GFP as a tool to analyze the organization, dynamics and function of nuclei and microtubules in *Neurospora crassa*

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Abstract

We report the construction of a versatile GFP expression plasmid and demonstrate its utility in *Neurospora crassa*. To visualize nuclei and microtubules, we generated carboxy-terminal fusions of sgfp to *Neurospora* histone H1 (\(hH1\)) and \(\beta\)-tubulin (Bml). Strong expression of GFP fusion proteins was achieved with the inducible *Neurospora ccg-1* promoter. Nuclear and microtubule organization and dynamics were observed in live vegetative hyphae, developing asci, and ascospores by conventional and confocal laser scanning fluorescence microscopy. Observations of GFP fusion proteins in live cells largely confirmed previous results obtained by examination of fixed cells with various microscopic techniques. H1-GFP revealed dynamic nuclear shapes. Microtubules were mostly aligned parallel to the growth axis in apical compartments but more randomly arranged in sub-apical compartments. Time-lapse imaging of \(\beta\)-tubulin-GFP in germinating macroconidia revealed polymerization and depolymerization of microtubules. In heterozygous crosses, H1-GFP and \(\beta\)-tubulin-GFP expression was silenced, presumably by meiotic silencing. H1-GFP was translated in the vicinity of \(hH1^{+}\)-sgfp\(^{+}\) nuclei in the common cytoplasm of giant Banana ascospores, but it diffused into all nuclei, another illustration of the utility of GFP fusion proteins.

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Index Descriptors: Green fluorescent protein; \(\beta\)-Tubulin; Microtubules; Histone H1; Meiotic silencing by unpaired DNA; Ascus development; Confocal microscopy

1. Introduction

Fluorescent proteins are powerful tools that have revolutionized cell biology over the last decade, particularly in studies of living cells. Since the initial report of heterologous expression of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* in *Escherichia coli* (Chalfie et al., 1994), GFP and other fluorescent proteins have been adapted (Tsien, 1998; Zimmer, 2002) for use in many experimental systems, including numerous species of fungi (Bourett et al., 2002; Lorang et al., 2001). Genetically encoded fluorescent proteins are used in a wide variety of applications in live-cell imaging, including measurement of dynamic gene expression, localization of DNA and proteins, interactions between proteins, enzyme activation, calcium and cAMP signalling, and pH homeostasis (e.g., Brandizzi et al., 2002; Czymmek et al., 2002; Heun et al., 2001; Lippincott-Schwartz et al., 2003; Miyawaki, 2003; Robinett et al., 2001; Zhang et al., 2002).

Although many attempts to express versions of GFP under the control of constitutive or inducible fungal
promoters at levels necessary for live-cell imaging in Neurospora crassa were unsuccessful, we achieved cytoplasmic expression of a GFP derivative (sgfp) in Neurospora (Freitag et al., 2001). The sgfp gene has been used in plants (Chiu et al., 1996) and fungi (Fernandez-Abalos et al., 1998; Fox et al., 2002). A serine to threonine substitution at position 65 (S65T; Heim et al., 1995) results in increased brightness, and mutations to shift the codon bias closer to that found in humans (Haas et al., 1996) and N. crassa (Borkovich et al., 2004; Galagan et al., 2003) made this GFP variant an attractive choice for studies in Neurospora. A mitochondrially targeted GFP fusion protein has been successfully expressed from a strong Neurospora promoter (Fuchs et al., 2002), but this construct is not easily adaptable for general use. For the present study, we aimed to build a versatile “cassette” plasmid, in which Neurospora promoters and genes, as well as fluorescent proteins can be easily exchanged and integrated at a precise location in the genome. Thus, our first aim was to develop methodology to achieve strong expression of GFP fusion proteins in N. crassa by constructing translational fusions of the N. crassa ccg-1 (formerly grg-1) promoter (McNally and Free, 1988) to sgfp and either the Neurospora histone H1 (hH1; Folco et al., 2003) or β-tubulin gene (Bml; Orbach et al., 1986). The second aim was to image nuclear and microtubule organization and dynamics in growing vegetative hyphae, developing ascii, and ascospores in strains transformed with these constructs, to confirm and extend results obtained previously by traditional microscopic techniques that principally made use of fixed cells.

Neurospora nuclei have been studied extensively using bright field light microscopy (Barry, 1972; Bianchi and Turian, 1967; Dodge, 1927; McClintock, 1945; Raju, 1980, 1984; Singleton, 1953; Wilson and Aist, 1967), fluorescence microscopy (Freitag et al., 2004; Heath et al., 2000; Lee et al., 2001; Marek et al., 2003; Minke et al., 1999a,b; Perkins et al., 1995; Raju, 1982, 1986; Riquelme et al., 2002; That et al., 1988; Thompson-Coffe and Zickler, 1994; Tinsley et al., 1996), and transmission electron microscopy (Bojko, 1988; Gillies, 1972, 1979; Lu, 1993; Riquelme et al., 2002). Most of these studies have used fixed cells. One study involved video-enhanced DIC light microscopy of living, unstained vegetative hyphae, and immunofluorescence and laser scanning confocal microscopy (Riquelme et al., 2002). Our recent studies employed conventional fluorescence (Folco et al., 2003; Freitag et al., 2004; Glass et al., 2004) and confocal imaging of GFP-labeled nuclei (Freitag et al., 2004). Similarly, studies of microtubules in Neurospora have used fixed cells (Bruno et al., 1996; Heath et al., 2000; Minke et al., 1999a,b; Riquelme et al., 2002; Shiu et al., 2001; That et al., 1988; Thompson-Coffe et al., 1999). The present study on live cells largely confirms previous results obtained with techniques that are more invasive. Visualization of H1-GFP and β-tubulin-GFP in meiosis revealed silencing in heterozygous crosses, presumably by meiotic silencing by unpaired DNA (Shiu et al., 2001). Our results illustrate the utility of GFP fusion proteins to study the cell biology of live Neurospora in real time.

