

Determination of cell polarity in germinated spores and hyphal tips of the filamentous ascomycete *Ashbya gossypii* requires a rhoGAP homolog

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SUMMARY

In the filamentous ascomycete *Ashbya gossypii*, like in other filamentous fungi onset of growth in dormant spores occurs as an isotropic growth phase generating spherical germ cells. Thereafter, a switch to polarized growth results in the formation of the first hyphal tip. The initial steps of hyphal tip formation in filamentous fungi, therefore, resemble processes taking place prior to and during bud emergence of unicellular yeast-like fungi. We investigated whether phenotypic similarities between these distinct events extended to the molecular level. To this end we isolated and characterized the *A. gossypii* homolog of the *Saccharomyces cerevisiae* *BEM2* gene which is part of a network of rho-GTPases and their regulators required for bud emergence and bud growth in yeast. Here we show that the AgBem2 protein contains a GAP- (GTPase activating protein) domain for rho-like GTPases at its carboxy terminus, and that this part of AgBem2p is required for complementation

of an *Agbem2* null strain. Germination of spores resulted in enlarged *Agbem2* germ cells that were unable to generate the bipolar branching pattern found in wild-type germ cells. In addition, mutant hyphae were swollen due to defects in polarized cell growth indicated by the delocalized distribution of chitin and cortical actin patches. Surprisingly, the complete loss of cell polarity which lead to spherical hyphal tips was overcome by the establishment of new cell polarities and the formation of multiple new hyphal tips. In conclusion these results and other findings demonstrate that establishment of cell polarity, maintenance of cell polarity, and polarized hyphal growth in filamentous fungi require members of rho-GTPase modules.

Key words: GTPase activating protein, GAP, Actin cytoskeleton, Morphogenesis, Polar growth

INTRODUCTION

The ability to establish cell polarity and induce polarized cell growth is fundamental for morphogenesis and development of unicellular and multicellular organisms. One of the best known examples of polar cell growth is provided by the hyphal growth of filamentous fungi. Fungal mycelia can be grown from spores. The first step in breaking the dormancy of a spore is the initiation of an isotropic non-polarized growth phase that generates a spherical germ cell. In germ cells switching from isotropic to polarized growth results in the formation of the first hyphae which continuously extend from their hyphal tips, proliferate by branching, and form radially spreading mycelia. Differing from filamentous fungi are unicellular yeast-like fungi in which polarized growth is restricted to a short phase of the cell cycle during bud emergence (Lew and Reed, 1993, 1995; Kron and Gow, 1995; Waddle et al., 1996; Karpova et al., 1998). Genetic approaches have been applied in the unicellular fungus *S. cerevisiae* to identify genes required in this process (Chant, 1994; Cid et al., 1995). Detailed analyses demonstrated that during bud emergence in *S. cerevisiae* a protein network integrates and performs the tasks of bud-site selection, polarity establishment, and bud growth (Chant and

Stowers, 1995; Cid et al., 1995; Drubin and Nelson, 1996; Cabib et al., 1998; Madden and Snyder, 1998; Johnson, 1999; Chant, 1999). The central parts of this protein network are constituted of ras/rho-like GTPases (Pringle et al., 1995; Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Ras/rho-like GTPases act as molecular modulators. Their signaling function depends on their association with GTP whereas hydrolysis of GTP to GDP renders them inactive. Guanine nucleotide cycling of rho-proteins between the GTP- or GDP-bound states is regulated by other proteins: GEFs (guanine nucleotide exchange factors) stimulate the exchange of GDP for GTP and GAPs (GTPase activating proteins) enhance the intrinsic GTPase activity of ras/rho-like GTPases and thus turn off their signaling (Tanaka and Takai, 1998). Therefore, a core GTPase module consists of at least three proteins. Establishment of a polarized actin cytoskeleton in yeast resulting in bud formation is controlled by the *CDC42* rho-module consisting of the rho-GTPase Cdc42p, its GEF Cdc24p and GAP Bem3p (Johnson, 1999; Toenjes et al., 1999). Growth at restrictive temperature of conditional mutants of either *CDC42*, *CDC24*, or *BEM3* results in the formation of large unbudded multinucleate cells which exhibit a delocalized deposition of chitin and a uniform distribution of actin cortical patches (Sloat et al., 1981; Adams

et al., 1990; Zheng et al., 1994). Bud growth and the actin cytoskeleton in *S. cerevisiae* are regulated by several other GTPase-proteins including *RHO1*, *RHO2*, *RHO3*, and *RHO4* (Tanaka and Takai, 1998). For the Rho1 protein the regulator proteins have been identified as Rom1/2-GEF and Bem2-GAP (Ozaki et al., 1996; Peterson et al., 1994). Temperature sensitive mutants of *ROM1* or *RHO1* become arrested as small budded cells under restrictive conditions. Whereas mutants in *BEM2* form large unbudded multinucleate cells at elevated temperatures (Bender and Pringle, 1991).

In sharp contrast to the detailed knowledge in *S. cerevisiae*, very little is known with respect to the molecular mechanisms that control the establishment of cell polarity and polarized cell growth in filamentous fungi. In *Neurospora crassa* a large number of colony morphology mutants have been isolated (Garnjobst and Tatum, 1967; Perkins et al., 1982). Two of those mutants have recently been characterized. Mutations in the protein kinase *cot-1* and in *mcb-1*, which codes for a regulatory subunit of cAMP-dependent protein kinase (PKA), were found to affect hyphal elongation and hyphal growth polarity, respectively (Yarden et al., 1992; Bruno et al., 1996). In *Aspergillus nidulans*, screens of a temperature sensitive mutant strain collection have been performed (Harris et al., 1994). Thereby three classes of mutants were isolated: (i) hypercellular mutants (*hypA-hypE*; Kaminskj and Hamer, 1998); (ii) polarity defective mutants (*podA/hypA*, *podB*, *podC*, and *podD*; Harris et al., 1999); (iii) swollen mutants (*swoA-swoH*; Momany et al., 1999). These mutants appear to be involved in different aspects of polar growth and polarity establishment in *A. nidulans*. However, except for *hypA*, which does not show homology to any known gene, none of these *A. nidulans* mutants has been characterized on the molecular level. Thus, we are not able to compare the molecular mechanisms underlying the yeast-like growth mode and polarized hyphal growth of filamentous fungi.

A. gossypii is a filamentous ascomycete that, based on rDNA sequences, is more closely related to *S. cerevisiae* than either *N. crassa* or *A. nidulans* (Wendland et al., 1999). We have chosen *A. gossypii* as a model to initiate molecular genetic studies on hyphal morphogenesis because this fungus allows the facile identification and manipulation of genes. Specifically (i) *A. gossypii* harbors an exceptionally small genome of 8.85 Mb coding for approximately 4500 genes (S. Steiner et al., unpublished). (ii) The identification of genes in *A. gossypii* can be facilitated based on the conserved gene order between *A. gossypii* and *S. cerevisiae* (Altmann-Jöhl and Philippsen, 1996). (iii) Due to the high homologous recombination efficiency in *A. gossypii*, one-step gene replacement can be done conveniently by PCR-based gene targeting (Steiner et al., 1995; Wendland et al., 2000). (iv) *A. gossypii* allows the extrachromosomal free replication of plasmids bearing an autonomous replicator (Wright and Philippsen, 1991). Stability of freely replicating plasmids can be increased under non-selective conditions by the introduction of centromeric-DNA of *A. gossypii* (J. Wendland et al., unpublished).

