Expression and visualization of Green Fluorescent Protein (GFP) in Neurospora crassa

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Abstract

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Expression and visualization of Green Fluorescent Protein (GFP) in *Neurospora crassa*

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We report the first successful imaging of GFP expression in *Neurospora crassa*. GFP was expressed under the control of the heterologous *ToxA* promoter from *Pyrenophora tritici-repentis* in transformants carrying multiple or single copies of the GFP construct. GFP was also detected in ascospores but not during earlier stages of the sexual cycle.

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Heterologous expression of Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* was first accomplished in *Escherichia coli* and *Caenorhabditis elegans* (M. Chalfie *et al.*, 1994, Science 263:802). GFP has quickly become one of the favorite protein markers in many experimental systems (M. Chalfie and S. Kain [Eds.], 1998, "Green Fluorescent Protein: Properties, Applications and Protocols", Wiley-Liss, 385 pp) including many fungi (for review see J. M. Lorang *et al.*, 2001, Appl. Env. Microbiol. 67:1987). Nevertheless, GFP constructs were not successfully expressed in *N. crassa*. Various forms of GFP under the control of constitutive or inducible fungal promoters or Neurospora-GFP fusion proteins have been tried but not found to be stably expressed at levels required for imaging.

In our most recent attempt to adapt GFP for *N. crassa*, we tested a transcriptional fusion of a strong fungal promoter with a GFP derivative of increased brightness ("S65T"; R. Heim *et al.*, 1995, Nature 373:663) and with the C+G-rich codon bias of humans and *N. crassa* (J. Haas *et al.*, 1996, Curr. Biol. 6:315). pCT74 contains the *E. coli hph* gene as selectable marker and a transcriptional fusion of the *Pyrenophora tritici-repentis ToxA* promoter (L. Ciuffetti *et al.*, 1997, Plant Cell 9:135) with the altered GFP, *sGFP*. *sGFP* (W. Chiu *et al.*, 1996, Curr. Biology 6:325) is identical to commercially available *eGFP* (Clontech). Transformation of other filamentous fungi with pCT74 resulted in GFP expression (J.M. Lorang *et al.*, 2001, Appl. Env. Microbiol. 67:1987).

We transformed a *N. crassa his-3* strain (FGSC# 6103) with pCT74 by electroporation (B.S. Margolin *et al.*, 1997, FGN 44:34 and 2000, FGN 47:112). Transformants were selected on minimal medium supplemented with histidine and hygromycin B (Hyg; 200 ug/ml). Variable expression of GFP was detected by microscopy in macroconidia and hyphae of transformants carrying randomly integrated copies of *ToxA-sGFP* (Fig. 1A). Germinated conidia accumulated enough GFP in hyphae to be detected after overnight incubation at 32° C. Expression in young hyphae was strong and fairly uniform in most single copy transformants, whereas older hyphae showed lower fluorescence. GFP appeared to accumulate in macroconidia. As has been observed in plants (J. Haseloff and B. Amos, 1995, Trends Genetics 11:328), GFP appeared to accumulate in the nucleoplasm and to be excluded from vacuoles.

GFP expression levels and patterns differed from transformant to transformant, perhaps because of varied copy number, integration site and/or gene silencing. We therefore targeted single copies of *sGFP* to the *his-3* locus (Fig. 1B). The resulting heterokaryotic transformants showed uniform and high-level expression of GFP.

To assess the expression of GFP in sexual tissues and the stability of GFP[+] transformants during the sexual phase, we crossed single copy GFP[+] strains to a wildtype strain (FGSC# 2490). GFP was expressed in protoperithicia and perithicia. Interestingly, GFP was not detected in dikaryotic and premeiotic tissue but was expressed in ascospores, regardless of whether GFP[+] strains were used as females or males in heterozygous crosses (Fig. 2). GFP was detected even in fully melanized ascospores (Fig. 2C). Expression of GFP in hyphae was first seen at approximately 24 hours after ascospore germination. GFP was detected in all vegetative tissues and mature ascospores in GFP[+] progeny from heterozygous crosses.

