BEM46 shows eisosomal localization and association with tryptophan-derived auxin pathway in *Neurospora crassa*

K. Kollath-Leiß, C. Bönniger, P. Sardar, F. Kempken

Abteilung Botanische Genetik und Molekularbiologie, Botanisches Institut und Botanischer Garten, Christian-Albrechts-Universität zu Kiel, Kiel, Germany

BEM46 proteins are evolutionarily conserved, but their functions remain elusive. We reported previously that the BEM46 protein in *Neurospora crassa* is targeted to the endoplasmic reticulum (ER) and is essential for ascospore germination. In the present study, we established a *bem46* knockout strain of *N. crassa*. This ∆*bem46* mutant exhibited a level of ascospore germination lower than that of the wild type but much higher than those of the previously characterized *bem46*-overexpressing and RNA interference (RNAi) lines. Reinvestigation of the RNAi transformants revealed two types of alternatively spliced *bem46* mRNA; expression of either type led to a loss of ascospore germination. Our results indicated that the phenotype was not due to *bem46* mRNA downregulation or loss but was caused by the alternatively spliced mRNAs and the peptides they encoded. Using the *N. crassa* ortholog of the eisosomal protein PILA from *Aspergillus nidulans*, we further demonstrated the colocalization of BEM46 with eisosomes. Employing the yeast two-hybrid system, we identified a single interaction partner: anthranyl synthase component II (encoded by *trp-1*). This interaction was confirmed *in vivo* by a split-YFP (yellow fluorescent protein) approach. The ∆*trp-1* mutant showed reduced ascospore germination and increased indole production, and we used bioinformatic tools to identify a putative auxin biosynthetic pathway. The genes involved exhibited various levels of transcriptional regulation in the different *bem46* transformant and mutant strains. We also investigated the indole production of the strains in different developmental stages. Our findings suggested that the regulation of indole biosynthesis genes was influenced by *bem46* overexpression. Furthermore, we uncovered evidence of colocalization of BEM46 with the neutral amino acid transporter MTR.

The bud emergence 46 (BEM46) protein is conserved across the eukaryotic kingdom, and the molecular evolution of members of the BEM46 family has been described in detail recently (1). Our group has demonstrated that while the majority of eukaryotic genomes include a single copy of *bem46*, vertebrates possess several paralogs, which originated through duplication events (1).

Studies with various model organisms have revealed limited data regarding the function of BEM46. The *bem46* gene of *Schizosaccharomyces pombe* (EMBL accession number U29892) is reportedly a suppressor of the *bem1 bud5* double mutant of *Saccharomyces cerevisiae* (1), which shows defects in cell polarization and budding (2, 3). BEM1 is a scaffold protein that interacts with BUD1 (4), actin (5), CDC42 (6), and BUD5 (2, 7), and it is reportedly required for the positioning of a protein complex involved in bud formation (2). BUD5 is a GDP-GTP exchange factor for BUD1 and is necessary for bud site selection (7). The *bem46* homolog of baker’s yeast (YNL320W) is not essential (8). Two-hybrid approaches with *Drosophila melanogaster* have shown that the BEM46 homolog interacts with the Rapsynoid protein (9), which is a putative GDP-GTP exchange factor for a G protein and is involved in controlling asymmetrical cell division (10). Wavy growth 2 (WAV2; encoded by *wav2*) is the BEM46 homolog in *Arabidopsis thaliana*, and a knockout mutant shows short-pitch waves related to root development (11). WAV2 is a common regulator involved in suppressing root bending caused by cell file rotation enhancement in response to touch stimuli, light, and gravity (12). The protein is expressed mainly in young seedlings and in the roots of adult plants, with subcellular localization in the plasma membrane and in compartment membranes (12).

All BEM46 proteins belong to an α/β hydrolase superfamily, characterized by the α/β hydrolase domain (13), comprising a β-sheet core of five to eight strands connected by α-helices. The α/β hydrolase domain is also found in several enzymes with diverse phylogenetic backgrounds, catalytic functions, and substrate specificities (14). The ESTHER database includes more than 30,000 members of this superfamily (15, 16).

Despite these hints, the exact function of BEM46 remains elusive. Therefore, *bem46* is considered one of the top 10 known genes encoding a protein with unknown function (17, 18). Previous results suggest that BEM46 may play a role in signal transduction or in the maintenance of cell polarity. Fungal hyphae serve as a model system for polarized growth (19); therefore, we have investigated the BEM46 protein of the ascomycete *Neurospora crassa*. We reported previously that BEM46 in *N. crassa* is localized to the perinuclear endoplasmic reticulum (ER), and in patches near the plasma membrane (20), as detected on the basis of an unusual ER retention signal at the C-terminal end of the protein (1). Either transcript overexpression or downregulation leads to a loss of ascospore germination. Using bioinformatic tools, we also previously predicted the native protein structure, which included an essential catalytic triad (1).

