Bgs2p, a 1,3-β-glucan synthase subunit, is essential for maturation of ascospore wall in *Schizosaccharomyces pombe*

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Abstract Previously we have reported that Drc1p/Cps1p, a 1,3-β-glucan synthase subunit, is essential for division septum assembly in *Schizosaccharomyces pombe*. In this report, we present evidence that *S. pombe* Bgs2p, a 1,3-β-glucan synthase that shows 56% identity to Drc1p/Cps1p, is essential for maturation of ascospore wall in *S. pombe*, but is not required for vegetative growth. Diploid cells homozygous for the *bgs2*-null mutation, as well as homothallic *bgs2*-null mutant haploids undergo meiosis normally. However, a 1,3-β-glucan containing spore wall is not assembled in these cells. The spores resulting from meiosis of a *bgs2*-null mutant lyse upon release from the ascus and are therefore inviable. Using a green fluorescent protein-tagged Bgs2p, we demonstrate that Bgs2p is localized at the periphery of the ascospores during meiosis and sporulation. However, Bgs2p is not detected in vegetative cells. We conclude that Bgs2p is required for 1,3-β-glucan synthesis during ascospore wall maturation.

Key words: Bgs2p; 1,3-β-Glucan synthase; Sporulation; Ascospore wall; *Schizosaccharomyces pombe*

1. Introduction

The polysaccharide 1,3-β-glucan is a major structural component of fungal cell wall that not only has a function in the strengthening of the cell wall but also plays a key role in morphogenesis and cell–cell communication. It has been demonstrated that 1,3-β-glucan is present in the inner portion of the wall as a fibrillar network that surrounds the *Schizosaccharomyces pombe* cell [1]. This fibrillar network structure is thought to be responsible for the mechanical strength of the cell wall. 1,3-β-Glucan is synthesized by 1,3-β-glucan synthases, the catalytic subunit of which are encoded by genes related to the *drc1/cps1* gene of *S. pombe* [2–4] and the FKS genes of *Saccharomyces cerevisiae* [5]. Proteins related to the *S. pombe* Drc1p/Cps1p have been identified in *S. cerevisiae*, *Aspergillus nidulans*, *Candida albicans* and several other fungi. From studies in *S. cerevisiae* and *S. pombe*, the GTPase Rho1p has been shown to be a key regulator of 1,3-β-glucan synthase function [6–9]. In addition, Pkc1p, a protein kinase C homolog, has also been shown to activate 1,3-β-glucan synthase activity in *S. cerevisiae* [10].

At least four genes encoding 1,3-β-glucan synthase catalytic subunits, *drc1*$^+$/*cps1*$^+$, *bgs2*$^+$ (for 1,3-β-glucan synthase, encoded in cosmid *C24C9*), *bgs3*$^+$ (encoded by the partially sequenced clone *AB027891*) and *bgs4*$^+$ (encoded in cosmid *C1840*), have been identified by various projects sequencing *S. pombe* genomic DNA and cDNAs. Of these, *drc1/cps1* is required specifically for division septum assembly but not for cell wall assembly during growth in interphase [2–4]. The presence of three more genes encoding 1,3-β-glucan synthase isoforms suggests that each might perform a distinct function. Alternatively, even though Drc1p/Cps1p is important only for septation, it is possible that the other three isoforms perform overlapping roles in cell wall assembly during cell elongation. To address these issues, we have undertaken a study of the function of each of these 1,3-β-glucan synthase isoforms. In this paper, we report the characterization of Bgs2p, which is dispensable for vegetative growth and is not required for cell elongation or division septum assembly. *bgs2*-null mutants, however, fail to assemble a 1,3-β-glucan containing spore wall following meiosis, resulting in the formation of inviable spores. The intracellular localization of Bgs2p supports its role in spore wall assembly.