2. Materials and methods

2.1. Construction of sgfp-containing plasmids

We first generated a plasmid with the bright S65T version of GFP by inserting the BamHI–BglII fragment of plasmid pFA6a-GFP(S65T)-kanMX6 (Longtine et al., 1998) into BamHI-digested pBM60 (Margolin et al., 1997) to yield pMF255. The GFP(S65T) gene was replaced by amplification of sgfp from pCT74 (Lorang, 2001) and insertion of the BamHI + EcoRI-digested fragment into pMF255. This yielded pMF267, a promoterless sgfp construct useful for generating translational fusions to genes driven by their endogenous promoters. To generate over-expression constructs, the inducible Neurospora ccg-1 (formerly grg-1) promoter (Pccg-1; McNally and Free, 1988) and 5′ untranslated region of the transcript directly preceding the ccg-1 AUG (nt 738–1659; GenBank Accession No. L14464) was amplified by PCR and inserted into NorI + XbaI-digested pMF267, yielding pMF272. To construct translational fusions to the N. crassa histone H1 gene (hH1) and the β-tubulin gene (Bml), we amplified the hH1 and Bml genes by PCR, digested with BamHI + PacI or XmaI + PacI, and inserted fragments into pMF272, to yield pMF280 and pMF309, respectively. All PCRs were performed with Herculase polymerase (Stratagene). Plasmids pMF272, pMF280, and pMF309 are available from the Fungal Genetics Stock Center, Kansas City, MO and their GenBank Accession Nos.: pMF272 (AY598428), pMF280 (AY598429), and pMF309 (AY598430).

2.2. Transformation protocols and transformant selection

Neurospora transformations were performed by electroporation as described previously (Margolin et al., 1997). Plasmids pMF272 and pMF280 were targeted to the his-3 locus of strain N623 yielding the primary heterokaryotic prototrophic His* transformants N226 and N227, and N227, respectively (Folco et al., 2003). Plasmid pMF309 was integrated at his-3 of N2240 and N2257, yielding N2509 and N2505, respectively (see Table 1). An Olympus SZX12 fluorescence stereo microscope with a GFP filter set (460–490 nm excitation, 505 nm dichroic mirror and 510–550 nm emission filter) was used to screen for GFP* transformants. Heterokaryotic transformants were crossed to obtain homokaryotic progeny of desired genotypes.
A novel ascospore maturation defect was identified in crosses of two independent transformants of N623 to either N1 or N2257 (N2276, N2276-1, N2281, N2281-8, N2281-3, N2280, N2281 (FGSC#9517), N2281-3 (FGSC#9518), N2524 (FGSC#9519), and N2526 (FGSC#9520). N2261 (mat A his-3) strain (N2276-1) was isolated from N2276 by serial targeting to the pMF272, pMF280, and pMF309) are designed for gene manipulation were according to standard Neurospora techniques (Davis, 2000). The heterokaryotic strain N2276 was crossed to wild type N1 to isolate mat A his-3::hH1\(^{+}\)-sgfp\(^{+}\) (N2282) and mat a his-3::hH1\(^{+}\)-sgfp\(^{+}\) (N2283) progeny, respectively. To obtain homokaryotic hH1\(^{+}\)-sgfp\(^{+}\) strains for subsequent crosses that would not be complicated by repeat-induced point mutation (RIP), we also crossed the heterokaryotic N2277 to a rid mat a his-3 strain (N2257; Freitag et al., 2002) and isolated mat A his-3::hH1\(^{+}\)-sgfp\(^{+}\) (N2280) and rid mat a his-3::hH1\(^{+}\)-sgfp\(^{+}\) (N2281), respectively. A homokaryotic mat A his-3::hH1\(^{+}\)-sgfp\(^{+}\) strain (N2276-1) was isolated from N2276 by serial conidial isolation. Two additional mat A his-3::hH1\(^{+}\)-sgfp\(^{+}\) strains (N2281-3 and N2281-8) were isolated from a cross of mat A; fluffy to N2281, because N2276-1, N2280, and N2282 carry a previously unknown ascospore maturation defect. N2281, N2281-3, and N2281-8 are free from the defect. Primary transformants N2505 and N2509 were crossed to isolate mat a rid\(^{RIP}\)his-3::Pccg-1-Bml\(^{+}\)-sgfp\(^{+}\) (N2524, N2525) and mat A rid\(^{RIP}\)his-3::Pccg-1-Bml\(^{+}\)-sgfp\(^{+}\) (N2526, N2527).

2.4. Fluorescent staining and preparation of cultures for live-cell imaging of vegetative hyphae

GFP-expressing strains were routinely grown on Vogel’s minimal salt medium, supplemented with 2% sucrose or on 2% malt extract medium. Live-cell imaging involved using the “inverted agar block method” of preparing and staining samples (Hickey et al., 2002; Hickey and Read, 2003). Hyphae were double-labeled for one hour with either 25 μM FM 4-64 (Molecular Probes, Eugene, OR), which stains the plasma membrane and organelle membranes (Fischer-Parton et al., 2000), or 10 μM DASPMI (Molecular Probes, Eugene, OR), which stains mitochondria (Bereiter-Hahn, 1990). Conidia from original H1-GFP\(^{+}\) transformants were counterstained with the DNA dye Hoechst 33258 to verify nuclear localization of the fusion protein (data not shown and Freitag et al., 2004).