In the present study, we show that BEM46 in *N. crassa* is part of...
the fungal eisosome and is an interaction partner of anthranilate synthase. We also demonstrate the colocalization of BEM46 with the putative tryptophan transporter MTR. Our present data indicate an influence of BEM46 on the auxin biosynthesis pathway of the fungus, and we use bioinformatic tools to predict a putative auxin biosynthesis pathway in N. crassa. Furthermore, we demonstrate that alternative splicing of the bem46 transcript is responsible for the loss of ascospore germination in the bem46 RNA interference (RNAi) lines.

**MATERIALS AND METHODS**

**Strains.** The present study used the following Neurospora crassa strains from the Fungal Genetics Stock Center (FGSC; Kansas City, MO, USA): FGSC 9718 (∆\textit{mts}-51:bar mat A), FGSC 9719 (∆\textit{ms}-52:bar mat A), FGSC 6103 [his-3 (Y234M723) mat A], FGSC 9716 [his-3 (Y234M723) mat a], FGSC 20870 [\textit{Δ}trp-1 (NCU00200.2) mat a], and FGSC 20871 [\textit{Δ}trp-1 (NCU00200.2) mat A]. Fungi were cultivated on Vogel’s minimal medium (21). All expression vectors carrying fusion constructs used in the present study were transformed into the histidine auxotrophic strains FGSC 6103 and FGSC 9716; therefore, these strains served as the “wild type” in control experiments. Strains carrying auxotrophic markers were grown on media supplemented with the required amino acids. For crosses, fungi were plated on Westergaard’s medium (22). In the colocalization studies, heterokaryons were formed as described previously (23).

For the propagation of vector constructs under standard culture conditions, we used \textit{Escherichia coli} strain XL1-Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacI42 lacZ58 is600 (F’ proAB lacZM15 Tn10 [Tet])] (Stratagene, La Jolla, CA). For the propagation of RNAi constructs, we used \textit{E. coli} strain SURE [recA1 (McrC-D) ∆(mrbc-hisDMK-mrr)]E1 endA1 supE44 thi-1 gyrA96 relA1 lacI42 recB recC umuC::Tn5 (Kan)] (Stratagene, La Jolla, CA). DNA and RNA isolation. DNA was isolated as described previously (24). Briefly, mycelia were ground under liquid nitrogen and were transferred to lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, and 2% SDS [pH 8.0]), followed by phenol extraction. Subsequently, the aqueous phase was incubated with 100 µg RNase A, followed by an additional phenol extraction and ethanol precipitation. Bacterial plasmid DNA was isolated using NucleoSpin reagent kits (Macherey-Nagel, Düren, Germany). Plasmids were isolated from yeast according to standard procedures (25).

RNA was isolated from mycelia as published previously (26). Vegetative mycelia of \textit{Neurospora crassa} strains were grown for 3 days in liquid Vogel’s minimal medium with 5.8 mM saccharose. For strain FGSC 6103, 1 mM l-histidine was added to the medium. The mycelia were ground and were cooled with liquid nitrogen.

**Gel electrophoresis, blotting, and hybridization.** Agarose gel electrophoresis, Southern and Northern blotting, and DNA-DNA and DNA-RNA hybridizations were performed as described previously (27). A DECAprime kit (Ambion, Austin, TX) was used to label 20 to 30 ng of template DNA with [α-\textit{32P}]dCTP. MBBL (Bielefeld, Germany) DNA markers were used to determine size in DNA gel electrophoresis.

**PCR and RT-PCR amplification.** PCR was performed as described previously (20). For quantitative reverse transcription-PCR (qRT-PCR), isolated nucleic acids were treated with DNase (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s recommendations. As a control for the success of DNase treatment, PCR was also performed using primers CB2480 (TACTTCCAGCGGCAAGGTC) and CB2481 (TGCGGAGAGGTGTTAAGAGAG) for the housekeeping gene \textit{L6_rRNA}.

The oligonucleotides used for quantitative real-time PCR were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Isolated and DNase-treated DNA (100 ng) was used as the template and was mixed with the contents of the Quantitect SYBR green RT-PCR kit (Qiagen, Hilden, Germany). The recommended program from Qiagen was modified to use an annealing temperature of 58°C. Quantitative real-time PCR was performed in a 7300 Real-Time PCR system (Life Technologies, Darmstadt, Germany). For each gene of interest, we calculated the level of expression by the fold difference from the expression level of the housekeeping gene \textit{tub2} and by using the 2^{−\DeltaΔCT} method (28). Statistical analysis was performed using SigmaPlot, version 12.

**Transformation and transformatant analysis.** Previously described methods were used for cloning and \textit{E. coli} transformation (27) and for \textit{N. crassa} transformation (29). All cloning and transformation experiments were conducted in accordance with the requirements of the German gene technology law (GenTG).