One explanation for past difficulties in creating strongly expressing Neurospora-GFP fusion proteins may be the relative strength of the promoters employed. The only published report of using a *N. crassa* promoter to drive GFP expression in fungi that we are aware of relates to experiments in *Mycosphaerella graminicola*, in which the *acu-3* (isocitrate lyase) promoter region was used as a carbon source-repressed promoter (E.A. Rohel *et al.*, 2001, Mol. Plant Microbe Interactions 14:156). In an attempt to find additional *N. crassa* promoters for GFP expression we performed a "promoter trap" screen. We co-transformed a *N. crassa his-3* strain (FGSC# 6103) with a selectable marker (*hph* or *his-3*[+]) and various *ToxA-sGFP* fragments lacking a functional promoter. We screened approximately 10,000 transformants but were unable to find strains that stably expressed GFP without the full *ToxA* promoter. While our screen was not saturating, i.e. GFP could not have properly inserted in every promoter region of the *N. crassa* genome, it appears unlikely that this approach will result in the identification of strong promoters in *N. crassa*.

Previous failures to detect GFP expression may also be explained by gene silencing, for example by quelling and/or DNA methylation (E. Selker, 1997, Trends Genetics 13:296). We did not find any evidence for quelling of GFP in transformations with either pCT74 or *ToxA-sGFP* fragments. To explore the possibility that methylation was responsible for complete or partial silencing of GFP genes in *N. crassa*, we transformed *N. crassa* (FGSC# 6103) with *his-3*[+] DNA from pNH60 (T.L. Legerton and C. Yanofsky, 1985, Gene 39:129) and DNA fragments with the promoterless *sGFP* (from pCT74) or jellyfish *GFP* (from pRSET_B-GFP or pGFP10.1) gene and isolated twenty transformants carrying each construct. Surprisingly, we found that neither GFP gene alone was efficiently methylated in vegetative tissue of *N. crassa* (Fig. 3 and data not shown). Jellyfish *GFP* was predicted to be methylated because it has relatively high A+T content (62%) and a high TpA/ApT ratio (0.71; GenBank accession number M62653), features that mimic RIP-mutated *N. crassa* DNA which is usually methylated (V. Miao *et al.*, 2000, J. Mol. Biol. 300:249). Modified *sGFP* and *eGFP* have low A+T content (39%) and a somewhat lower TpA/ApT ratio (0.67; accession number U43284) and thus were predicted to be unmethylated. In some transformants with *ToxA-sGFP* or promoterless *sGFP* some methylation was observed (e.g. TMF166-1, -5 and -8 in Fig. 3A) but presence of methylation did not always correlate with

gene silencing. As expected, methylation was detected in *sGFP* genes that had undergone RIP (Fig. 3A, lanes 15 and 16) because sequences mutated by RIP can serve as efficient *de novo* methylation signals in vegetative tissues (M. Singer *et al.*, 1995, Mol. Cell. Biol. 15:5586). We also found methylation in some multicopy pCT74 inserts that nevertheless expressed GFP at high levels (Fig. 3B). In these transformants, *hph* and plasmid fragments apparently promoted methylation, consistent with earlier results with chimeric plasmids (E. Selker *et al.*, 1987, Science 238:48).   These results encourage us to predict that transcriptional or translational fusions of fungal promoters or protein-coding regions to *GFP* will not be silenced by methylation in *N. crassa*.

　　　　Our results show that expression of GFP in *N. crassa* can be achieved by using transcriptional fusions of a strong fungal promoter and *sGFP*. Under our conditions, GFP had to accumulate for at least 16 hours in hyphae before visualization was possible. We created a set of GFP plasmids that allow *sGFP* targeting to the *his-3* locus. It is unlikely that *sGFP* in future Neurospora-GFP fusion constructs will be silenced by methylation if single copies of genes are integrated into the genome by replacement strategies. Likewise, we have not found evidence for efficient quelling of GFP. GFP should immediately prove useful as an ascospore color marker, in genetic screens and for gene expression studies. Neurospora-GFP fusion proteins under construction in our lab will allow studies on the subcellular localization of organelles and proteins and will facilitate cell biology research with *N. crassa*.