### Table 1

Neurospora crassa strains used in this study

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1 (74-OR8-1)</td>
<td>mat a</td>
<td>FGSC#988</td>
</tr>
<tr>
<td>N150 (74-OR23-IV)</td>
<td>mat A</td>
<td>FGSC#2489</td>
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<tr>
<td>fluffy</td>
<td>mat A; fl (RL); scot</td>
<td>FGSC#6682</td>
</tr>
<tr>
<td>fluffy</td>
<td>mat a; fl (RL); scot</td>
<td>FGSC#6683</td>
</tr>
<tr>
<td>Banana</td>
<td>mat a Ban mei-3; int</td>
<td>FGSC#2990</td>
</tr>
<tr>
<td>per-1</td>
<td>mat A esp-2; per-1</td>
<td>FGSC#6662</td>
</tr>
<tr>
<td>N223</td>
<td>mat A his-3</td>
<td>FGSC#103</td>
</tr>
<tr>
<td>N2240</td>
<td>rid(^{RIP}) mat A his-3</td>
<td>Freitag et al. (2002)</td>
</tr>
<tr>
<td>N2257</td>
<td>rid(^{RIP}) mat a his-3</td>
<td>Freitag et al. (2002)</td>
</tr>
<tr>
<td>N2261</td>
<td>(mat A his-3::Pccg-1-sgfp(^{+}) + mat A his-3)</td>
<td>Folco et al. (2003)</td>
</tr>
<tr>
<td>N2276, N2277</td>
<td>(mat A his-3::Pccg-1-hH1(^{+})-sgfp(^{+}) + mat A his-3)</td>
<td>Folco et al. (2003)</td>
</tr>
<tr>
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<td>mat A his-3::Pccg-1-hH1(^{+})-sgfp(^{+})</td>
<td>This study</td>
</tr>
<tr>
<td>N2280</td>
<td>mat A his-3::Pccg-1-hH1(^{+})-sgfp(^{+})</td>
<td>This study</td>
</tr>
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<td>mat A his-3::Pccg-1-hH1(^{+})-sgfp(^{+})</td>
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</tr>
<tr>
<td>N2526, N2527</td>
<td>rid(^{RIP}) mat A his-3::Pccg-1-Bml(^{+})-sgfp(^{+})</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Selected strains are available from the Fungal Genetics Stock Center: N2240 (FGSC#9014), N2257 (FGSC#9015), N2261 (FGSC# 9516), N2281 (FGSC#9517), N2281-3 (FGSC#9518), N2524 (FGSC#9519), and N2526 (FGSC#9520).

All Neurospora plasmids based on pBM60 (e.g., pMF272, pMF280, and pMF309) are designed for gene targeting to the his-3 locus (Margolin et al., 1997). No additional selectable marker for fungal transformation is contained on these plasmids, but co-transformations with hph, a gene that confers resistance to hygromycin B (Staben et al., 1989), have been successfully performed with N. crassa (M. Freitag and E. U. Selker, data not shown) and Magnaporthe grisea (J-R. Xu, Purdue University, personal communication).

### 2.3. Strains and culture conditions

Strains used in this study are listed in Table 1. Strains were maintained on Vogel’s minimal medium and all manipulations were according to standard Neurospora techniques (Davis, 2000). The heterokaryotic strain N2276 was crossed to wild type N1 to isolate mat A his-3::hH1\(^{+}\)-sgfp\(^{+}\) (N2282) and mat a his-3::hH1\(^{+}\)-sgfp\(^{+}\) (N2283) progeny, respectively. To obtain homokaryotic hH1\(^{+}\)-sgfp\(^{+}\) strains for subsequent crosses that would not be complicated by repeat-induced point mutation (RIP), we also crossed the heterokaryotic N2277 to a rid mat a his-3 strain (N2257; Freitag et al., 2002) and isolated mat A his-3::hH1\(^{+}\)-sgfp\(^{+}\) (N2280) and rid mat a his-3::hH1\(^{+}\)-sgfp\(^{+}\) (N2281), respectively. A homokaryotic mat A his-3::hH1\(^{+}\)-sgfp\(^{+}\) strain (N2276-1) was isolated from N2276 by serial conidial isolation. Two additional mat A his-3::hH1\(^{+}\)-sgfp\(^{+}\) strains (N2281-3 and N2281-8) were isolated from a cross of mat A; fluffy to N2281, because N2276-1, N2280, and N2282 carry a previously unknown ascospore maturation defect. N2281, N2281-3, and N2281-8 are free from the defect. Primary transformants N2505 and N2509 were crossed to isolate mat a rid\(^{RIP}\)his-3::Pccg-1-Bml\(^{+}\)-sgfp\(^{+}\) (N2524, N2525) and mat A rid\(^{RIP}\)his-3::Pccg-1-Bml\(^{+}\)-sgfp\(^{+}\) (N2526, N2527).
2.5. Confocal microscopy and image processing

Confocal laser scanning microscopy was performed using a Bio-Rad Radiance 2100 system equipped with an argon ion laser, and mounted on a Nikon TE300 inverted microscope. GFP and FM4-64 or GFP and DASPMI, respectively, were imaged simultaneously by excitation with the 488 nm laser line and fluorescence detection at 515–530 nm (for GFP), and >600 nm (for FM4-64 or DASPMI). Oil immersion 60× (N.A. 1.4) or dry 20× (N.A. 0.75) plan apochromatic objective lenses were used for imaging. The laser intensity and laser scanning of individual hyphae were kept to a minimum to reduce dye photobleaching and phototoxic effects. The imaging parameters used produced no background signal from any source other than from GFP or dye fluorescence (data not shown). Time-lapse imaging was performed at scan intervals of 3–10 s for periods up to 15 min. Confocal images were captured using Lasersharp software (version 5.1; Bio-Rad) and were initially viewed using Confocal Assistant software (version 4.02). These images were transferred into Paintshop Pro software (version 7.0; JASC) for further processing. Much of the analysis of time-lapse sequences involved converting them into animation movies. For this purpose, time-lapse sequences were re-sampled to convert them from “.pic” to “.avi” files in order to be processed by Adobe Premiere software (version 6.0). File compression was often necessary to prevent “stuttering” playback due to the limited data transfer rate (i.e., the time taken to transfer data from hard disk to the video display hardware). Final animation movies (Supplementary Movies 1–3) accompany this report and can be downloaded from http://www.Neurospora.org and www.sciencedirect.org in MPEG-1 format.