In this report, we present the first isolation, deletion, and phenotypic characterization of a rhoGAP gene in a filamentous fungus. We demonstrate the requirement of *AgBEM2*, which is a homolog of the *S. cerevisiae* *BEM2* gene for the determination of cell polarity during germination and hyphal growth in *A. gossypii*. Our results imply that polarized hyphal

growth of filamentous fungi relies, at least in part, on the same regulatory networks, i.e. rho-like GTPase modules, as, e.g. in the yeast *S. cerevisiae*.

MATERIALS AND METHODS

Strains and media

The *Ashbya gossypii* wild-type strain (ATCC10895) and a derivative of it deleted for the *AgLEU2* and *AgTHR4* genes was used (Altmann-Jöhl and Philippsen, 1996; C. Mohr and P. Philippsen, unpublished). For selection of G418/geneticin resistant transformants geneticin (GIBCO BRL) was included in the complete medium (AFM: 20 g/l glucose, 10 g/l yeast extract, 10 g/l peptone, 1 g/l myo-inositol) at a final concentration of 200 µg/ml. For selection of *LEU*⁺ transformants minimal medium was used lacking leucine (AMM: 20 g/l glucose, 0.69 g/l CSM-LEU (BIO101, Inc.; Vista CA), 1.7 g/l yeast nitrogen base without amino acids and (NH₄)₂SO₄ (Difco), 0.3 g/l myo-inositol, 1.0 g/l L-asparagine). Mycelia were routinely grown at 30°C unless otherwise specified. The *Escherichia coli* strains DH5α and XL1-Blue were used as hosts for plasmids.

General methods

DNA- and colony hybridization, as well as standard recombinant DNA techniques were performed according to the methods of Sambrook et al. (1989). Colony hybridization was done to isolate the complete *AgBEM2* gene from a YE352-based plasmid library (Maeting et al., 1999). Sequencing was done on an ABI377 automated sequencer according to the manufacturer's instructions. Sequence comparisons were done by BLAST searches against available databases (Altschul and Lipman, 1990). Profile searches were done at the ISREC profile-scan server (<http://www.isrec.isb-sib.ch>). To generate the pairwise and the dot-matrix comparisons of *AgBem2p* and *ScBem2p* the GCG-software package from the University of Wisconsin was used (Devereux et al., 1984).

Plasmids

For the amplification of the *ScLEU2* gene pRS415 was used (Sikorski and Hieter, 1989), using the primers LEU2a-5'-GACAGATCTCTTAGCAACCATTATTTTTTCTCAAC-3' and LEU2b-5'-ACGGGATCCTTATCACGTTGAGCCATTAGTATCAAT-TTG-3' (restriction sites underlined). The PCR fragment containing the entire *LEU2* gene was cloned into the *Bam*HI-site of pFA100 (Thierry et al., 1990) as a *Bg*II-*Bam*HI-fragment generating pLEUMX1 and pLEUMX2 in both orientations. Plasmid pBEM2 was created by inserting a 1.7 kb *Sal*I-fragment containing the *ScLEU2* gene excised from pLEUMX1 into the unique *Xho*I site of pBEM2-A (see results). In-frame deletions were produced based on pBEM2. Plasmid pBem2Δ885-1250, carrying *Agbem2Δ2*, was created by deleting a *Spe*I restriction fragment from pBEM2, plasmid pBem2Δ211-1436, carrying *Agbem2Δ3*, by deleting a *Nru*I/*Msc*I fragment from pBEM2, and plasmid pBem2Δ1152-2071, carrying *Agbem2Δ4*, by deleting an *Aoc*I fragment from pBEM2. The resulting plasmids were checked by restriction digest and the newly created junctions were sequenced. In plasmid pBem2Δ1152-2071 a frameshift at the new junction was found altering the *Sau*I restriction site 'CCTNAGG' into 'CCTAGG'. This frameshift led to a stop codon after the addition of a few amino acids as would have also been the case in a non-frameshifted clone followed by the original *AgBEM2* terminator.

Deletion of *AgBEM2*

Complete deletion of the *AgBEM2* open reading frame was achieved by using a PCR-based one-step gene replacement approach recently established in *A. gossypii* similar to the method used in *S. cerevisiae* (Wach et al., 1994; Wendland et al., 2000). In short, as a selectable marker gene we used the *GEN3* module which consists of the

kanamycin-resistance gene under the control of the *S. cerevisiae* *TEF2* promoter and terminator sequences and confers resistance to G418/geneticin to *A. gossypii* transformants. In a PCR-step the module was amplified adding short target guide sequences that allow specific gene targeting in *A. gossypii*. For amplification *BEM2*-S1 (5'-ctacttgctactcttctcgcgtcctcagccaccgaacaacgcagGCTAGGGATAACA GGGTAAT-3') and *BEM2*-S2 (5'-gattaagaatgataagaacacacacacac-gagcttgcataacaGGCATGCAAGCTTAGATCT-3') primers (homology to the marker cassette in upper case letters and homology to the target locus in lower case letters) were used generating a PCR product that was used directly in transformation of either the *A. gossypii* wild-type strain or the *A. gossypii* *leu2*, *thr4* strain. Preparation of *A. gossypii* mycelia for transformation was done as described previously (Maeting et al., 1999). After electroporation mycelia were resuspended in 1.0 ml AFM, plated on AFM plates, and incubated for 6 hours at 30°C to allow regeneration of the mycelia and expression of the resistance marker. Subsequently plates were first overlaid with 7.0 ml 0.5 % agarose containing 1.4 mg G418 and after an incubation period of 40 hours at 30°C a second overlay of 7.0 ml 0.5% agarose was applied containing 7.5 mg G418. Primary heterokaryotic transformants were isolated and via a sporulation step homokaryotic mutant *Agbem2Δ1* strains were obtained according to a procedure described previously (Steiner et al., 1995). Correct deletion of *AgBEM2* was shown by analytical PCR using the primers: G1: 5'-gctgagcctcccgcctag-3'; G2: 5'-gtttagtctgaccatctcatctg-3'; G3: 5'-tcgcagaccgataaccaggatc-3'; G4: 5'-gcacatagttcaaaagcgcg-3'; I1: 5'-gtataagtacttgagaaaaag-3'; and I2: 5'-gcgagatcatcggtgaagtc-3'. Additionally DNA-hybridization was employed to verify locus specific gene targeting. Transformations of plasmid DNA were performed following the same transformation protocol. For selection of *LEU*⁺ transformants transformation mixtures were directly plated onto selective minimal medium plates.

Cytological techniques and microscopy

For calcofluor (0.1 µg/ml) or DAPI staining (1.0 µg/ml) cells were grown either in liquid medium or on microscopy slides covered with thin layers of full medium solidified with 0.7% agarose. For calcofluor staining untreated germ cells or mycelia were directly incubated with the dye for up to 20 minutes at room temperature. To eliminate background fluorescence samples were washed with water. For DAPI staining, cells were fixed in 70% ethanol for two minutes, stained, and washed with water. For actin staining, cells were fixed in 3.7% formaldehyde containing 0.4% Triton X-100 (fixation for less than 30 minutes gave best results). Mycelia or germinated spores were collected by centrifugation and washed several times in PBS (9.4 g/l Na₂HPO₄, 1.8 g/l NaH₂PO₄, and 4.4 g/l NaCl) and resuspended in PBS containing 1-2 µl rhodamine-conjugated phalloidin (77 µM in methanol). After an incubation of 1-2 hours at room temperature samples were washed five times in PBS and finally resuspended in mounting medium (90% glycerol, 10% PBS including 0.1% *p*-phenylene-diamine). Microscopy was performed using a Zeiss Axioskop microscope with the appropriate filter combinations. For picture acquisition either a TE/CCD-1000PB back-illuminated cooled CCD camera (Princeton Instruments, Inc, Trenton, NJ, USA) or a VI-470 CCD camera (Optronics, Goleta, CA, USA) was used. With the former image acquisition and picture processing was done using Metamorph 3.51 software (Universal Imaging Corp., West Chester, PA, USA) and with the latter images were processed by the NIH-Image software package (<http://rsb.info.nih.gov/nih-image>).