**Microscopy.** For the germination assays, ejected ascospores were harvested and were incubated for 90 min in 300 µl sterile water at 60°C to inactivate contaminating macroconidia. Ascospore concentrations were adjusted to 4 × 10^3 spores/ml. All light microscopy was performed using a Zeiss Axiopt microscope equipped with a SONY 3CCD digital camera. Confocal fluorescence analysis was performed using a confocal laser scanning microscope (CLSM) (TCS SP5; Leica). Fusion constructs containing enhanced green fluorescent protein (eGFP), enhanced yellow fluorescent protein (eYFP), or tag red fluorescent protein (eRFP) were excised at 488 nm, 514 nm, or 543 nm, respectively, and emission was detected at 500 to 550 nm, 520 to 550 nm, or 570 to 620 nm, respectively. Images were analyzed using Leica LAS AF Lite software. Fungi were cultivated for microscopy as described previously (30).

**Vector construction.** The construction of the pMM532 (bem46-overexpressing), pMM532 (bem46 RNAi), pMM536 (bem46:eGFP fusion), and pUH280 (used for the bem46 repeat induced point mutation [RIP] mutation assay) vectors has been described previously (20). In the supplemental material, Fig. S1 presents a schematic representation of all other vectors used, and Table S1 lists the sequences of the oligonucleotides used in the present work. The vectors used for the yeast two-hybrid approach are described below.

The pKK790 and pKK791 vectors were used in the bimolecular fluorescence complementation assay. pKK790 consists of the \textit{bem46} open reading frame (ORF) and the portion of \textit{eyfp} encoding the C-terminal sequence (31), amplified in two steps by overlapping extension PCR. In the first step, the \textit{bem46} ORF was created by using oligonucleotides KK2212 and KK2352. In parallel, the portion of \textit{eyfp} encoding the C-terminal sequence was amplified using oligonucleotides KK2231 and KK2215. In the second step, the two fragments were united in a PCR using oligonucleotides KK2212 and KK2215. The fusion construct was ligated in an expression vector under the control of the \textit{cgg1} promoter.

The pKK791 vector contains the \textit{trp-1} ORF (amplified by KK2333 and KK2334) cloned into the portion of \textit{eyfp} encoding the N-terminal sequence (amplified by KK2335 and KK2336) (31). The frame was corrected in an \textit{in vitro} deletion step using oligonucleotides KK2388 and KK2389. For eisosomal localization studies, we created vector pQ771, comprising the sequence encoding the \textit{N. crassa} PILA homolog (NCU07495) amplified by KK2347 and KK2348 and fused to \textit{trp} amplified by KK2350 and KK2349 (32). The expression of this fusion construct was controlled by the \textit{cgg1} promoter. The final frame was corrected by \textit{in vitro} deletion using JQ2413 and JQ2414.

Three additional expression vectors with \textit{eyfp} fusion constructs under the control of the \textit{cgg1} promoter were also created. Vector pCB779 contains the 0.5-kb alternative splicing product of \textit{bem46}, amplified using oligonucleotides KK2433 and KK2434. The gene sequence encoding the MTR homolog in \textit{N. crassa} (NCU006619.5) was generated by PCR with oligonucleotides CB2562 and CB2563, using \textit{N. crassa} genomic DNA as the template. The restriction sites required for directed ligation into the final vector, pCB794, were added in a subsequent PCR with oligonucleotides CB2508 and CB2514.

**Construction of the \textit{N. crassa} \textit{Δbem46} mutant.** A \textit{Δbem46} mutant strain was generated according to previously described procedures (33). The construction of the vector is described in Fig. S1 in the supplemental material. The final vector carries the full-length \textit{hph} ORF under the con-
control of the *Aspergillus nidulans* trpC promoter (amplified by FK547/FK548) and the *Neurospora crassa* arg2 terminator (amplified by FK541/FK542), flanked by 1.0- to 1.3-kb fragments downstream and upstream of the bem46 genomic DNA sequence (amplified by AS944/AS945 and AS946/AS947, respectively). The resulting vector, pHS606, was transformed into *Neurospora crassa* strains FGSC 9718 and FGSC 9719 by electroporation according to standard protocols (29). Transformants were selected on Vogel’s minimal medium with 5.8 mM saccharose, supplemented with 200 μg/ml hygromycin B. Homokaryotic bem46 strains were obtained by isolation of single microconidia and were tested for homologous single-copy integration of the transformation cassette by Southern blot hybridization. Homokaryotic transformants free of the mus mutation were generated by crossing with the histidine auxotrophic strains FGSC 6103 and FGSC 9716.

**Sequence analysis.** All sequence analyses were performed by Eurofins MWG Operon (Ebersberg, Germany) and GATC Biotech (Constance, Germany).

**Yeast two-hybrid assay.** To identify proteins putatively interacting with BEM46, we applied the yeast two-hybrid approach (34, 35) by using Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA) according to standard protocols. The following yeast strains were utilized: *Saccharomyces cerevisiae* AH109 (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ lys2::GAL1 UAS-GAL1 TATA-HIS3 GAL2 UAS-GAL2 TATA-AD2 ura3::MEL1 UAS-MEL1 TATA-lacZ) and *Saccharomyces*

![FIG 1](image)

**FIG 1** Ascospore germination in different *N. crassa* strains. Germination rates are expressed in relation to that of the wild-type (wt) strain (100%). wt, FGSC 6013 × FGSC 9016; OE, overexpression transformant strain; ko, knockout mutant strain; RNAi, RNAi-directed knockdown transformant strain; 0.5 kb, transformant strain containing the 0.5-kb alternative splicing product of bem46; 1.2 kb, transformant strain containing the 1.2-kb alternative splicing product of bem46. Strains were crossed with histidine auxotrophic strains FGSC 6103 and FGSC 9716. Statistical analysis was accomplished with SigmaPlot, version 12. Two asterisks indicate a *P* value of <0.01.