Figure 1 (Following page): Expression of GFP in *N. crassa*. (A) GFP in 3-day old hyphae and macroconidia of a heterokaryotic primary transformant (TMF153-9) grown on minimal medium supplemented with Hyg. (B) Expression of sGFP was uniform in GFP[+] His[+] transformants in which *ToxA-sGFP* had been targeted to the *his-3* locus (N2002). pMF245 (*ToxA-sGFP* without terminator) and pMF246 (*ToxA-sGFP* with the *nos* terminator), respectively, were created by inserting *Apa*I - *Not*I or *Apa*I - *Eco*RI fragments of pCT74 into the same sites of pBM61 (B.S. Margolin *et al.*, 1997, FGN 44:34). As expected, we found that the nopaline synthase (*nos*) terminator did not affect expression of GFP in *N. crassa* (data not shown). To detect GFP microscopically, we used a Zeiss Axioscope with a 100X oil immersion Plan-Neofluar objective (N.A. 1.3 mm), a Zeiss filter set (green excitation filter 450-490 HB) and a Zeiss AttoArc2 HB-100W mercury lamp. Images were captured with a Micromax EBF512 camera (Princeton Instruments) and processed with the MetaMorph Imaging System (Universal Imaging Corp.) and Adobe Photoshop software. Typical exposure times were 5 - 15 ms for both GFP and Nomarski images. Color reproductions of figures shown will be made available with the on-line version of this article on the FGSC website (http://www.fgsc.net/).