RESULTS

Identification and isolation of *AgBEM2*

The *Ashbya gossypii* *BEM2* gene was initially identified in a screen of 650 end-sequenced plasmid clones. Two identical clones (pAG1060 and pAG1637) revealed sequence similarity

to the 5' end of *S. cerevisiae* *BEM2*. Colony hybridization was performed to isolate the complete *AgBEM2* gene (using a library kindly provided by K. P. Stahmann). Two overlapping clones (pBEM2-A and pBEM2-B) were isolated which together cover a region of 9.4 kb (Fig. 1). The complete double strand sequence was determined and the entire *AgBEM2* gene including its promoter was located on pBEM2-A. Searches for other open reading frames and BLAST searches in the available databases revealed two complete and two partial genes on the 9.4 kb fragment. Interestingly, this four-gene cluster is conserved between *A. gossypii* and *S. cerevisiae* (Fig. 1). The degree of conservation among the *A. gossypii* and *S. cerevisiae* proteins ranges between 26% and 57% identity. *AgBem2p* is 44% identical and 66% similar to *ScBem2p*. The *AgBEM2* open reading frame is predicted to encode a protein of 2071 aa whereas *ScBem2p* is a 2167 aa protein. Conservation between *AgBem2p* and *ScBem2p* can be found almost along their entire lengths (Fig. 2A). Only the amino-terminal regions of both proteins, which are rich in serine/threonine residues, are more divergent and account for most of the size difference (Fig. 2B).

Domain structure of *AgBem2p*

A pairwise alignment of *AgBem2p* and *ScBem2p* revealed nine blocks of greater than 65% aa identity compared to the 44% overall aa identity (Fig. 2B). A profile search on both *Bem2* proteins using the PROSITE Database assigned specific domains to three of these conserved blocks. The carboxy-terminal 250 aa residues of both *Bem2* proteins show similarity to pleckstrin homology (PH)- and GTPase activating protein (GAP)-domains found in a large family of proteins (Lemmon et al., 1996; Lamarche and Hall, 1994); including e.g. *S. cerevisiae* *Bem3p* (Zheng et al., 1994), human *Bcr* (Heisterkamp et al., 1985; Hariharan and Adams, 1987; Lifshitz et al., 1988), and human *rhoGAP* (Lancaster et al., 1994). Sequence identity of the *AgBem2p* GAP-domain with the GAP-domains of human *Bcr*, *ScBem3p* and *rhoGAP* is 36%, 26%, and 23%, respectively (Fig. 3A). Alignment of these GAP-domains shows several highly conserved amino

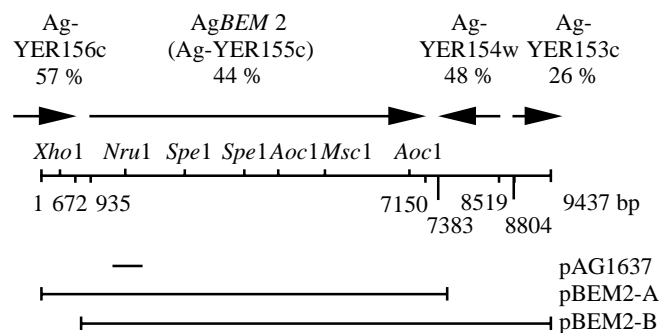


Fig. 1. The *A. gossypii* *BEM2*-locus. The cloned and sequenced *AgBEM2*-locus is shown. Genes that were identified based on blast searches are depicted by arrows indicating their relative length and transcriptional orientation. The nomenclature used assigned hybrid gene names consisting of an Ag-prefix followed by the systematic name of the homologous yeast gene (described as percentage identity at the aa level). The numbering within the scale corresponds to start and stop codons of the genes. Restriction sites used for cloning and genomic library plasmid clones used in this study are listed.

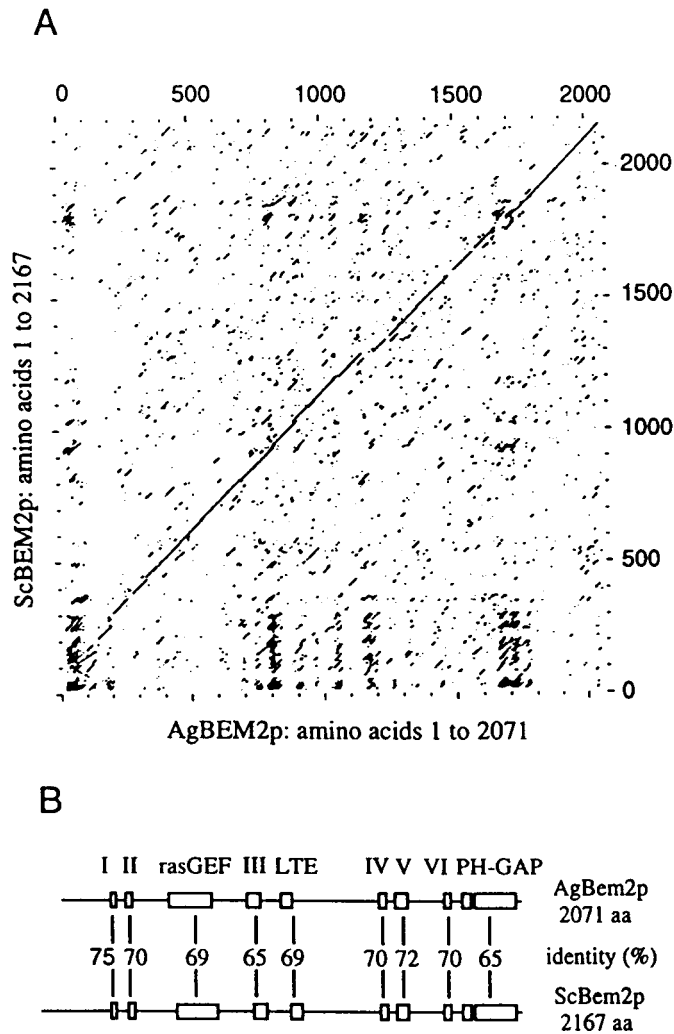


Fig. 2. Comparison of the Bem2 proteins of *A. gossypii* and *S. cerevisiae*. (A) Dot-matrix comparison of AgBem2p and ScBem2p. The alignment was performed with the COMPARE/DOTPLOT programs of the GCG software package using default parameters. (B) Domain structure of the Bem2 proteins. Blocks of homology with >65% identity at the aa level between AgBem2p and ScBem2p are indicated. Domains with homology to entries in the PROSITE database are shown others are numbered from I to VI. The location of blocks of homology is indicated by boxes along the lines representing the Bem2 proteins.