![FIG 2](image)

**FIG 2** Alternative splicing of bem46 in *N. crassa*. (A) RT-PCR amplification of different alternatively spliced bem46 fragments. cDNAs from different strains and tissues were used as the templates. In addition to the full-length (0.9-kb) cDNA sequence, two alternative fragments (1.2 kb and 0.5 kb [indicated by black and white arrows, respectively]) were amplified using bem46-specific oligonucleotides. myc., mycelium; mcon., macroconidia; wt, wild-type strain; RNAi, bem46 knockdown strain. (B) Schematic presentation of different spliced products of bem46. The bem46 genomic DNA sequence consists of four exons divided by three introns (DNA). In most cases, splicing resulted in a 0.9-kb mRNA fragment (spliced mRNA). Two other bem46 mRNA variants were generated by alternative splicing, i.e., 1.2-kb mRNA (which retains intron 1) and 0.5-kb mRNA (using the 5′ alternate splice site of intron 3), in the RNAi line. Gray rectangles, exons; black rectangles, introns. The alternate intron 3 resulting from the use of an alternate 5′ site is represented by a black rectangle with a white border.
*Saccharomyces cerevisiae* Y187 (MATa ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δ metα gal80Δ URA3::GAL1UAS-GAL1TATA-lacZ) (both from Clontech, Mountain View, CA). Figure S1 in the supplemental material depicts the vectors. The bait vector (pEH646) was generated by cloning the bem46 cDNA (which was amplified by oligonucleotides EH1132 and EH1133) into the pGBKT7 vector (Clontech, Mountain View, CA) in frame with the GAL4 DNA-binding domain. The prey vector carried an *N. crassa* cDNA bank (provided by S. Seiler, Göttingen, Germany) cloned into the pGADT7 vector (Clontech, Mountain View, CA), containing the GAL4 transcription activation domain.

Yeast transformation was performed by electroporation. First, 50 μl of an electrocompetent *Saccharomyces cell* suspension was mixed with 100 ng DNA, and the mixture was kept on ice for 5 min. Subsequently, the cells were transferred to a cold electroporation cuvette (Gene Pulser cuvette; Bio-Rad, Munich, Germany), and transformation was performed in a Gene Pulser system (Bio-Rad, Munich, Germany) at 200 μF and 25 μF with 1.5 kV. Directly after transformation, 1 ml of 1 M sorbitol at 4°C was added, and the mixture was incubated at room temperature for 5 min. Finally, 100 μl of the sample was plated on a solid medium, and the plates were incubated at 30°C until colony development.

Putative positive colonies were identified on selective minimal medium (SD/-Ade/-His/-Leu/-Trp), and the putative interaction was confirmed using the β-galactosidase assay. Colonies were plated on selective minimal medium supplemented with 80 mg/liter 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-β-Gal). After incubation at 30°C for 8 days, blue colonies were further tested for their β-galactosidase activity in a colony lift filter assay (36).

Plant material, growth conditions, and GUS assay. The β-glucuronidase (GUS) assay was performed with transgenic 5-day-old *Arabidopsis thaliana* (ecotype Col-0) seedlings carrying the synthetic auxin response element DR5 (37) coupled to the GUS reporter gene. In these plants, the glucuronidase activity is auxin dependent. Seeds were sterilized with 96% ethanol according to the standard procedure (38). Plants were grown on solidified half-strength MS nutrient medium (39) containing 1% sucrose.
RESULTS

**bem46 knockout mutant.** We previously demonstrated the localization of *Neurospora crassa* BEM46 protein in the ER and in areas near the plasma membrane, as well as the loss of ascospore germination in *bem46* RNAi and *bem46*-overexpressing transformants (20). Here, to confirm the *bem46* mutant phenotype, we established a *bem46* knockout strain by replacing the *bem46* gene with the *hph* gene, carrying hygromycin B resistance, using *mus-51* and *mus-52* mutants provided by the Fungal Genetic Stock Center. Figure S2 in the supplemental material shows that we successfully generated several *bem46* knockout strains. These knockouts exhibited normal vegetative growth and produced micro- and macroconidia. The rate of ascospore germination was somewhat lower than that in the wild type (Fig. 1); however, the phenotype was much weaker than that of the RNAi or overexpressing transformant, neither of which exhibits ascospore germination.