2. Materials and methods

2.1. Yeast strain construction

Fission yeast was grown on YES medium or EMM with appropriate supplements as previously described [11]. For sporulation, diploid cells or h^{90} haploids were grown on YPD plates or EMM-N liquid. Bacterial growth media were as described [12]. Strains used in this study were constructed as follows. A 10.5 kb DNA fragment harboring *bgs2*$^+$ gene was liberated by *Sac*I digestion of cosmid *SPAC24C9* and ligated onto pHL plasmid to yield pHL-*bgs2*$^+$ plasmid (Fig. 1). About 500 bp upstream and downstream sequences of *bgs2* open reading frame (ORF) were synthesized by polymerase chain reaction (PCR) using sequence-specific primers. Two PCR fragments were subsequently cloned at either side of a *ura4*$^+$ gene in a pBluescript vector to yield a pBS-*bgs2*-null::*ura4*$^+$ plasmid (Fig. 2A). The *bgs2*-null strain was constructed using the one-step replacement method by transformation of *Bam*HI and *Xho*I double-digested pBS-*bgs2*-null::*ura4*$^+$ DNA. Four out of 20 colonies were positive in a PCR test indicative of successful replacement of one of the two copies of *bgs2* with *ura4* (data not shown). A 1 kb PCR fragment containing the 3′-end of *bgs2* ORF sequences (stop codon omitted) fused with the *egfp* gene was cloned into an integration vector, pJK210, to yield a pJK-*bgs2tail::egfp* plasmid (Fig. 1). Strains containing a chromosomal copy of *bgs2*$^+$*::egfp* were constructed by transformation of *Sph*I-digested pJK-*bgs2tail::egfp* DNA. A homozygous *bgs2*-null/*bgs2*-null diploid strain was obtained by crossing opposite mating type strains of haploid *bgs2*-null mutants. A homothallic *bgs2*-null strain was obtained by crossing a heterothallic *bgs2*-null strain to a homothallic *bgs2*$^+$ strain.

2.2. Microscopy

Fluorescence microscopy was used essentially as described previously [13]. Formaldehyde fixation was used for visualization of DNA using DAPI and 1,3-β-glucan using aniline blue. For transmission electron microscopy (EM), cells were first fixed in 2.5% glutaral-

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dehyde/phosphate-buffered saline pH 7.2 and then post-fixed in 1% osmium tetroxide. Subsequently, cells were dehydrated through graded ethanol and embedded in Spurr resin. Ultra thin sections were cut, mounted on copper grids and stained with saturated aqueous uranyl acetate. Sections were viewed and photographed using a JEOL transmission electron microscope at 100 kV.

3. Results

3.1. Bgs2p, a 1,3-β-glucan synthase subunit, is dispensable for vegetative growth

Bgs2p (*SPAC24C9.07C*) was identified upon searching the databases of the *S. pombe* genomic DNA and cDNA sequencing projects for Drc1p/Cps1p-like sequences. Bgs2p was also related to two other sequences related to 1,3-β-glucan synthase subunits, which we refer to as Bgs3p (*AB027891*) and Bgs4p (*SPCC1840.02C*). The primary amino acid sequence of Bgs2p shows 56% identity to that of Drc1p. To study the cellular role of Bgs2p, we constructed a strain bearing the *bgs2*-null mutation (see Section 2 and Fig. 1). The *bgs2*-null mutant did not show any vegetative growth defect under normal growth conditions. Furthermore, the *bgs2*-null mutant was not hypersensitive to cell wall degrading enzymes suggesting that Bgs2p likely did not play an overlapping role with other 1,3-β-glucan synthases in cell wall or division septum assembly (data not shown).

3.2. Bgs2p is essential for sporulation

Previous studies have shown that the *S. cerevisiae* Fks2p is required for sporulation [14]. We therefore tested if Bgs2p in *S. pombe* was required for mating as well as for proper sporulation. To address the first question, *bgs2*-null mutant cells of the opposite mating type were mixed and assessed for the ability to form diploid colonies, as formation of diploid colonies involves cell fusion. Diploid colonies were readily obtained in these crosses, suggesting that Bgs2p is also dispensable for mating of cells prior to meiosis. To address if Bgs2p was required for sporulation following meiosis, a diploid strain of the genotype *bgs2*-null/*bgs2*-null h^+/h^- was induced to undergo meiosis. Meiosis I and II occurred normally in the *bgs2*-null mutant diploid cells as judged by the observation of four nuclei in many cells (data not shown). Interestingly, however, the asci produced following meiosis of a *bgs2*-null/*bgs2*-null h^+/h^- strain were abnormal. Whereas spores obtained following meiosis of wild-type diploid cells are bright and spherical under bright field optics, spores obtained from *bgs2*-null/*bgs2*-null h^+/h^- cells were dull in appearance and were constructed of very weak looking walls. Furthermore, the spores were seldom spherical in shape (Fig. 2A). Spores obtained from *bgs2*-null/*bgs2*-null h^+/h^- cells lysed upon release from the ascus and therefore resulted in lethality of spores.