acids residues including an arginine residue at position 85 of the rhoGAP sequence which according to its resolved structure plays a critical role for GAP-funtion (Rittinger et al., 1997). ScBem2p, for example, has been shown to function specifically as a GTPase activating protein for ScRho1p (Peterson et al., 1994). Two other domains that were indicated by the profile search in both the *A. gossypii* and *S. cerevisiae* Bem2 proteins were a putative rasGEF-domain between the aa residues 451 and 598 and a LTE-domain between positions 989 and 1029 of the AgBem2p. Protein alignments of rasGEF domains of proteins from yeast, mouse and *Drosophila* suggested the presence of three 'structurally conserved regions', SCR1, SCR2, and SCR3, respectively (Boguski and McCormick, 1993). Alignment of the AgBem2p and ScBem2p rasGEF-

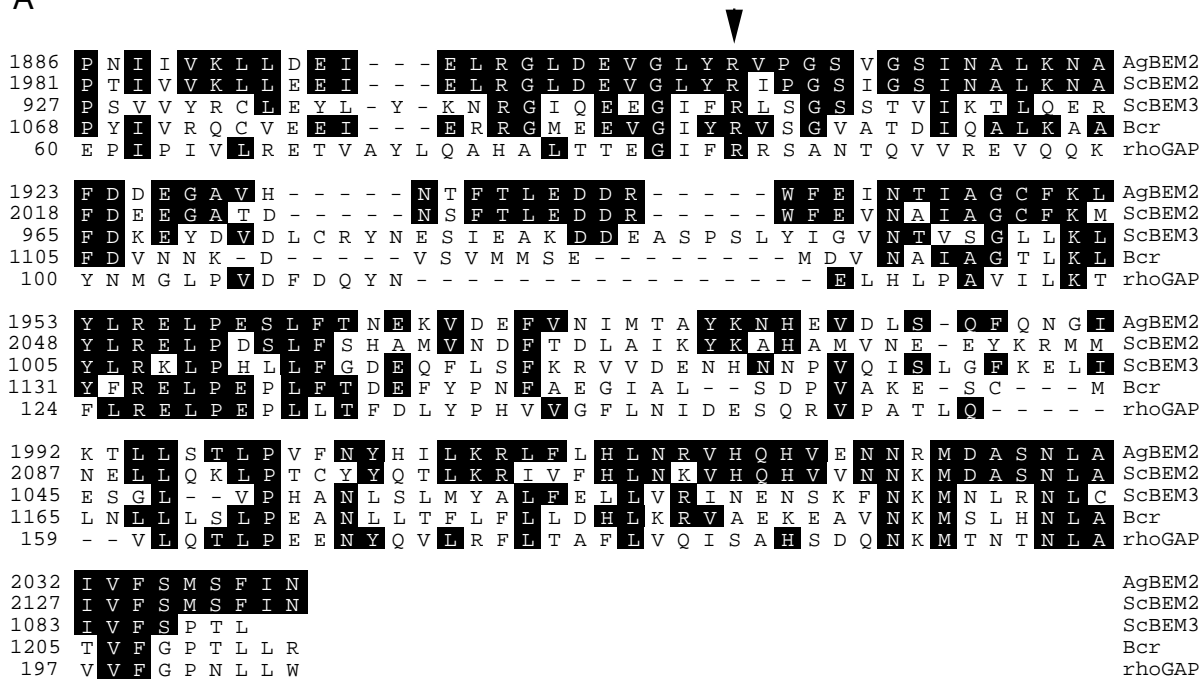
domains with these SCRs showed recognizable agreements to the SCR2 whereas manual alignment of the AgBem2p sequence to SCR1 and SCR3 showed only moderate conservation (Fig. 3B). Since this putative rasGEF domain is located in a well conserved region between AgBem2p and ScBem2p suggests a functional role for this domain even if it does not correspond to the bona fide rasGEF consensus represented by the PROSITE annotation. For the LTE1/rasGRF-associated domain named after the *S. cerevisiae* *LTE1* gene only a preliminary entry is available in PROSITE which does not implicate a specific function of this domain. Six additional blocks (I to VI) of high homology between AgBem2p and ScBem2p were found. Profile and blast searches with these short aa sequences revealed no motif and did not identify other proteins with similar sequences which could have indicated some functional relationship.

Deletion of AgBEM2

The open reading frame of AgBEM2 was completely deleted from the genome by one-step gene replacement using the recently established PCR-based gene targeting protocol for *A. gossypii* (see Materials and Methods). The deletion was constructed both in an *A. gossypii* wild-type and in an *A. gossypii leu2, thr4* strain using the dominant selectable marker *GEN3* that confers resistance to G418/geneticin (Fig. 4A). The hyphae of *A. gossypii* consist of multinucleate septate compartments. The primary transformation event generated a heterokaryon consisting of nuclei that carried either the wild-type AgBEM2 or the *Agbem2Δ1* deletion (Fig. 4B). The heterokaryotic transformants were indistinguishable from wild type as far as hyphal and colony morphology were concerned but were able to grow on selective medium. In order to obtain homokaryotic mutant strains, in which all nuclei carried the mutant allele, spores (which are mononucleate and haploid) of the heterokaryotic strains were isolated and germinated under selective conditions (see Materials and Methods). Viable homokaryotic *Agbem2Δ1* transformants could be obtained indicating that AgBEM2 is not essential. The constructed deletions were confirmed by analytical PCR (Fig. 4B). Additionally, DNA hybridization experiments on chromosomal DNA of the progenitor strains, as well as on the heterokaryotic and homokaryotic mutants indicated that the selectable marker module had integrated correctly only once into the genome at the *BEM2* locus (data not shown).

Mycelial growth assays were performed comparing the radial growth rates of wild-type and *Agbem2Δ1* strains at 20°C, 30°C, and 37°C (Fig. 5). *Agbem2Δ1* shows a slow growth phenotype at all temperatures. The ability of *Agbem2Δ1* mutant strains to grow above 33°C is in contrast to the *Scbem2* strains which are inviable above this temperature (Kim et al., 1994; Peterson et al., 1994). Restoration of wild-type-like growth-rate was achieved in an *A. gossypii bem2Δ1, leu2, thr4* strain by complementation with the freely replicating plasmid, pBEM2, containing the wild-type AgBEM2 and the *ScLEU2* gene as a selection marker (Fig. 5). Freely replicating plasmids are stably maintained under selective conditions in *A. gossypii* but can be lost in the mycelium under non-selective conditions (Wright and Philippsen, 1991). Interestingly, pBEM2 is maintained in the mycelium of *Agbem2Δ1* strains even without selective pressure suggesting that fast growing hyphae have

A



B

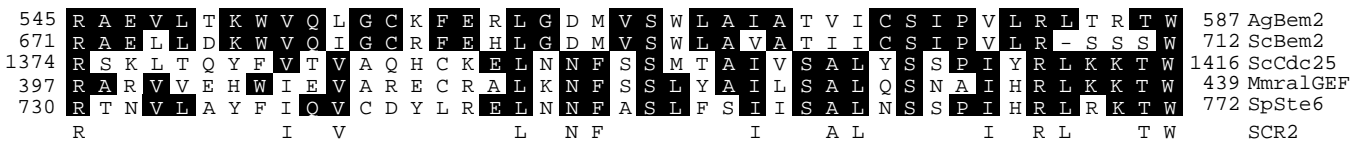


Fig. 3. Alignment of domains identified in Bem2 proteins. (A) Sequence alignment of rhoGAP-domains. Identical aa between AgBem2p and any other protein sequence are shaded. The conserved arginine residues corresponding to Arg85 of rhoGAP are indicated by an arrowhead. (B) Partial sequence alignment of AgBem2p and ScBem2p to the 'structurally conserved region 2' of Ras-GEF and related domain containing proteins according to Boguski and McCormick (1993). Amino acids corresponding to a majority of aligned sequences or to the AgBem2p are shaded. Accession numbers: ScBem2p: P39960; ScCdc25p: P04821; MmralGEF:L07924; SpSte6p: P26674. Sc: *S. cerevisiae*; Mm: *Mus musculus*; Sp: *Schizosaccharomyces pombe*.

become dependent on AgBEM2. Two lines of evidence indicated the free replication of the complementing plasmid. (i) Repeated rounds of spore isolation and re-growth experiments from non-selectively grown mycelia of Agbem2Δ1 transformants harboring the pBEM2 plasmid indicated differential plasmid distribution in the spores. Plasmid loss resulted in the reoccurrence of the slow growth phenotype that was observed in Agbem2Δ1 whereas fast-growing colonies had maintained pBEM2. (ii) Transformation of DNA obtained from the pBEM2 bearing *A. gossypii* strains into *E. coli* cells resulted in the formation of ampicillin resistant colonies.