2.6. Crosses for conventional fluorescence microscopy and imaging of asc and ascospores

Croses for cytology were routinely made in Petri plates on synthetic crossing medium supplemented with 1% sucrose and 2% agar (Davis, 2000). For each cross, the protoperithecial parent was first grown on medium for 5 days at 25°C and then fertilized by adding conidia from the second parent. The H1-GFP and β-tubulin-GFP strains were examined both in homozygous and heterozygous crosses, often made reciprocally. The developing perithecia (unfixed, unstained) were dissected at 12–24 h intervals from 3 to 10 days post-fertilization on a glass slide in a drop of 10% glycerol, lightly squashed under a cover glass and sealed with melted dental wax.

Asci were examined with an upright Nikon Microphot FX microscope equipped with epifluorescence and dark-field illumination. A filter set specifically suited for sGFP imaging was obtained from Chroma (#41012:480/40 nm excitation filter, 505 nm dichroic mirror, 510 nm long pass emission filter). This combination of filters produced no fluorescence signal from any source other than from GFP. Low intensity back-lighting through a dark-field condenser provided the necessary contrast and made ascus and ascospore outlines visible. Three objective lenses were used for observation and microphotography: Zeiss Neofluor 10× (N.A. 0.30), Nikon Plan 20× (N.A. 0.50), and Nikon Fluor 40× Oil (N.A. 1.30). For microphotography, a Nikon Coolpix 5000 or 5400 digital camera was attached to the video port with adapters from Microscope World (http://www.microscopeworld.com). The 5 megapixels sensor in these cameras is capable of recording high-quality fluorescence images of asc, but it was often necessary to frame and focus cells on the camera’s LCD screen in brightfield first, and then switch the filter setup to fluorescence for 2–8 s manual exposures, depending upon the numerical aperture of the objective lens.

3. Results

3.1. Construction of sGFP plasmids for expression of translational fusions

We generated a series of versatile plasmids to express tagged fusion proteins in N. crassa, based on a system available for S. cerevisiae (Longtine et al., 1998). The plasmid backbone was designed as a simple cassette system that allows targeting to the N. crassa his-3 locus and to accept various promoters, coding regions to be expressed, and various fluorescent marker genes (Fig. 1 and data not shown). Building on results obtained with the strong ToxA promoter from Pyrenophora tritici-repentis (Freitag et al., 2001), we attempted to express translational fusions of Neurospora genes to sgfp driven by different Neurospora promoters.

When under the control of the Neurospora ccg-1 promoter, primary His+ transformants with either hH1*-sgfp* or Bml*-sgfp* showed GFP expression after ~2–5 days on minimal medium plates when visualized under the fluorescence stereo microscope. The native H1+ promoter and the inducible Neurospora qa-2 and arg-2 promoters, however, did not result in visible H1-GFP expression (data not shown). For successful constructs, typically >75% of all His+ transformants showed strong GFP expression on the initial plate. Primary transformants were transferred to slants and grown overnight at 32°C, after which GFP was easily detectable in the aerial hyphae and developing macroconidia under the dissecting microscope. GFP expression was stable in strains kept at room temperature for a month and for at least a year in strains kept at −20°C. We observed increased autofluorescence in hyphae from month-old cultures, which can obscure low levels of GFP fluorescence. Nevertheless, growth on
fresh medium fully restored expression of H1-GFP and β-tubulin-GFP of strains kept in slants at −20°C for more than one year.

Primary transformants are usually heterokaryotic. Thus, transformants were either crossed to obtain homokaryotic progeny or purified by serial conidial isolation (Davis, 2000). The \textit{hH1}+ -sgfp+ component of strain N2276, for example, was purified by serial isolations to generate a homokaryon (N2276-1). Microscopic observations of heterokaryotic mycelia and conidia revealed that all nuclei showed green fluorescence, indicating import of mature H1-GFP protein into all nuclei.

The \textit{Neurospora ccg-1} promoter is strongly induced by glucose deprivation or stress (McNally and Free, 1988). Accordingly, we observed significant induction of fusion proteins after growth in liquid starvation medium. Cultures were grown for 2 days in liquid medium supplemented with Vogel’s minimal salts and 2% sucrose, harvested by filtration and the mycelial pads split in half. The “uninduced” pad was quick-frozen in liquid nitrogen and stored at −80°C, while the remaining tissue was resuspended in Vogel’s minimal salts without added carbon source for six hours. GFP was detected in the uninduced tissue, albeit at much lower levels than under induced conditions (data not shown). On solid medium, however, no increase in fluorescence was detected when strains were transferred to water agar after growth on Vogel’s minimal salts and 2% sucrose or on malt extract medium. The relative fluorescence of cultures grown on all solid media tested was comparable to that observed from induced liquid cultures, which suggests that the \textit{ccg-1} promoter is activated, and remains active, when aerial hyphae are formed.