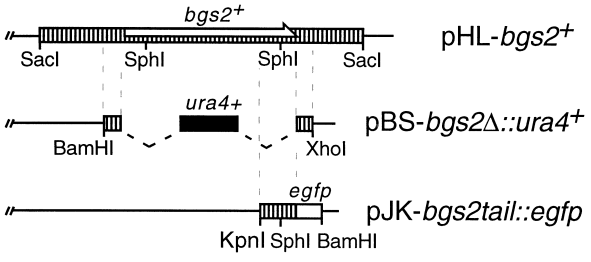

Fig. 1. DNA constructs used in this study. The pHL-*bgs2*$^+$ plasmid contains a 10.5 kb *Sac*I fragment from cosmid *SPAC24C9* in which a *bgs2* ORF resides. pBS-*bgs2*-null::*ura4*$^+$ and pJK-*bgs2tail::egfp* are used in construction of *bgs2*-null mutant and *bgs2::egfp* strains, respectively. Hatched boxes represent sequences of *Sac*I fragment and a residing *bgs2* ORF is indicated by an open arrow. Thin lines represent vector sequences. A solid box and an open box indicate *ura4* ORF and *egfp* ORF, respectively.

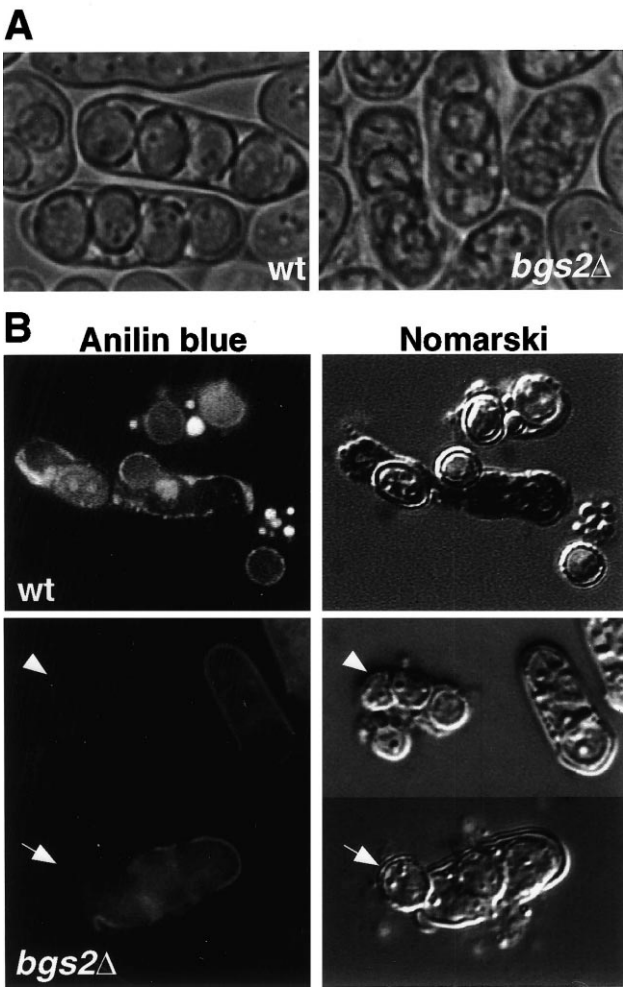

Fig. 2. Ascospores from the *bgs2*-null mutants are abnormal in morphology and lack 1,3-β-glucan. (A) A bright field image of ascospores from wild-type and *bgs2*-null mutant. Note that the spores in wild-type are bright and spherical compared to the withered looking *bgs2*-null mutant spores. (B) Aniline blue staining of wild-type and *bgs2*-null ascospores. Arrows point to *bgs2*-null spores devoid of aniline blue staining.