The carboxy terminus of AgBem2p is necessary for complementation of Agbem2Δ1

In an attempt to identify subdomains within the 2071 aa of AgBem2p that are required for the complementation of Agbem2Δ1 we constructed internal deletions of AgBEM2 creating Agbem2Δ2, Agbem2Δ3, and Agbem2Δ4 based on pBEM2 (Fig. 6). Agbem2Δ2 fully complemented the

Agbem2Δ1 strain whereas Agbem2Δ4 could not. Deletion of approximately 60% of the BEM2 open reading frame in Agbem2Δ3 was partially able to complement the BEM2 deletion. Complementation resulted in the restoration of elongated hyphal morphology, which led to increased rates of radial growth compared to the deletion strain transformed with a ScLEU2 control plasmid. Radial growth rates, however, were only 45% of that of wild type and microscopic inspection revealed an increased proportion of hyphae exhibiting an Agbem2Δ1-associated phenotype (data not shown). These results indicated that the carboxy-terminal part of AgBem2p containing the rhoGAP-domain is critical for its function. The partial complementation obtained with Agbem2Δ3 could be due to either the loss of function of the putative rasGEF-domain or a decrease in protein stability of AgBem2Δ3p since a large part of AgBEM2 (60%) had been removed. Similarly, it was shown in *S. cerevisiae* that the temperature sensitive growth defect of Scbem2 cells could be complemented by overexpressed carboxy-terminal fragments of ScBem2p containing the PH and GAP-domains (Peterson et al., 1994; Kim et al., 1994).

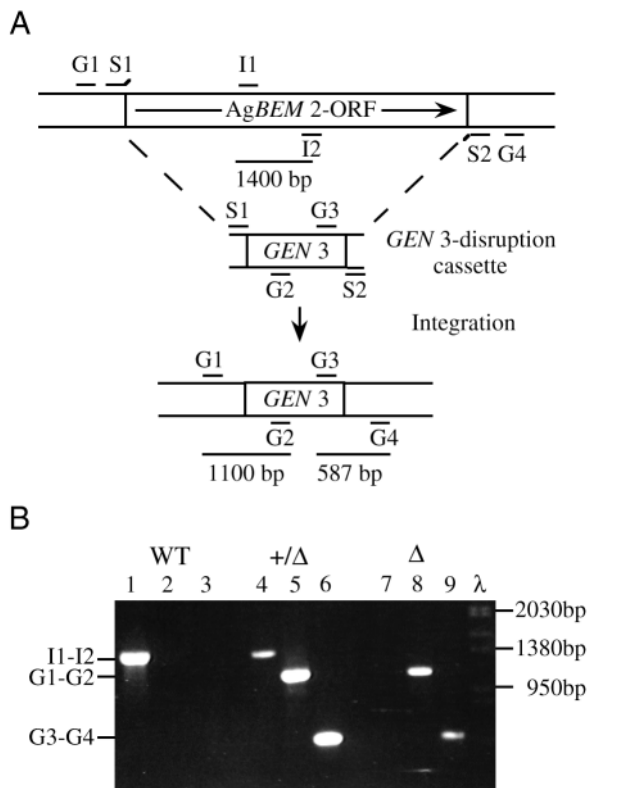


Fig. 4. Deletion of *AgBEM2*. (A) Schematic diagram showing the disruption procedure. The orientation of the *AgBEM2*-ORF (boxed) is indicated by an arrow. The positions of the primers used are indicated according to the strand of DNA they are homologous to. The *GEN3*-module was amplified with the S1 and S2 primers which carried 45 bp homology to the *AgBEM2* gene directly upstream and downstream of the start and stop codon, respectively. Transformation of the amplified *GEN3*-module led to the replacement of the *AgBEM2*-ORF by *GEN3* generating *Agbem2Δ1*. I1 and I2 represent internal primers that were used to indicate the presence of the *AgBEM2* gene. Upon successful disruption of *AgBEM2* amplification products of indicated sizes were expected with the given primer combinations. (B) Ethidium bromide-stained agarose gel showing the products of the analytical PCR. Genomic DNA of the wild-type (lanes 1-3), primary heterokaryotic (lanes 4-6), and homokaryotic (lanes 7-9) *Agbem2Δ1* strains was analyzed with the primer combinations I1-I2, G1-G2, and G3-G4, respectively. No product was obtained in lanes 2, 3, and 7 as expected. Fragment sizes of the λ -marker are as indicated.

Defects during germination of *Agbem2Δ1* spores

Germination of *A. gossypii* spores occurs as an isotropic growth phase that results in the formation of spherical germ cells. Wild-type spores germinate into germ cells that reach on average 8 μm in diameter ($n=100$; s.d.=0.5 μm ; Fig. 7A). Seven to eight hours after inoculation of spores into rich medium at 30°C a switch from isotropic to polarized growth leads to the formation of the first hyphal tube (Fig. 7B). After formation of the first hypha a second hypha is produced opposite of the first resulting in a bipolar germ cell branching pattern (100/100 observed cases; Fig. 7C). From these initial hyphae a mycelium is generated first by lateral branching and later (16-18 hours post inoculum) by dichotomous tip branching (Fig. 7D,E).

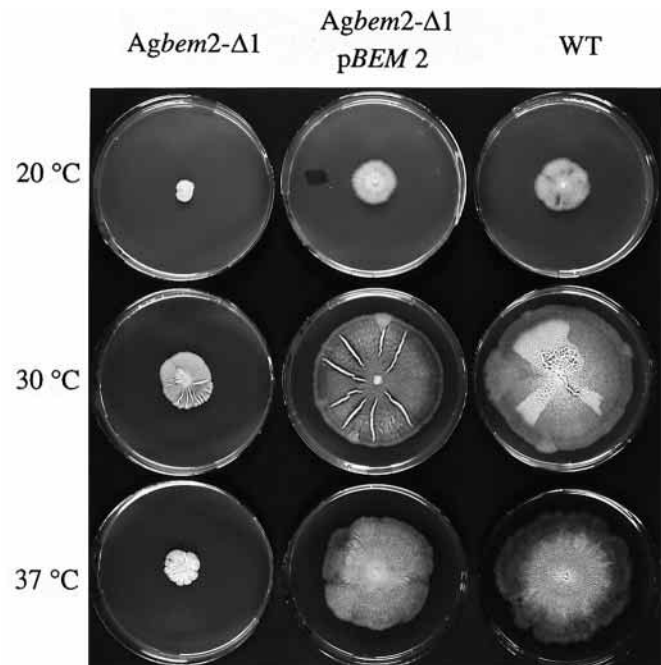


Fig. 5. Growth comparison of wild-type and *Agbem2Δ1* mutant strains. *Agbem2Δ1*, an *Agbem2Δ1* mutant transformed with the freely replicating plasmid pBEM2, carrying the complete *AgBEM2* gene, and an *A. gossypii* wild-type strain were grown at the indicated temperatures for one week on full medium plates.