3.2. Nuclear organization and dynamics in hyphae monitored with H1-GFP

Nuclei labeled with H1-GFP fluoresced brightly (Fig. 2). As expected (e.g., Davis, 2000), nuclei were typically absent in the tips (first 20–25 μm) of growing hyphae (Figs. 2A–C; see Supplementary Movie 1 at \url{http://www.Neurospora.org} and \url{www.sciencedirect.com}). This anucleate apical hyphal region was occupied by a Spitzenkörper in the very tip (as shown by double-labeling with FM4-64, Figs. 2A and B) and mitochondria (as shown by double-labeling with DASPMI, Fig. 2C). Nuclei were more plentiful in apical hyphal compartments than in older subapical hyphal compartments (cf. Figs. 2A and E). Rather than being diffusely localized in the nuclear matrix, H1-GFP stained nuclei unevenly and was found in stable fluorescent foci (Fig. 2D), which suggests differential association with chromatin. The shapes of nuclei in transport varied from oval to pear-shaped (Figs. 2B–E). A bright fluorescent region was often observed at the leading end of a moving nucleus (see Supplementary Movie 2 at \url{http://www.Neurospora.org}).

Fig. 1. Construction of GFP expression plasmids for use in \textit{N. crassa}. (A) Expression of the GFP variant gene (sgfp) was driven by the \textit{Neurospora ccg-1} (P\textit{ccg-1}) promoter (McNally and Free, 1988). Plasmid pMF272 (GenBank Accession No. AY598428) is a versatile cassette vector with a multiple cloning site (MCS) for construction of translational fusions of \textit{Neurospora} genes to sgfp. Because the ATG start codon of sgfp was included, expression of this construct results in nuceloplasmic expression (Folco et al., 2003). The P\textit{ccg-1}-sgfp cassette is contained on a pBM60 backbone to allow \textit{his}-3 targeting by gene replacement (see Section 2; Margolin et al., 1997). The NdeI and Drai restriction endonuclease sites are useful for linearizing constructs before transformation. (B) Partial maps of pMF280 (AY598429) and pMF309 (AY598430), carrying the histone H1 (hH1) and β-tubulin (Bml) gene, respectively.
Mitotic nuclei appeared relatively immobile, while interphase nuclei were in transport to the apex. We also observed retrograde transport of nuclei (Supplementary Movies 1 and 2 at http://www.Neurospora.org and www.sciencedirect.com).

3.3. Microtubule organization and dynamics in hyphae monitored with β-tubulin-GFP

Microtubules labeled with β-tubulin-GFP were readily visible and were more concentrated in the cytoplasm of young apical hyphal compartments than in old sub-apical compartments (cf. Fig. 3A with Fig. 3D). In apical compartments, microtubules showed pronounced longitudinal orientation and extended into the Spitzenkörper when visualized after co-labeling with FM4-64 (Fig. 3B), but they were less longitudinally orientated (Fig. 3C) in subapical compartments close to the colony periphery, and appeared randomly arranged further back still (Fig. 3D). Superficially, microtubules in apical hyphal compartments appeared longer (Figs. 3A and B) than those further back from the colony periphery (Fig. 3D). The short appearance of microtubules in optical sections of subapical hyphal compartments is expected, however, because the random orientation of microtubules result in shorter segments of the individual microtubules in the image plane when compared with an apical hyphal compartment. Microtubules extended through septal pores and were forced into closer proximity with each other as the septum grew towards the center of the hypha (Fig. 3C). What are assumed to be bundles of microtubules formed spindles in nuclei undergoing mitosis, and these were most obvious in subapical hyphal compartments (Figs. 3D and E). These dynamic spindles appeared randomly orientated and positioned within hyphae. We observed astral ray microtubules extending from each end of a spindle (Figs. 3D and E), and sometimes these appeared to connect with the plasma membrane (Fig. 3D). Microtubules were extremely dynamic and exhibited growth and shrinkage. This was best observed in germ tubes, which were narrower and possessed fewer microtubules than leader hyphae (cf. Fig. 4 with Fig. 3A; Supplementary Movie 3 at http://www.Neurospora.org and www.sciencedirect.org).

3.4. Expression of H1-GFP and β-tubulin-GFP during ascus development

Ascus development in Neurospora has been previously studied by conventional light microscopy (e.g., Raju, 1980, 1992). In 3-day-old perithecia, most of the hymenial tissue is composed of a non-ascogenous, multinucleate, paraphysal network of cells. Embedded within this tissue are the ascogenous pre-crozier and crozier branches, which give rise to a population of asynchronously developing asci (~200–400 per perithecium). Various stages of nuclear division and ascospore delimitation can be observed in the developing ascis from 3 to 5 days after fertilization. The second postmeiotic mitosis occurs in the young ascospores shortly after they are delimited. Within the next 2–3 days, the ascospores enlarge, form striations, and become pigmented. Four to five additional mitoses occur in the mature black ascospores, which may contain 32 or more nuclei prior to their discharge from perithecia. We examined the expression of H1-GFP and β-tubulin-GFP during ascus development in homozygous and heterozygous crosses by conventional wide-field fluorescence microscopy.
Heterozygous crosses of *his-3*::*hH1*-*sgfp* with *per-1* (perithecial color) allowed observation of postmeiotic mitoses in the unpigmented *per-1* ascospores. Crosses with *Banana* (giant ascospore) revealed expression of *hH1*-sgfp* in maturing heterokaryotic ascospores with a common cytoplasm. We found marked differences in the expression of H1-GFP and β-tubulin-GFP during early ascus development when comparing heterozygous and homozygous crosses.

### 3.4.1. Homozygous hH1-GFP and β-tubulin-GFP crosses

In homozygous *hH1*-sgfp* perithecia, the nuclei in the asogenous pre-crozier and crozier cells as well as those in the non-ascogenous, multinucleate, paraphysal network of cells fluoresced brightly and it was often difficult to distinguish the asogenous nuclei at the base of the ascus rosette from the ubiquitous paraphysal nuclei in the background (Fig. 5A). Although the developing asci showed a moderate level of background H1-GFP in the cytoplasm, the general features of nuclear divisions could be readily followed throughout ascus development (Fig. 5B). Nuclei fluoresced during meiosis, postmeiotic mitosis, and ascospore delimitation and maturation. Condensed chromosomes were resolved in some metaphase/anaphase nuclei (data not shown). Because of the high expression levels of H1-GFP, which resulted in strong out-of-focus fluorescence, we found H1-GFP unsuitable for detailed chromosome analysis or for examination of division spindles and spindle pole bodies.