To further characterize this sporulation defect, we stained sporulated *bgs2*-null/*bgs2*-null h^+/h^- diploid cells and sporulated wild-type diploid cells with aniline blue, a compound that specifically detects 1,3-β-glucans (Fig. 2B). Whereas aniline blue-positive material was detected in the periphery of spores produced from wild-type cells, such staining was not observed in *bgs2*-null/*bgs2*-null h^+/h^- cells (Fig. 2B). Interestingly, aniline blue reactive material was detected on the ascus walls of both strains. Identical results were obtained when homothallic haploid *bgs2*-null cells that were allowed to proceed through meiosis following mating were stained with aniline blue (data not shown). Thus, Bgs2p is essential for assembly of 1,3-β-glucan on the spore wall but not for assembly of 1,3-β-glucan on cell walls or ascus walls.

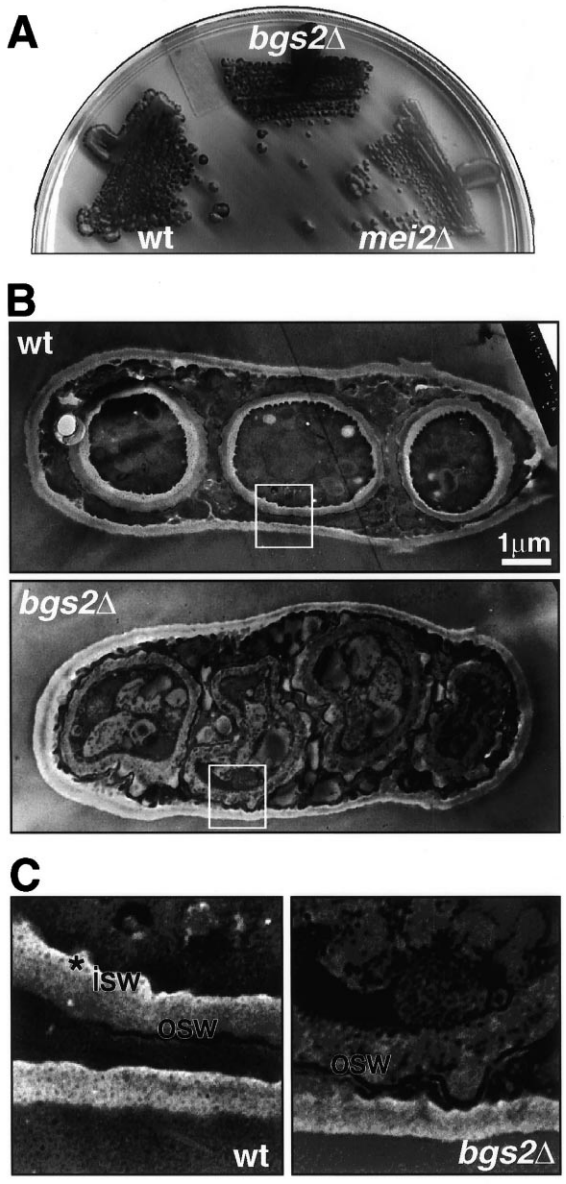

Fig. 3. (A) *bgs2*-null spores react positively to iodine vapor staining. Ascospores generated from sporulation-defective mutants, such as *mei2*$^-$, *mes1*$^-$ and *spo5*$^-$ (not shown), are iodine-negative. But *bgs2*-null spores show dark brown staining to iodine similar to wild-type. (B) No 1,3-β-glucan in inner wall of *bgs2*-null spores. EM images of asci from wild-type and *bgs2*-null mutant are shown. Three out of four spores in the wild-type ascus are shown in this section. The electron-light material in the inner spore wall is present in wild-type spores, but absent in *bgs2*-null mutant spores. (C) Magnified sections from image (B). **isw** and **osw** stand for inner and outer spore wall, respectively. The asterisk marks the inner spore wall.