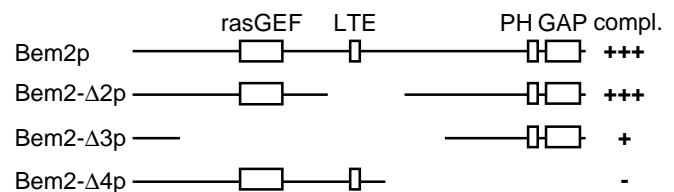
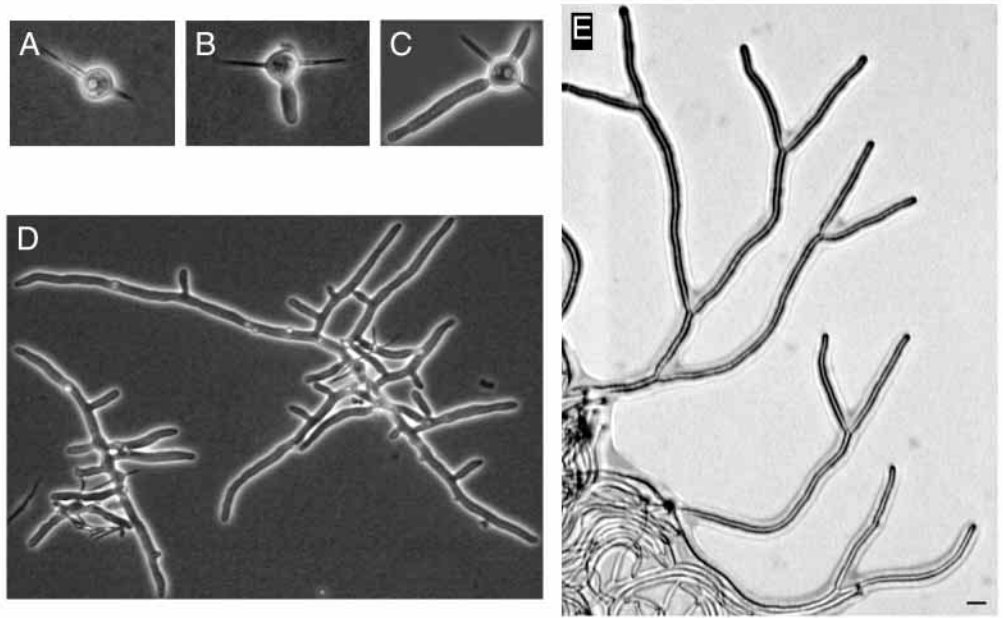


Fig. 6. Complementation analysis of *Agbem2Δ1*. The ability (+) or inability (-) of different freely replicating plasmids encoding either wild-type Bem2p or alleles lacking the indicated portions of *AgBEM2* to complement the growth defect of an *Agbem2Δ1* strain at 30°C is listed. For detailed cloning procedures see Materials and Methods.

Dichotomous tip branching is usually not observed during the initial period of mycelium formation suggesting that a second switch is needed in *A. gossypii* cells to progress from unipolar hyphal tip growth to dichotomous tip growth which then occurs at regular intervals (Fig. 7E). At the edge of an *A. gossypii* colony almost all new tip cells were found to be produced by dichotomous tip branching compared to lateral branching of subapical hyphal cells.

In *Agbem2Δ1* mutant strains the isotropic growth phase during spore germination was prolonged and produced enlarged germ cells with cell diameters of up to four times the size of wild-type germ cells. Interestingly, on the surfaces of *Agbem2Δ1* germ cells protuberances were noted that might represent multiple randomly distributed initiation sites of polarized hyphal growth (Fig. 8A). This indicated the inability of *Agbem2Δ1* germ cells to correctly initiate the switch from

Fig. 7. Growth patterns of *A. gossypii* wild type. Needle-shaped spores were inoculated on coverslips containing a thin layer of full medium and incubated at 30°C for up to 36 hours. (A) Germ cell. (B) Germ cell with first hyphal tube. (C) Germ cell displaying bipolar germ cell branching pattern. (D) Small mycelia proliferating by lateral branches. (E) Adult mycelium proliferating predominantly by dichotomous tip branching. Bars: 5 µm for A-C; 10 µm for D,E.

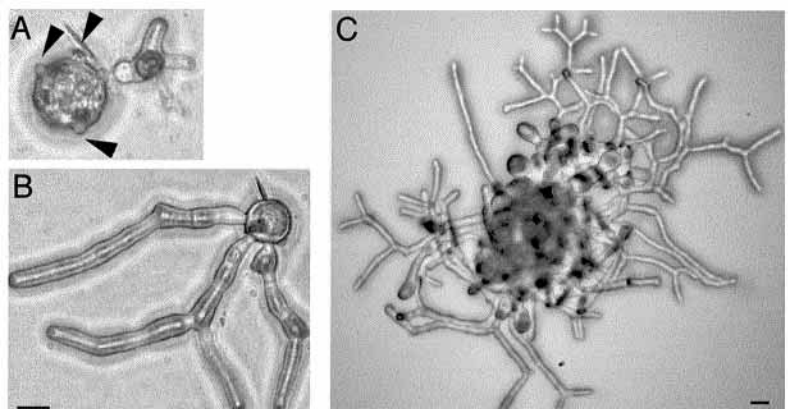


isotropic to polar growth. Eventually germ cells were either unable to produce hyphae and oversized cells lysed or *Agbem2Δ1* germ cells switched to polarized growth. In contrast to wild-type germ cells, however, these *Agbem2Δ1* cells were defective in generating the bipolar germ cell branching pattern (0/200 observations). *Agbem2Δ1* germ cells were found to produce either only one or multiple growing hyphae (Fig. 8A,B). The dichotomous tip branching at later stages of mycelium development was not affected in *Agbem2Δ1* mutants. However, the distance between two consecutive tip branches were found to be much shorter in *Agbem2Δ1* hyphae than in wild-type hyphae (Figs 7E, 8C).

Defects in the maintenance of polarized hyphal growth and loss of cell polarity in *Agbem2Δ1* hyphae

Fluorescence microscopy was used to analyze the distribution of chitin (and other β-linked polysaccharides) by calcofluor staining. In wild-type hyphae calcofluor decorated sites of septation that occur at regular intervals and showed that hyphal tips stain more brightly compared to subapical cell wall regions indicating areas of polarized cell growth (Fig. 9A). In *Agbem2Δ1* hyphae staining at sites of septation was found to be comparably weak and in tip cells of *Agbem2Δ1* hyphae calcofluor staining was most often uniformly distributed along the hyphal walls (Fig. 9B). This indicated a defect in polarized cell growth and a complete loss of cell polarity in those hyphal tip cells that had become spherical (Fig. 9B). The diameter of *Agbem2Δ1* hyphae was up to three times larger than that of wild-type hyphae. We therefore asked whether the positioning of nuclei in *Agbem2Δ1* hyphae was also different from that of the wild type. DAPI-fluorescence showed that wild-type nuclei are rather evenly distributed whereas nuclei in *Agbem2Δ1* hyphae accumulated to large numbers in swollen areas of the hyphae (Fig. 9C,D).

Fig. 8. Growth patterns of *Agbem2Δ1*. (A) Germ cell with one branch and additional protuberances of which three are indicated by arrowheads. (B) Germ cell with multiple branches. (C) Small mycelium with dichotomous tip branches. Growth conditions as described in Fig. 7. Bars, 10 µm (A,B are at the same magnification).