The expression of H1-GFP was most consistent and striking in the developing ascospores. After the first

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Fig. 4. Confocal images showing microtubule dynamics in a germ tube labeled with β-tubulin-GFP and visualized over a 70s period. Note the growth and shrinkage of three microtubules (numbered 1–3). The likely location of a spindle pole body (s) associated with a nucleus is also evident. Also see Supplementary Movie 3 at http://www.Neurospora.org and www.sciencedirect.com. Bar = 5 μm.

Fig. 5. Conventional fluorescence images showing expression of H1-GFP in homozygous asci. (A) A partial rosette of developing asci at 5 days after fertilization. Nuclei fluoresce throughout meiosis in the young asci and in developing ascospores. Nuclei in non-ascogenous paraphysal cells in the background fluoresce when the protoperithecial parent carries the hH1*-sgfp* gene. (B) An ascus soon after spore delimitation; ascospores are uninucleate at inception. (C) A partial rosette of maturing asci at 8 days. The ascospores are binucleate at this stage; all eight ascospores show glowing nuclei (A–C: N2281×N2281-3). (D) Mature black ascospores contain 50 or more fluorescing nuclei resulting from 4–5 mitoses (N2283×N2280). Scale bars in (A) and (C) = 100 μm, in (B) and (D) = 25 μm.
mitosis in the young ascospores, the two nuclei fluoresced brightly until the ascospores formed striations, began to pigment, and matured. The maturing ascospores remained binucleate during this two to three-day-long maturation period (Fig. 5C). The two fluorescing nuclei in the newly pigmented mature ascospores did not show up well through the melanized cell walls. Ascospore nuclei became more conspicuous again following four or five additional mitoses, however (Fig. 5D).

As with H1-GFP, the homozygous crosses of β-tubulin-GFP showed high levels of β-tubulin-GFP expression during meiosis, postmeiotic mitoses, and ascospore delimitation and development. Developing asci through meiotic prophase showed strong β-tubulin-GFP fluorescence throughout the ascus cytoplasm. Fluorescence was excluded from putative vacuoles. Spindles fluoresced brightly during various divisions in the ascus and during the first mitosis in the ascospores (Fig. 6A). Spindle pole bodies were sometimes visible at one end of each delimiting ascospore (data not shown). All immature ascospores fluoresced with β-tubulin-GFP, but microtubule filaments were difficult to discern by conventional fluorescence microscopy (Fig. 6B). Nuclei were sometimes visible in the ascus and in young ascospores (Fig. 6B). β-Tubulin-GFP fluorescence remained clearly visible until the ascospores became pigmented (data not shown).

3.4.2. Expression of H1-GFP and β-tubulin-GFP in heterozygous crosses

In his-3+:hH1⁺-sgfp⁺ × wild type crosses, H1-GFP expression was markedly different from that of homozygous crosses, regardless of whether the hH1⁺-sgfp⁺ strain was used as the male or the female parent. In heterozygous crosses, no H1-GFP fluorescence was observed throughout meiosis, postmeiotic mitoses, and ascospore delimitation. Between 12 and 24h after the nuclei were sequestered into ascospores and after one mitosis, the hH1⁺-sgfp⁺ nuclei in four of the eight ascospores began to fluoresce, albeit faintly. As the ascospores continued to develop and mature during the next two days, the H1-GFP nuclei fluoresced more brightly (Figs. 7A and B). Apparently, the expression of H1-GFP was silenced in the heterozygous crosses during meiosis until after the ascospores were delimited, presumably by meiotic silencing by unpaired DNA (Shiu et al., 2001). The silencing did not extend into the autonomously developing ascospores. These observations were confirmed in crosses of several his-3+:hH1⁺-sgfp⁺ strains with wild type and other mutant strains (N.B. Raju, M. Freitag, and R.L. Metzenberg, unpublished results).

The original mat A his-3⁺::hH1⁺-sgfp⁺ strains (N2276A, N2280A, and N2282A) showed an unexpected ascospore maturation defect both in heterozygous crosses with wild type and in homozygous crosses with mat A his-3⁺::hH1⁺-sgfp⁺ strains. When heterozygous with wild type, the four H1-GFP ascospores, failed to melanize and continued to glow brighter for an extended period as the four non-GFP ascospores pigmented and matured normally (Figs. 7A and B). This maturation defect allowed us to obtain images of large rosettes of asci showing 4:4 ascospore patterns for H1-GFP. The mutant ascospores were viable but germinated at lower frequencies than wild type or mat a his-3⁺::hH1⁺-sgfp⁺ ascospores (data not shown). None of the mat a strains inspected showed this ascospore maturation defect. We have not yet investigated the genetic basis for this defect.