**Alternative splicing of the *bem46* transcript.** This unexpected discrepancy between the knockout mutant and the RNAi and overexpression transformants could have been caused by nonspecific downregulation of some other RNA, but there was no evidence of any such occurrence (20). It was also possible that alternate RNAs had accumulated in the RNAi transformant, since alternative splicing has been reported for a number of *N. crassa* transcripts (43). To investigate this possibility, we performed RT-PCR with oligonucleotides located at the beginning and end of the *bem46* open reading frame in order to generate full-length cDNA. RT-PCR amplification from the wild-type and RNAi strains was performed using RNA isolated from mycelium and macroconidia. The amount of the full-length *bem46* amplicon was somewhat reduced in the RNA from mycelium, and strongly reduced in the RNA from macroconidia, for the RNAi transformant (Fig. 2A). More importantly, both RNA samples from the RNAi transformant produced additional amplicons, including a 1.2-kb amplicon, slightly larger than that from the wild type, and, in one sample, a smaller amplicon of about 0.5 kb.

These amplicons were eluted from the gel and were sequenced. Figure 2B presents a schematic view of the sequencing data, which provided direct evidence of alternative splicing. The small (0.5-kb) amplicon resembled an alternatively spliced form that was missing exon 3. The larger (1.2-kb) transcript still contained the first intron, suggesting a case of intron retention, a frequent alternative splicing event in fungi (43). Neither transcript would encode the full-length *bem46* protein. Assuming that translation was initiated at the same AUG start codon used for translation in the wild-type sequence, the 1.2-kb mRNA could encode a 40-amino-acid truncated BEM46 protein, and the 0.5-kb mRNA could encode a 123-amino-acid truncated BEM46 protein. Both truncated proteins would include the same N terminus as that in the 320-amino-acid truncated BEM46 protein, and the 0.5-kb mRNA could encode a 0.5-kb mRNA could encode a 0.5-kb DNA probe.

**Indole extraction and quantitative analysis.** To determine indole production, fungi were cultivated in Vogel’s minimal liquid medium supplemented with 0.5 mM tryptophan (incubation at 25°C and 180 rpm in darkness). Samples were collected at various time points and were either centrifuged at 7,000 × g for 15 min or filtered through one layer of Whatman paper to remove fungal residue. The supernatant was collected, and the pH was adjusted to 2.8 with 10% HCl.

The total indole content was determined quantitatively by using the Salkowski method (40, 41). In a light-protected tube, 500 μL Salkowski reagent was mixed with an equal volume of the collected supernatant. The probes were incubated at 30°C for 15 min, and the absorbance at 540 nm was then determined. Standard curves were prepared from serial dilutions of a 100 mM IAA stock solution.

Indoles were qualitatively analyzed by thin-layer chromatography (TLC). After pH adjustment, aliquots of the supernatant were extracted by the addition of a double volume of ethyl acetate, followed by vigorous shaking for 10 min. After phase separation, the ethyl acetate fraction was collected in light-protected tubes, the solvent was evaporated, and the solid indole-containing residue was dissolved in 30 μl methanol. Samples were spotted onto silica gel plates (TLC Silica gel 60 F254; Merck, Darmstadt, Germany) and were developed with an ethyl acetate-isopropanol-ammonia solution (45:35:20). Subsequently, the plates were dried, stained with Ehrmann’s reagent (42), and heated to 90°C until spots were clearly visible. We applied 1 mM (each) IAA and tryptophan solutions as standards.

**FIG 5** Bimolecular fluorescence complementation assay. (A) *N. crassa* transformant strain overexpressing the BEM46 protein coupled with the C-terminal portion of eYFP. (B) *N. crassa* transformant strain overexpressing the putative interaction partner (anthranilate synthase [AS]; identified by the yeast two-hybrid approach) fused to the N-terminal portion of eYFP. (C) Macroconidia of *N. crassa* strains overexpressing both the BEM46::eYFP-C and AS::eYFP-N vectors. (i) CLSM images; (ii) calculated bright field images.
those of the wild-type, bem46 RNAi, bem46-overexpressing, and bem46 knockout strains. Expression of each type of truncated BEM46 caused complete loss of ascospore germination (Fig. 1), suggesting a possible inhibitory effect of truncated BEM46, which could be due to incorrect cellular localization (see below) or to a wrong or incomplete structure.

BEM46 colocalizes with the eiososomal PILA homolog protein. BEM46 localizes to the perinuclear ER and to spots near the plasma membrane (20), which are not actin patches (46). Microscopic analyses showed that these spots did not re-form within 15 min of investigation (Fig. 3A). Fungal eisosomes are reportedly stable protein complexes connected to the plasma membrane (47). Thus, we investigated the potential colocalization of BEM46 with the Neurospora crassa homolog of PILA (NCU07495), which has been described as an eiosomal core protein in Aspergillus nidulans (48). After identification using a bioinformatics approach, the full-length coding sequence of the PILA homolog was coupled to the reporter gene construct. A heterokaryon comprising a transformant expressing BEM46 coupled to eGFP was investigated by confocal laser scanning microscopy. The BEM46::GFP reporter gene construct showed colocalization with the PILA::tRFP protein in germinating macroconidia of N. crassa (Fig. 3B).