Analysis of the polarity of the actin cytoskeleton in wild-type hyphae by staining with rhodamine-phalloidin showed that actin cortical patches were clustered in hyphal tips, indicating regions of active growth, and in ring like structures at sites of developing septa (Fig. 10A,B). Actin cables could be resolved in wild-type only after strongly overexposing actin patches but were invisible in *Agbem2* mutants using this staining protocol. In *Agbem2Δ1* hyphae actin-ring formation at sites of developing septa was found to occur (Fig. 10C). Actin cortical patches still localized to the hyphal apices, although the area of patch distribution was found to be much broader corresponding to the enlarged size of the tips of *Agbem2Δ1* hyphae. Hyphae that had swollen at their tips during a period of isotropic growth, however, showed a uniform distribution of actin cortical patches (Fig. 10C). Thus, the defects in polarized cell surface growth and loss of cell polarity visualized by chitin staining were connected with a failure in maintaining the polarity of the actin cytoskeleton. Surprisingly, swollen *Agbem2Δ1* hyphal tip compartments could establish new cell polarities resulting in the formation of multiple hyphal tips at randomly positioned sites (Fig. 10C). These ‘protuberances’ which had already been noted in *Agbem2Δ1* germ cells were shown to contain accumulations of cortical actin patches and therefore corresponded to newly

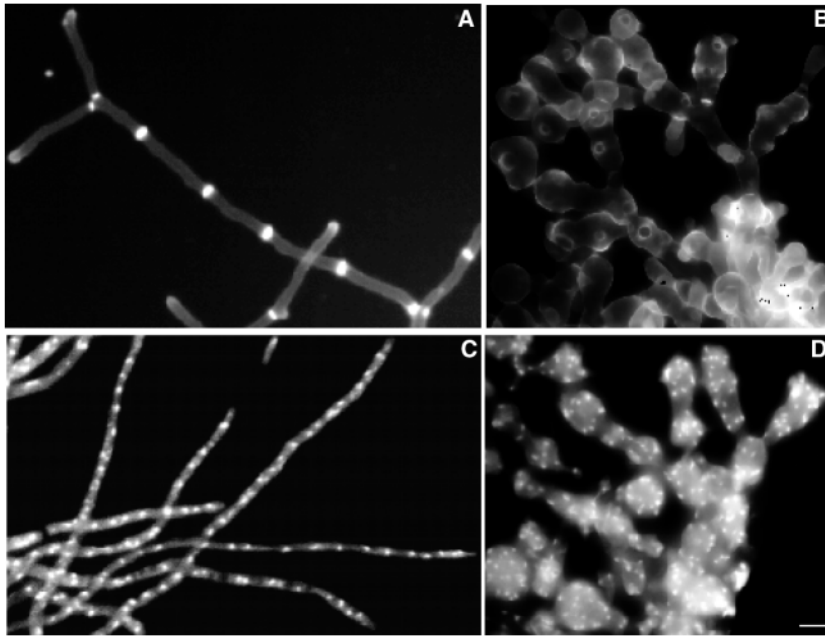


Fig. 9. Chitin and nuclear distribution. Fluorescent images of calcofluor (A,B) and DAPI (C,D) stained hyphae of wild type (A,C) and *Agbem2Δ1* (B,D). Hyphae were grown exponentially at 30°C in liquid medium. Bar, 10 μm.

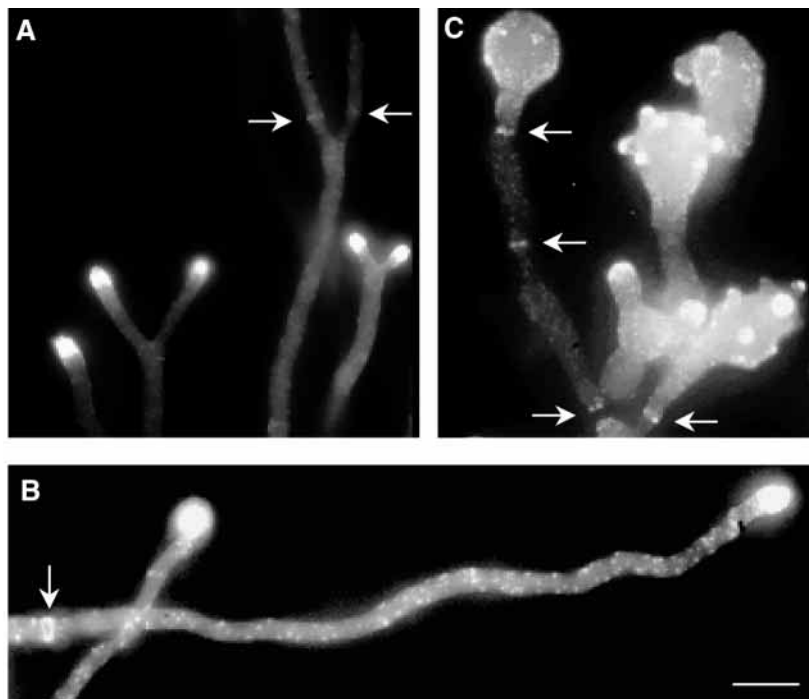


Fig. 10. Localization of actin cortical patches. Fluorescent images of rhodamine-phalloidin stained hyphae of wild type (A,B) and *Agbem2Δ1* (C). Arrows indicate sites of developing septa. Growth conditions as in Fig. 9. Bar, 10 μm.

established sites of polarized growth (Fig. 10C). Initiation of multiple new cell polarities in *Agbem2Δ1* mutants occurred at two different stages: (i) In *Agbem2Δ1* germ cells after prolonged periods of isotropic growth (ii) in vegetative hyphae after loss of cell polarity. The observation that in germ cells not all newly established sites of growth continued to form

hyphae might suggest the requirement of other factors whose supply could be limited.

DISCUSSION

We chose the filamentous ascomycete *A. gossypii* as a model to investigate polarized hyphal growth on the molecular level due to its very small genome and the facile applicability of powerful molecular genetic tools. During an initial sequencing screen, we tried to identify *A. gossypii* homologs of genes known to be part of rho-GTPase modules in other organisms. In this study we have presented the characterization of an *A. gossypii* rhoGAP homolog. We could show, that a member of a rho-GTPase module is involved in the maintenance of hyphal growth and cell polarity. Several lines of evidence suggested that the rhoGAP homolog isolated from *A. gossypii* is the functional homolog of the *S. cerevisiae* *BEM2* gene. (i) Both proteins show sequence similarity along their entire length. (ii) The function of both proteins depends on their carboxy termini carrying PH- and GAP-domains. (iii) The *AgBEM2*-locus displays synteny to the *S. cerevisiae* *BEM2*-locus. The order of at least four genes including their transcriptional orientation has been conserved. Another example for the conservation of a four-gene cluster has been reported for the *A. gossypii* and *S. cerevisiae* *THR4*-loci (Altmann-Jöhl and Philippsen, 1996). (iv) Phenotypic similarities were observed in the *A. gossypii* and *S. cerevisiae* mutant strains concerning heterogeneity in cell size, cell lysis, depolarized growth, and defects in the organization of the actin cytoskeleton. Preliminary experiments indicated that the *AgBEM2* gene does not complement a *S. cerevisiae* *BEM2* deletion. This might be due to the inability of the *AgBEM2* promoter to function in *S. cerevisiae*. Analysis of the inter-ORF regions upstream of the *AgBEM2* and *ScBEM2* open reading frames, which is only 262 bp in *A. gossypii* and therefore 215 bp shorter than in *S. cerevisiae* revealed no conserved promoter elements. Biochemical studies have shown that *ScBem2p* shows GAP-activity towards *ScRho1p* but not towards *ScCdc42p* (Zheng et al., 1993, 1994; Peterson et al., 1994). Similar experiments with *AgBem2p* could delineate its function in *A. gossypii*.

