1 μg of GST-MCM1 fusion protein. The detection of GST was carried out with a polyclonal anti-GST antibody (Amersham Biosciences, 1:50 000) and with the BM chemiluminescence Western blotting kit (Roche Diagnostics, Germany) according to the supplier's recommendations.

Fluorescence and light microscopy

Zeiss Axiophot or Zeiss Axio imager microscope, respectively, were used for light microscopy. Pictures were captured with the AxioCam (Zeiss) or Cool SNAP HQ camera (Roper Scientific, USA). Recorded images were edited using Adobe Photoshop CS2™. Transmission images were recorded using DIC optics. Staining of cell walls with calcofluor (1 μg ml^{-1}) and visualizing was performed as described by Nolting and Pöggeler (2006).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. *Sordaria macrospora* STE12 protein.

Table S1. Two-hybrid interactions with the Gal4p-AD-MCM1d2-BD bait.

This material is available as part of the online article from http://www.blackwell-synergy.com