Subcellular localization of the truncated BEM46 protein encoded by the 0.5-kb alternatively spliced fragment. We also analyzed the subcellular localization of the truncated protein encoded by the small (0.5-kb) alternative splicing product of bem46. To this end, the cDNA fragment was joined to the egfp reporter gene under the control of the ccg1 promoter. The full-length BEM46 protein contains an unusual ER retention signal (1), which leads to the localization of the full-length protein to the perinuclear ER. The alternative splicing event results in a 123-amino-acid protein lacking the amino acids for the retention signal. The confocal microscopic image in Fig. 3C shows the subcellular localizations of the 0.5-kb::eGFP and PILA::tRFP proteins. As expected, the small peptide did not localize to the perinuclear ER but was found in several small spots close to the plasma membrane, similar to the spots observed for the full-length BEM46.
However, most of these patches showed no colocalization with the eisosomal PILA protein.

**Anthranilate synthase component II is an interaction partner of BEM46.** Using yeast two-hybrid analysis, we identified about 100 proteins specifically interacting with BEM46 (Fig. 4). Plasmid isolation and sequencing revealed that all plasmids analyzed contained one of four cDNA fragments from the same trp-1 gene sequence, encoding anthranilate synthase component II (49). Among these four cDNAs (Fig. 4B), the two smaller ones encoded only the F domain, which resembles an N-(5'-phospho-ribosyl)anthranilate isomerase. Since these cDNAs enabled a specific two-hybrid interaction with the BEM46 protein, it is likely that the F domain is sufficient for protein-protein interaction with BEM46. A bimolecular fluorescence complementation assay (31) was used to confirm the in vivo interaction of BEM46 and anthranilate synthase (Fig. 5).

**The Δtrp-1 mutant.** Our results described above indicated a link between BEM46 and the tryptophan biosynthesis pathway. Tryptophan reportedly may act as a signal molecule and inhibit conidial anastomosis tube fusion in *N. crassa* (50). It is also an important precursor for secondary metabolites and plant hormones, such as auxin (51, 52), which is produced both in plants and in fungi (53).

Further investigation revealed that the Δtrp-1 strains (FGSC 20870 and FGSC 20871) showed single ascospores developing no directed hyphae but rather growing bubble-like structures, similar to those observed in the bem46 RNAi line (20), at the point of germination (Fig. 6A, arrow). The rate of germination in the Δtrp-1 strains was reduced to about 50% of that in the wild-type ascospore (Fig. 6C), with conidia germinating earlier and building longer young hyphae. Since indole acetic acid reportedly promotes conidial germination and the elongation of young hyphae (54–57), we tested whether the observed phenotypic effect might be caused by auxin. In the medium, we determined the indole content released by germinating conidiospores. Indeed, the Δtrp-1 mutants produced 10-fold-larger amounts of indoles than the wild-type strain when the medium was supplemented with 0.5 mM tryptophan (Fig. 6E). Determination of the indole content produced by ascospores revealed no differences between the Δtrp-1 and wild-type strains (Fig. 6D).

**A pathway for indole production in N. crassa.** Auxin production has long been known to occur in filamentous fungi (58, 59) and has also been reported recently in symbiotic and phytopathogenic fungi (see, e.g., reference 60). Several pathways have been suggested for auxin production in plants, but not all are confirmed (52). The most studied such pathway is the indole-3-pyruvic acid (IPA) pathway (61), in which the main enzymes are tryptophan aminotransferase (TAM), indole-3-pyruvate decarboxylase (IPD), and indole-3-acetaldehyde dehydrogenase (IAD). Previous studies (58, 62) have shown the presence of TAM1 and IAD1 in *Ustilago maydis*. Here we used bioinformatic tools to identify the entire set of ortholog genes for the IPA pathway in *N. crassa*: NCU09166.7 (tam1), NCU02397.7 (ipd), and NCU03415 (iad1) (see Fig. S3 in the supplemental material).

**Regulation of transcription of the auxin biosynthetic pathway in the bem46 transformant and mutant strains of N. crassa.**

![FIG 7 qRT-PCR results showing the relative levels of expression of the three genes involved in a putative auxin biosynthesis pathway of *N. crassa*, evaluated in different bem46 transformant and mutant strains. The transcript quantities were calculated relative to that of the housekeeping gene *tub2*. NcT288, bem46-overexpressing strain; NcT289, bem46 RNAi strain; NcT411, bem46 knockout strain; NcT419, strain overexpressing the 0.5-kb alternatively spliced bem46 fragment.](http://ec.asm.org/)
We investigated the expression of *tam1*, *ipd*, and *iad1* in the wild-type, *bem46* mutant, and different transformant strains. Figure 7 shows the quantitative RT-PCR results for the mRNAs of these genes. The first gene of the pathway (*tam1*) was significantly downregulated in the *bem46* knockout (NcT411) and knockdown (NcT289) strains. Interestingly, overexpression of the 0.5-kb alternatively spliced fragment (NcT419) had the same effect. The *bem46*-overexpressing line (NcT288) showed no altered regulation of the *tam1* gene but was the only strain to show altered regulation of the *ipd* gene (3-fold upregulation). The last gene of the pathway, *iad1*, was upregulated in all strains investigated, except for the line expressing the 0.5-kb fragment. Expression of *iad1* was more than 10-fold higher in the *bem46*-overexpressing strain.