Defects in germination and the germ cell branching pattern in *Agbem2Δ1*

The bipolar branching pattern of *A. gossypii* wild type was abolished in *AgBEM2* mutants. In *A. nidulans* temperature-sensitive mutant strains interfering with a similar branching pattern have been isolated recently (Harris et al., 1999; Momany et al., 1999). Germination and growth of *Agbem2Δ1* strains was not restricted at elevated temperatures, although at all temperatures prolonged periods of isotropic growth led to heterogeneity in the sizes of germ cells as well

as to increased germ cell lysis. In contrast, *Scbem2* cells become arrested as large unbudded multinucleate cells at temperatures above 33°C (Adams et al., 1990; Peterson et al., 1994; Kim et al., 1994). In *S. cerevisiae* heterogeneity in cell-size as well as a cell lysis phenotype has been described previously for the *LYT3* gene of *S. cerevisiae* which was later found to be allelic to *BEM2* (Cid et al., 1994, 1998).

Depolarized growth and loss of cell polarity in *Agbem2Δ1*

Polarity defects in *Agbem2* mutants eventually caused completely isotropic growth resulting in the deposition of chitin over the entire cell surface. Depolarized growth generated large balloon-shaped tip cells. In *A. gossypii* as well as in other filamentous fungi cortical actin is concentrated at active sites of growth and secretion, i.e. in hyphal apices and at sites of developing septa (Harris et al., 1994; Salo et al., 1989; Runeberg et al., 1986). In *Agbem2Δ1* hyphae actin patches were depolarized as could be shown by their uniform distribution. A similar phenotype has been described for *Scbem2* strains and also for *A. nidulans* and *Saprolegnia ferax* wild-type after treatment with cytochalasin A or latrunculin B, respectively, which both disrupt cortical actin organization (Wang and Bretscher, 1995; Torralba et al., 1998; Gupta and Heath, 1997). In *S. cerevisiae* genetic interactions of *BEM2* with a number of cytoskeletal genes have been shown. Among these are synthetic lethality of *BEM2* with alleles of *TPM1* (tropomyosin), *MYO1* (myosin II), *MYO2* (myosin V), *ACT1* (actin) and *SAC6* (fimbrin; Wang and Bretscher, 1995). With respect to the polarity of the actin cytoskeleton it was shown that tropomyosin is a stabilizing component of actin cables. Conditionally defective tropomyosins lead to a disassembly of actin cables under restrictive conditions which results in a depolarization of actin cortical patches and isotropic growth in *S. cerevisiae* cells (Pryne et al., 1998). Therefore a genetic interaction between *BEM2* and *TPM1* provides a route via which a Rho1-GTPase module could modulate the actin cytoskeleton (Pryne et al., 1998). An alternative route was provided by showing that ScRho1p interacts with ScBni1p in a GTP-dependent manner (Kohno et al., 1996). ScBni1p interacts directly with actin and *ScBNII* is synthetic lethal with *ScRHO1* (Kohno et al., 1996; Evangelista et al., 1997). Overproduction of N-terminally truncated *ScBNII* (452-1953) interestingly leads to depolarized growth resulting in large round cells (Evangelista et al., 1997) as does overproduction of ScRho1p (Peterson et al., 1994; Madaule et al., 1994) resembling *Scbem2* mutant cells. It will be of interest to examine the role of *AgBEM2* in the morphogenetic network of *A. gossypii*.

Surprisingly, we found that growth of *Agbem2Δ1* hyphal tips did not become arrested after an isotropic growth phase. Instead, repolarization of the actin cytoskeleton occurred generating multiple sites of activated polarized growth. Several observations suggested that multiple branch points were produced simultaneously from swollen hyphal tips: (i) In a few cases simultaneous outgrowth of new hyphal tips could be observed via time lapse video microscopy. (ii) Samples from logarithmically grown cultures contained hyphae with apical swellings from which already multiple elongated hyphae of comparable length had emerged suggesting that their growth had started at around the same time. (iii) Cyclic reoccurrence

of rounds of polarity establishment, hyphal growth, and loss of cell polarity was observed in a few instances.

Possible function of *AgBem2p* in a rho-GTPase morphogenetic network

We could show that the carboxy terminus of *AgBem2p* containing a PH- and rhoGAP-domain is critical for its function. Additionally both, *AgBem2p* and *ScBem2p*, contain a domain with moderate homology to rasGEF domains as well as seven other conserved sequence elements of unknown function. The possession of both a putative rasGEF- and a rhoGAP-domain in *AgBem2p* and *ScBem2p* might provide a link between ras- and rhoGTPase modules. Such a link could be important to control the isotropic growth phase during germination of spores. It was shown in the zygomycete *Phycomyces blakesleeanus* that a RhoA homolog was expressed during germination and in *S. cerevisiae* that during spore germination signaling through the Ras protein pathway but not via Cdc28p is required (Ramirez-Ramirez et al., 1999; Herman and Rine, 1997). Ras proteins have been isolated from various ascomycetous fungi and were shown to be regulators of growth and morphology (Truesdell et al., 1999; Kana-uchi et al., 1997; Som and Kolaparthi, 1994).

The role of *AgBem2p* in the morphogenetic network might also suggest an explanation for the fact that at a given time only a portion of *Agbem2Δ1* hyphae (<5%) displayed completely isotropic growth resulting in spherical swellings of hyphal tips. The amount of Rho1p-GTP increases upon stimulation of GDP-GTP exchange by Rho1p-GEF. Signaling via Rho1p-GTP in an *Agbem2Δ1* background is not turned down in *A. gossypii* which might initially result in delocalized but still polar growth generating hyphae with an enlarged diameter compared to wild-type cells. Eventually, if signaling via Rho1p-GTP surpassed an overflow level, cell polarity would be lost. Interestingly, loss of cell polarity can be overcome in *A. gossypii* both at the germ cell stage and during vegetative growth. An important role in the process of re-establishment of polarity might be exerted by *A. gossypii* homologs of genes required for polarizing the actin cytoskeleton, e.g. the homologs of the *S. cerevisiae* genes *CDC42* and *CDC24*. Therefore, we have recently isolated and started to characterize the *AgCDC42* and *AgCDC24* genes (J. Wendland et al., unpublished). Deletion of both genes generated similar phenotypes as have been described for the temperature-sensitive mutant strains of *S. cerevisiae* at the non-permissive temperature (Bender and Pringle, 1989; Adams et al., 1990). *A. gossypii* spores of heterokaryotic mutant strains germinated but then failed to initiate the switch from isotropic to polarized growth and became arrested as large, unbranched, multinucleate germ cells (J. Wendland et al., unpublished). This indicates a role of the *AgCdc42p*-GTPase module during establishment of polarity, which is taking place in a deregulated manner both in *Agbem2Δ1* germ cells and swollen hyphal tips.

Our results suggest that in filamentous fungi rhoGTPase modules such as driven by the rho-GTPases *CDC42* and *RHO1* are key regulators for polarized hyphal growth in a similar way as has been shown in yeast and animal cells. Further studies may therefore reveal the peculiarities of the different morphogenetic regulatory machineries that result in the generation of distinct growth forms.

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