Almost all asci (>90%) showed first-division segregation for hH1⁺-sgfp⁺ because of the close proximity of the his-3⁺::hH1⁺-sgfp⁺ locus to the centromere of linkage group I. Thus, four his-3⁺::hH1⁺-sgfp⁺ ascospores with fluorescing nuclei were usually found at one end of the ascus and four non-GFP ascospores at the opposite end. In his-3⁺::hH1⁺-sgfp⁺ × per-l crosses, nuclear divisions and multiple H1-GFP nuclei were observed more clearly in the mature, unpigmented, per-l ascospores. In contrast to his-3⁺::hH1⁺-sgfp⁺, per-l often segregated at the second division of meiosis resulting in 2:2:2:2 or 2:4:2 ascospore patterns. Fig. 7C illustrates six spores of an 8-spored ascus showing the first-division segregation pattern (4:4 ascospore pattern) for his-3⁺::hH1⁺-sgfp⁺ and the second-division segregation pattern (2:2:2:2 ascospore pattern) for per-l (two GFP⁺ per- spores to the left are not shown). As expected, the his-3⁺::hH1⁺-sgfp⁺ nuclei appeared much brighter, and nuclear divisions were observed more clearly in the
unpigmented per-I ascospores than in the black wild type ascospores. In β-tubulin-GFP × wild type crosses, early perithecial development was normal and nearly normal numbers of asci were produced. The asci underwent karyogamy and proceeded into meiotic prophase. They usually elongated to their normal length but they often showed characteristic subapical bends (see image in Shiu et al., 2001). Most of these ascis (95%) did not show β-tubulin-GFP fluorescence, however, and they were usually arrested prior to metaphase I. A few rare ascis (5%) did show β-tubulin-GFP and these ascis, though abnormally shaped, were capable of undergoing one or more nuclear divisions. Clearly, the expression of β-tubulin-GFP is silenced during meiosis in heterozygous crosses, as described above for hH1*-sgfp+. Unlike the histone H1 silencing, the absence of β-tubulin seriously impaired ascus development and production of ascospores (see also Shiu et al., 2001). Consequently, the heterozygous perithecia were completely barren. Results of further studies on meiotic silencing that utilize GFP fusions will be described elsewhere (N.B. Raju, M. Freitag, and R.L. Metzenberg, manuscript in preparation).

3.5. H1-GFP is translated in the vicinity of hH1*-sgfp+ nuclei but diffuses throughout a common cytoplasm

The initial heterozygous crosses of his-3*:::hH1*-sgfp+ with wild type were expected to provide information on whether H1-GFP is nucleus-limited or shared by other nuclei in the common cytoplasm of the ascus. This information could not be obtained from heterozygous crosses because the his-3*:::hH1*-sgfp+ was silenced throughout meiosis in heterozygous asci. We next crossed his-3*:::hH1*-sgfp+ with the giant ascospore mutant Banana (Banana (Ban)), in which all eight nuclei of the ascus are enclosed in a single giant ascospore. After a single mitosis, eight nuclei at one end first showed H1-GFP, followed soon thereafter by fluorescence of the remaining sgfp− nuclei at the other end. As the ascospores formed striations and pigmented within the next two days, all 16 nuclei fluoresced equally brightly. Apparently, H1-GFP is translated in the immediate vicinity of the hH1*-sgfp+ nuclei but the protein diffuses through the cytoplasm and becomes incorporated into all nuclei in the spore (N2281-8 × Ban). Scale bar in (A) = 100 μm, in (B–D) = 25 μm.
ascospores retain their first-division segregation alignment, and that H1-GFP fusion protein is produced in the immediate vicinity of his-3\(^{-}\):::hH1\(^{+}\)-sgfp\(^{+}\) nuclei. Furthermore, the fusion protein readily diffuses throughout the cytoplasm and is eventually imported into all nuclei.

4. Discussion

We have shown that histone H1-GFP and β-tubulin-GFP can be expressed under the control of the Neurospora ccg-1 promoter at levels that are useful to study the organization, dynamics, and function of nuclei and microtubules in living cells of N. crassa. Approximately half of our attempts to construct carboxy-terminal GFP fusions with Neurospora genes have been successful (M. Freitag, G.O. Kothe, K. Adhvaryu, T.K. Khalfallah, J.G. Murphy, H. Tamaro, and E.U. Selker, unpublished data). There are a number of reasons why expression of GFP fusions may be inefficient or even impossible. For example, the large, barrel-shaped GFP adduct may interfere with folding of the protein to be tagged or the carboxy-terminus of the original protein may be buried within the folded protein (e.g., Fox et al., 2002). To address the latter problem, we have constructed plasmids to express amino-terminal GFP fusions (M. Freitag and E.U. Selker, unpublished data). In general, we find that small proteins with little defined tertiary structure at the amino- or carboxy termini, such as histone H1 (Folco et al., 2003) and heterochromatin protein 1 (HP1; Freitag et al., 2004), are easier to visualize than larger fusion proteins. Nevertheless, we had success with a 1127 amino acid ATPase subunit of a putative chromatin remodeling factor, CRF4-1 (M. Freitag and E.U. Selker, unpublished data). Over-expression of large GFP fusion proteins in Neurospora can result in green exudate or crystals on hyphae or macroconidia. In such transformants, no GFP fluorescence is detectable under the fluorescence stereo microscope, but in some cases properly localized, weakly fluorescing fusion protein is observed by confocal laser scanning microscopy (M. Freitag, G. Kothe, and E.U. Selker, unpublished data). Over-expression of GFP fusion proteins may interfere with the normal function of tagged proteins. We found that this is not usually the case; for example, HP1-GFP complements Neurospora hpo growth and DNA methylation phenotypes (Freitag et al., 2004). We conclude that small proteins (<500 amino acids) can be productively tagged with GFP for localization studies in Neurospora.

Because strains with tagged genes are useful for genetic studies, we prefer to use host strains that are defective in repeat-induced point mutation (RIP; Selker, 1990), even though RIP does not directly affect transgenes in vegetative cells. We routinely insert GFP-tagged transgenes into two strains of opposite mating types that each carry a mutation in the rid gene, which is essential for RIP (Freitag et al., 2002). On the other hand, when using RIP to intentionally mutate genes, GFP tags can facilitate the identification of strains in which RIP has occurred. For example, to identify likely RIP-mutated candidates with duplicated hpo genes from a cross of a rid his-3\(^{-}\):::hpo- sgfp\(^{+}\) strain and a his-3 strain, non-fluorescing prototrophs were selected. Approximately 30% of the duplication progeny showed hallmarks of RIP (i.e., RFLPs and DNA methylation; Freitag et al., 2004).