Indole production by the *bem46* transformant and mutant strains compared to that by the wild type. Figure 8 shows data indicating auxin production in *N. crassa*, which had been observed previously as well (63). Transgenic *Arabidopsis thaliana* plants expressing the DR5::GUS fusion protein (37, 64) were incubated either with water (Fig. 8A), with 100 μM IAA (Fig. 8B), or with germinating wild-type ascospores (Fig. 8Ci and ii). Seedlings incubated with either IAA or germinating ascospores showed positive staining for β-glucuronidase in cotyledon tips. Because the expression of the fusion protein is auxin dependent, our data provide indirect evidence for auxin production by germinating ascospores. Next, auxin was detected directly by thin-layer chromatography (Fig. 8D). Figure 9 shows the concentrations of auxin from germinating macroconidia and germinating ascospores in growth medium. Auxin concentrations did not differ significantly in 4-day-old mycelium, but we observed significant differences between auxin concentrations in the germinating macroconidia of different strains. Overexpression of the full-length *bem46* gene under the control of the *cgl*1 promoter did not increase the auxin concentration over that in the wild type; however, the *bem46* knockout and RNAi lines showed significantly increased auxin concentrations. Auxin production during ascospore germination was also investigated (Fig. 9B). No significant differences from production by the wild type were observed for the *bem46* knockout and RNAI strains, but the overexpression line showed reduced indole production by germinating ascospores.

Investigation of indole production by germinating macroconidia also revealed differences in the time flow of macroconidial germination and in the rate of hyphal elongation. The micrographs in Fig. 9C show that most wild-type macroconidia germinated after 5 h, while the macroconidia of the *bem46*-overexpressing line germinated later. Macroconidia from the *bem46* knockout strain germinated within 5 h and produced longer germ tubes than the wild type. Determination of the growth rate of young hyphae within the first 4 days after germination (Fig. 9D) clearly showed strongly inhibited hyphal elongation in the *bem46* overexpression strain. In contrast, the knockout and RNAI strains showed growth rates somewhat higher than that of the wild type.

The MTR protein of *N. crassa*: a specific tryptophan transporter. The H⁺-driven tryptophan and tyrosine permease TAT2 (YOL020W) of *Saccharomyces cerevisiae* is reportedly localized in eisosomes (65). MTR (NCU06619) is a neutral amino acid transport protein (66, 67) that we identified as the TAT2 homolog in *N. crassa*. We used qRT-PCR to analyze MTR expression in the different *bem46* transformant and mutant strains. Figure 10A shows 4-fold upregulation of the gene in the *bem46* overexpression strain (NcT288) relative to the wild type. The subcellular localization of MTR was studied using a trfp reporter gene construct. Figure 10B shows the localization of the protein in the perinuclear ER and in several additional small spots. The trFP-coupled MTR protein colocalized with the BEM46::eGFP protein in swollen and germinating macroconidiospores and in mature hyphae.

**DISCUSSION**

BEM46 interacts with anthranilate synthase. Here we identified the anthranilate synthase encoded by the *trp-1* gene (49, 68) as an interaction partner of BEM46 in *N. crassa*. This result was unexpected, because in other model organisms, BEM46 interacts with proteins involved in developing/maintaining polar growth (9, 12, 69). Phenotypic analyses of the *N. crassa trp-1* mutant showed reduced ascospore germination, with spores developing the same loss-of-polarity phenotype as that described for the Δ*bem46* mutant (20). These results suggested a situation similar to that in the...
model organism A. thaliana, where mutations in the asal gene encoding anthranilate synthase result in a wavy root growth phenotype (70). The same phenotype was observed in the bem46 homolog (wavy growth 2) knockout mutant (12). However, IAA was unable to rescue the wavy root phenotype in A. thaliana (70); thus, we considered that BEM46 might influence polar growth through an effect on IAA biosynthesis in the fungus. We observed that conidiospores of the H9004 trp-1 mutant germinated earlier and developed longer young hyphae than those of the wild type. The same effects were reported previously for N. crassa following external addition of IAA (54, 57). Therefore, we investigated the auxin biosynthesis and indole production of the different N. crassa bem46 mutant and transformant strains.

Auxin biosynthesis in Neurospora crassa and its connection to BEM46. Although it is not widely known, auxin biosynthesis in fungi was reported many decades ago (59). These fungi include N. crassa (53, 63), and studies investigated the effects of externally applied auxins (56, 57). However, we discovered this information only after our present analyses of indole production in N. crassa wild-type and bem46 knockout, RNAi, and overexpression lines. Recent research has focused mainly on auxin production by phytopathogenic fungi, e.g., Ustilago maydis, Fusarium species, and Colletotrichum gloeosporioides (58, 60, 71–73). In these phytopathogenic fungi, auxin is believed to affect host plant growth. In N. crassa, IAA at a concentration of 10⁻⁶ M enhanced the conidial germination rate after 2 h of incubation (54). At higher concentrations, an inhibitory effect was shown. It has been reported previously (55) that IAA removes the “conidial density effect” (74), and 10⁻⁶ M auxin reportedly induces the elongation of young hyphae germinating from conidia, while higher concentrations are inhibitory (57).