3.3. The bgs2 mutant lacks inner spore wall

Previous studies have shown that *S. pombe* ascospores are stained dark brown by iodine vapor. Cells defective in genes that are required for sporulation have been isolated by screening for mutants that fail to stain with iodine vapor [15]. Interestingly, the spores that were generated from the *bgs2*-null/*bgs2*-null h$^+$/h$^-$ mutant exhibited positive dark brown staining with iodine, which was unique among other known sporulation-defective mutants, such as *mei2*$^-$, *mes1*$^-$ and *spo5*$^-$, which showed negative staining to iodine (Fig. 3A, data not shown). It has been suggested that the amylose-like rich outer spore wall was responsible for the dark brown staining with iodine [16]. Therefore, we examined the fine structure of *bgs2*-null/*bgs2*-null h$^+$/h$^-$ spore wall by EM analysis. The spore wall of wild-type spores appeared to have both electron-light and -dense materials (Fig. 3B,C). The electron-light material represents the inner spore wall and contains 1,3-β-glucan, whereas the electron-dense material represents the outer spore wall that contains amylose-like material (Fig. 3B,C, see asterisk). In contrast, only the electron-dense amylose-like material was observed in *bgs2*-null/*bgs2*-null h$^+$/h$^-$ spore walls consistent with its staining positive with iodine vapor (Fig. 3B,C). Thus, loss of Bgs2p function prevents assembly of the 1,3-β-glucan containing inner spore wall.

3.4. Bgs2p is localized to the periphery of the ascospores

The localization of Bgs2p was examined utilizing an enhanced green fluorescence protein (eGFP) fusion to Bgs2p.

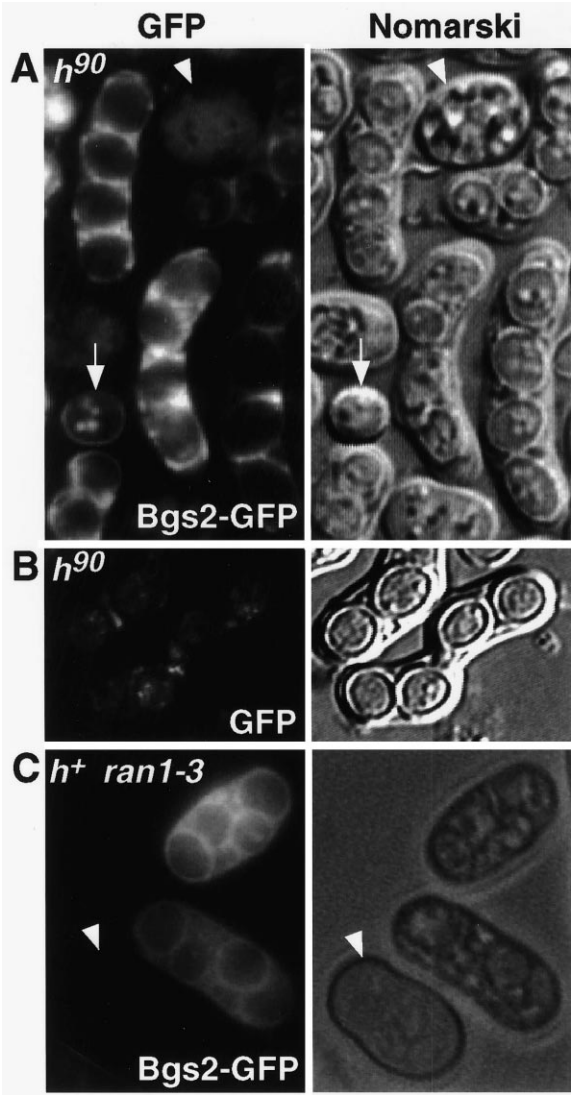

Fig. 4. Bgs2p is localized to the periphery of zygotic and azygotic ascospores. (A) GFP and Normaski images of a homothallic strain containing a chromosomal copy of *bgs2*$^+$*::egfp* to visualize Bgs2-GFP. (B) A homothallic strain containing pREP42GFP used as a control. (C) Localization of Bgs2-GFP in azygotic asci produced from a *ran1*-3 mutant. Arrows indicate non-sporulating cells with no GFP localization and the arrowhead indicates a free spore with a peripheral localization of GFP.