4.1. GFP imaging of nuclei and microtubules in living cells

Vegetative hyphae of Neurospora are multinucleate, as illustrated in this study. It has been estimated that the apical compartments of leader hyphae of Neurospora commonly contain 200–400 nuclei (Davis, 2000). We have not been able to image complete mitoses in vegetative hyphae of Neurospora, because nuclear division in Neurospora is asynchronous (Minke et al., 1999b) and because nuclei move continuously. Mitosis is easier to image in fungi with thinner hyphae and in which nuclear division is synchronous or occurs in waves (e.g., Aspergillus nidulans, see movie in Hickey and Read, 2003).

Nuclei move along microtubules propelled by the motor protein dynein (Han et al., 2001; Xiang and Plaumann, 2003). A bright focus of HI-GFP fluorescence in nuclei adjacent to the leading edge of moving nuclei is presumably associated with the region of the nuclear envelope attached to the dynein motor. This focus may reflect the location of chromosomes within the nucleus, where centromeres cluster opposite of the spindle pole body with the remainder of the chromosomal DNA extended behind the centromeres (“Rab1 orientation,” e.g., see Loidl, 2003). Alternatively, histone H1 may be closely associated with the spindle pole body, which acts as a microtubule organizing center for both cytoplasmic and spindle microtubules (e.g., Oakley and Morris, 1983; Oakley et al., 1990). In a study on A. nidulans in which the spindle pole body was labeled with GFP (Fox et al., 2002), nuclei moved with the spindle pole body orientated in the direction of movement. We observed similar behavior in Neurospora strains in which heterochromatin was labeled with an HP1-GFP fusion (Freitag et al., 2004). A bright focus of fluorescence has also been described in Neurospora nuclei stained with DAPI (Thompson-Coffe and Zickler, 1994) and densely stained regions in nuclei and microtubules have been colocalized in studies on fixed cells (Riquelme et al., 2002). As observed in earlier studies with several fungi (Robinow, 1957; Wilson and Aist, 1967; Minke et al., 1999b), nuclei changed shape from being more- or less spherical to pear-shaped; rapidly moving nuclei tended to be pear-shaped.
We used GFP fused to β-tubulin to visualize microtubules in living hyphae of Neurospora. Our results are comparable to what was found with α-tubulin-GFP in a study on the dynamic behavior of microtubules in A. nidulans (Han et al., 2001). Microtubule organization depends on filament growth by polymerization of α- and β-tubulin subunits and shrinkage by depolymerization (Desai and Mitchison, 1997). By altering different parameters of this “dynamic instability,” animal and plant cells can rearrange the microtubular network and quickly respond to stimuli to regulate cell morphogenesis (Desai and Mitchison, 1997; Dhonukshe and Gadella, 2003). Both the orientation and distribution of microtubules observed in living hyphae matched those observed in fixed mature hyphae examined by immunofluorescence (Riquelme et al., 2002). We commonly observed the spindle of mitotic nuclei as a fluorescent “bar,” which represents a bundle of microtubules (Aist and Morris, 1999). We also observed apparent astral ray microtubules emanating from the ends of spindles and these sometimes appeared to be connected to the plasma membrane as in other species (Aist, 2002).

4.2. GFP expression in ascis and ascospores

Expression patterns of H1-GFP in giant Banana ascospores showed that H1 is not nucleus-limited during ascus development. Exchange of histone H1 from chromat in has been observed by fluorescence recovery after photobleaching (FRAP; Lever et al., 2000). We provide evidence for a protein gradient, resulting from the location of the hH1-sgfp+ nuclei in the giant Banana ascospores. This suggests that nuclei stay positioned and that hH1-sgfp transcripts are processed by ribosomes in the vicinity of hH1-sgfp+ nuclei.

Unlike previous immunofluorescence studies on fixed cells (Shiu et al., 2001; Thompson-Coffe et al., 1999), we were unable to visualize individual microtubules by conventional fluorescence microscopy. One explanation for this discrepancy is that overexpression of β-tubulin-GFP causes fluorescent “noise” or mislocalization. Alternatively, the fusion protein may not have been fully functional. Nuclei were sometimes discernable in the ascus and in young ascospores, apparently because of higher concentrations of β-tubulin-GFP in the vicinity of nuclei (e.g., see Thompson-Coffe and Zickler, 1992; Tran et al., 2001).

Both the GFP-tagged hH1 and Bml (β-tubulin) genes are expressed throughout ascus development when homozygous, but their expression is silenced in heterozygous crosses, at least until after ascospores are formed. Thus, both GFP fusions can serve as markers for meiotic silencing (Shiu et al., 2001). Silencing of β-tubulin arrested ascus development; almost all ascii were aborted at meiotic prophase, as observed previously (Shiu et al., 2001). Fission yeast with a defective β-tubulin gene also arrests in meiosis (Paluh et al., 2004). We found that silencing of H1-GFP did not interfere with ascus development; meiosis, postmeiotic mitoses, and early ascospore development all appeared normal. That histone H1 is not essential for ascus development and meiosis is consistent with previous findings with Neurospora (Folco et al., 2003) and A. nidulans (Ramon et al., 2000) mutants, and with strains of Asco bolus immersus in which the histone H1 gene was silenced by DNA methylation (Barra et al., 2000).

5. Conclusion

As demonstrated here by our studies with histone H1 and β-tubulin fusions, the potential applications of GFP fusions in Neurospora are numerous. Applications are not limited to studies of the subcellular localization of proteins (Freitag et al., 2004) or organelle dynamics (Fuchs et al., 2002). Clearly GFP will be a useful reporter for gene expression in Neurospora—perhaps in combination with genetic screens—and as an indicator for monitoring signal molecule dynamics, enzyme interactions, and protein–protein interactions.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2004.06.008
References