Here, using bioinformatic tools, we have presented evidence for a tryptophan-dependent IAA biosynthesis pathway in N. crassa. We showed that the expression of the three genes involved in this pathway (tam1, ipd, and iad) was altered in the different bem46 transformants and mutant strains, indicating a connection between bem46 and auxin biosynthesis in the fungus. However, the effects of auxin and its connection to bem46 appear to differ in the different developmental stages of N. crassa. In germinating conidiospores, bem46 downregulation led to iad1 overexpression and higher indole production, resulting in earlier conidial germination and increased hyphal elongation. Interestingly, bem46 overexpression also led to very high iad1 expression. While indole content did not differ from that of the wild type, conidial germination was delayed and hyphal elongation strongly reduced,
which may result from negative regulation, since high levels of IAA are inhibitory.

We further detected colocalization of BEM46 with the neutral amino acid transporter MTR. BEM46 may act at the crossing point between tryptophan synthesis and uptake. Indeed, mtr expression was significantly higher in the bem46-overexpressing strain, which may lead to high internal levels of indole, resulting in the inhibitory effects described above. The indole production of vegetative mycelia of bem46 transformant and mutant strains did not differ significantly from that for the wild type; however, the expression of genes involved in the putative auxin biosynthesis pathway was affected. It is possible that indole content is additionally regulated by inactivation of the end product, as described for A. thaliana (75).

**Alternative splicing of bem46.** In multicellular organisms, alternative splicing is a mechanism by which gene expression is regulated on the mRNA level (76). Alternative splicing can affect the transcriptome both quantitatively and qualitatively, e.g., by degradation of alternatively spliced forms over the nonsense-mediated mRNA decay (NMD) pathway (77, 78). Such changes on the transcript level can potentially affect almost all areas of protein function (76). The transcript isoforms can be translated into various proteins with different sequences and/or domain arrangements (79). The resulting truncated proteins can act as dominant negative regulators of the authentic protein (80). Alternative splicing itself can be regulated by riboswitches or by different environmental cues (81, 82), thus making alternative splicing a flexible tool that allows adaptation to the environment (83). Alternative splicing also occurs in ascomycetes, though at a rate significantly lower than that in mammals (43). In N. crassa, 162 of 9,733 protein-coding genes exhibit evidence of alternative splicing. The most common form of alternative splicing in fungi is intron retention (43).

In the present study, we identified 0.5- and 1.2-kb alternatively spliced bem46 fragments that encoded truncated proteins of 123 and 40 amino acids, respectively. There are two main reasons to assume that these are alternative splicing products rather than splicing intermediates. First, if they were splicing intermediates,
the fragments would have been detected in the wild-type strains; this was not the case in our investigations. Second, the fragment sequences were not simple combinations of exons; the 1.2-kb product resulted from intron retention (the most common method of alternative splicing in fungi), while the 0.5-kb product showed a very unusual splicing pattern that did not match any splicing intermediate. The alternatively spliced fragments accumulated in the bem46 RNAi lines (20), which exhibited no ascospore germination. Since overexpression of either full-length or truncated BEM46 protein led to a loss of ascospore germination, it is possible that the alternatively spliced fragments (or rather, the translated truncated proteins) act as dominant competitors of the full-length protein. This would explain how overexpression of the small fragments leads to the same effect as overexpression of the full-length protein, i.e., the inhibition of ascospore germination.

It remains unknown how BEM46 may influence the mechanism of ascospore germination. Indole production was reduced in ascospores from strains overexpressing either the full-length or the truncated BEM46 protein. Hence, it is possible that BEM46 directly affects indole production, which negatively influences ascospore germination. However, indole production by the RNAi and knockout strains did not differ significantly from that by the wild type. With the knockout strain, a considerable number of ascospores will normally germinate, and even in the RNAi strain, one out of a few thousand ascospores may germinate (20). It is possible that indole production by these germinated spores could falsify the measurement of indole content. This may also explain the relatively high standard deviation found for ascospore germination. This may also explain the potential presence of several possible auxin biosynthetic pathways in N. crassa (P. Sardar, unpublished data), some of which may share the last aldehyde dehydrogenase, encoded by iad. The existence of different pathways resulting in the same final product may enable precise regulation of the internal auxin level, which is advantageous considering the fact that the effect of auxin is concentration dependent. Phytosphingosine treatment—which induces programmed cell death in N. crassa—strongly upregulates iad1 expression and slightly upregulates bem46 expression (84). This could suggest the involvement of the two gene products in general stress response reactions.

Germination and hyphal elongation are critical points in the life of an ascomycete and are strongly dependent on the variable environment of the fungus. Therefore, flexible and effective regulation of these processes is required. It appears possible that one aspect of this mechanism may include regulation of the internal indole level of the fungus, which results from uptake as well as intracellular production. Our present results indicate that BEM46 may act at the crossing point of this regulation.

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