A homothallic strain that contained an eGFP C-terminal tagged chromosomal copy of *bgs2*$^+$ gene was constructed (see Section 2, Fig. 1). Bgs2p-eGFP is functional since cells bearing Bgs2p-eGFP produced viable spores that were normal in morphology. Homothallic *bgs2*$^+$*::egfp* strains produced ascospores that exhibited GFP signal outlining the spore periphery (Fig. 4A). Expression of GFP alone in homothallic wild-type cells did not result in similar staining of the spore wall periphery (Fig. 4B), establishing the authenticity of localization of Bgs2p-GFP to the periphery of the spore. Homothallic *S. pombe* cells produce zygotic asci, due to the fusion of cells and nuclei. To assess the localization of Bgs2p-GFP following azygotic meiosis, we introduced Bgs2-GFP into a *ran1*-3 mutant strain [17], which initiates meiosis without cell fusion upon temperature shift to the restrictive temperature. The *ran1*-3 mutants expressing Bgs2p-GFP were shifted to the restrictive temperature and the localization of Bgs2p assessed. Again, Bgs2p-GFP was detected on the spore periphery in these azygotic asci (Fig. 4C). Thus, Bgs2p is a component of the spore periphery. Given that Bgs2p contains several transmembrane domains, it is likely that Bgs2p is indeed a component of the ascospore membranes which lie below the ascospore walls.

4. Discussion

1,3-β-Glucan is a major polysaccharide component of fungal cell/spore wall and division septum. Based on the data from the *S. pombe* genomic DNA and cDNA sequencing projects, at least four genes encoding 1,3-β-glucan synthase subunits have been identified. Of these, Drc1p/Cps1p has been shown to be essential for division septum assembly [3,4]. In this study, we demonstrate that Bgs2p is essential for the development of the ascospore wall but not for the assembly of cell wall during cell elongation and cell division. Currently, the functions of Bgs3p and Bgs4p are unknown. It is likely that these proteins are important for cell elongation.

Consistent with its role in ascospore development, Bgs2p localizes to the periphery of the ascospores (Fig. 4). Future studies should precisely establish if Bgs2p is a component of the ascospore membrane and the role it performs in directing ascospore wall maturation. No Bgs2p signal is detected in vegetative cells, which is consistent with the fact that Bgs2p is dispensable for mitotic growth. We have demonstrated here that mutant spores are not stained with aniline blue, indicating the lack of 1,3-β-glucan in the mutant spore wall (Fig. 2). EM analysis shown in Fig. 3 has revealed that the *bgs2*-null mutant spores contain only the outer spore wall and lack the inner wall which is composed of 1,3-β-glucan [18]. Whelan and Ballou have previously shown that *gfa1* mutants, which are defective in glucosamine fructose-6-phosphate aminotransferase, generate spores without outer spore wall in *S. cerevisiae* [19]. It is likely that the *S. pombe* homolog of *S. cerevisiae GFA1* (58% identity between the two proteins) would also be required for the development of outer spore wall, since the sporulation processes are similar in both yeasts.

The GTPases of the Rho family are known regulators of the activity of 1,3-β-glucan synthases in both *S. cerevisiae* and *S. pombe*. Currently, it is unclear if Rho-like molecules specific for the regulation of sporulation exist in *S. pombe*. It is also interesting to note that Rho proteins are modulators of the actin cytoskeleton. Thus, analysis of the role of Rho proteins in sporulation might also reveal a role for F-actin in sporulation.

Nutrient deprivation initiates meiosis and sporulation in *S. pombe*. At anaphase of meiosis II, the morphogenic transformation of the spindle pole body (SPB) is thought to be essential for initiation of spore formation. Mutations that affect SPB re-structuring are known to abolish spore formation [20]. Mutants of *spo4*, *spo15* and *spo18* undergo meiosis I and II, however, the SPB morphogenic transformation fails to occur resulting in an inability to form the forespore membrane. The forespore membrane grows from the SPB by fusion of vesicles and eventually encloses the divided nuclei. We propose that Bgs2p acts at this step to allow assembly of the inner spore wall containing 1,3-β-glucan. Future studies should assess the detailed mechanisms that coordinate the assembly of the inner and outer spore walls and their regulation in the meiotic cell cycle.

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