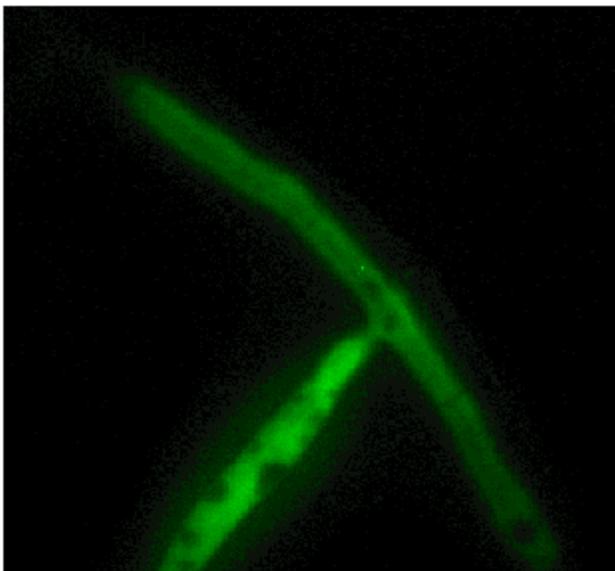
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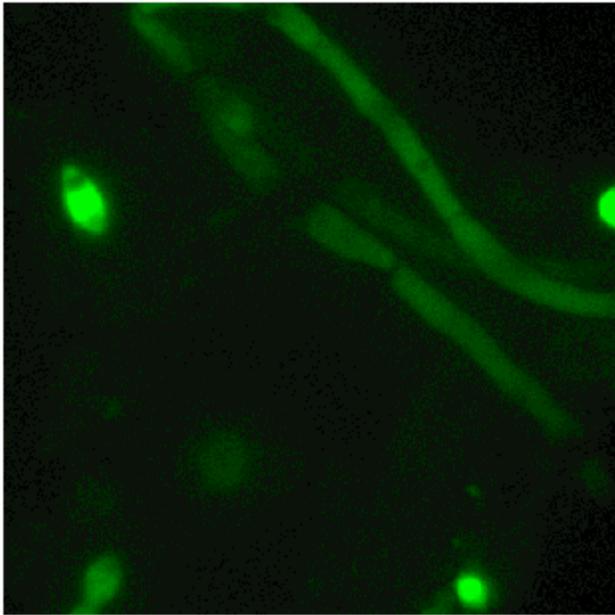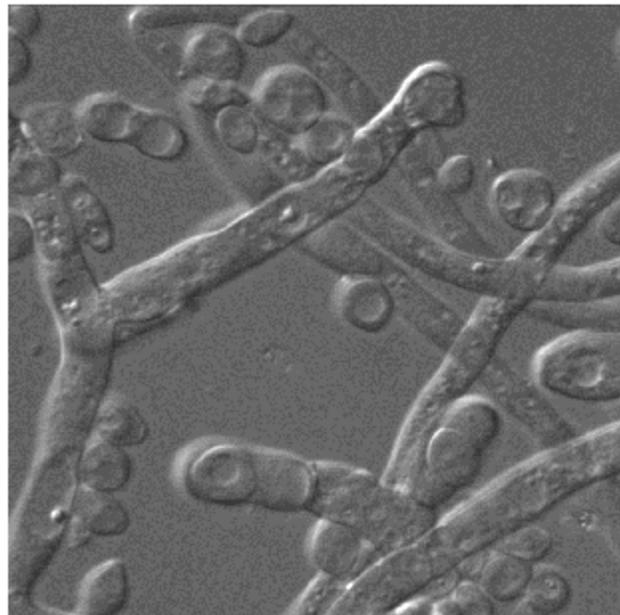
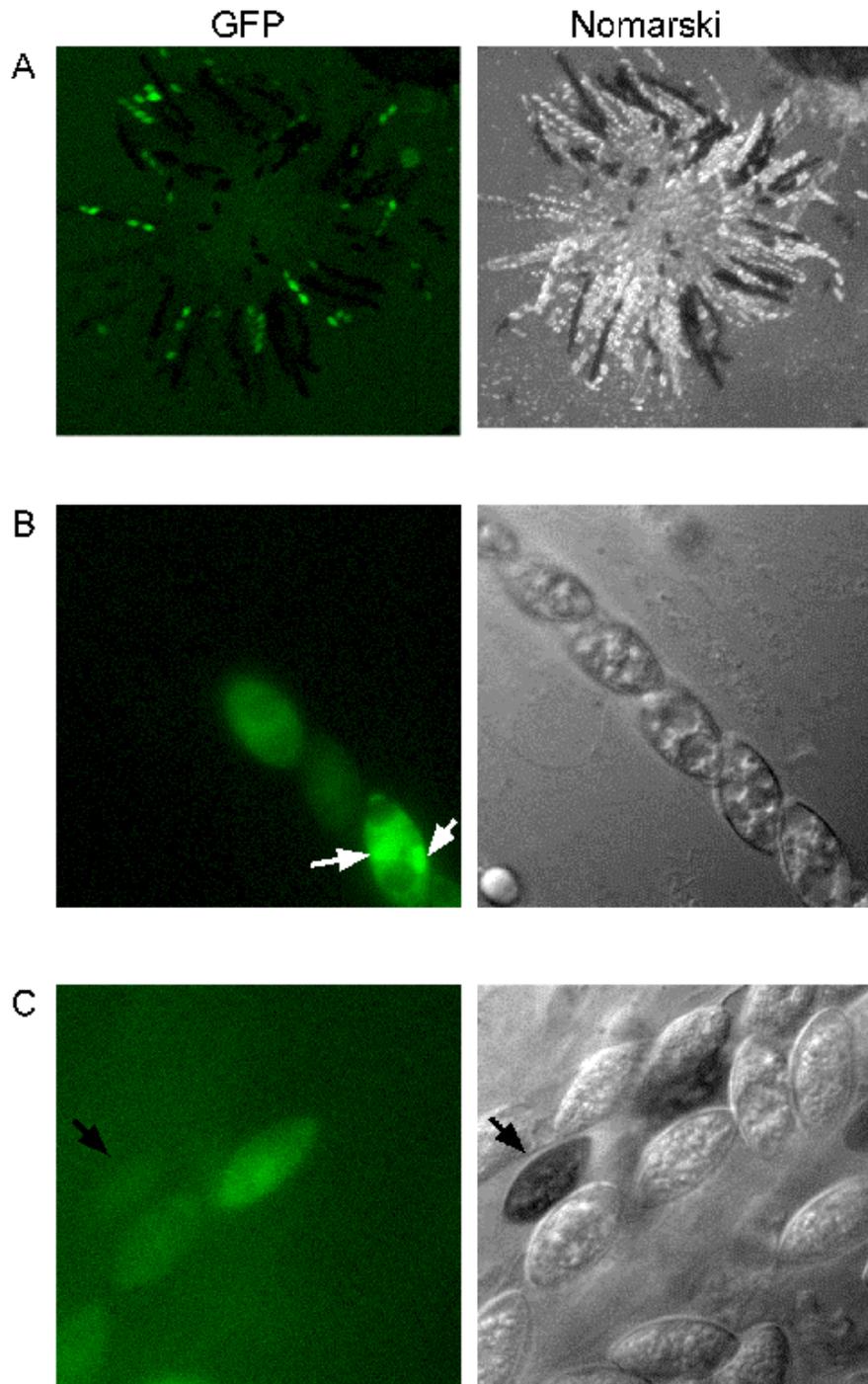GFP   Nomarski

B

Figure 2: Expression of GFP in sexual tissues. (A) Perithicia from a heterozygous cross of the heterokaryotic GFP$^+$ strain TMF153-9 (male) to FGSC #2490 (female) were squashed at approximately 10 - 14 days post fertilization to extrude developing asci and examined for GFP expression. Tissue in early stages of post fertilization development apparently did not express GFP. GFP$^+$ / GFP$^-$ segregation patterns are evident. Images were captured as described in Fig.1, except that a 5X Plan-Neofluar objective (N.A. 0.15 mm) was used. Exposure times had to be increased to 100 ms; exposure times for the Nomarski images were approximately 10 ms. (B) Segregation of GFP$^-$ / GFP$^+$ ascospores in one ascus. GFP appears to accumulate in nuclei (white arrows). (C) GFP can be detected even in melanized ascospores (black arrow). Images in (B) and (C) were captured as described in Fig. 1.
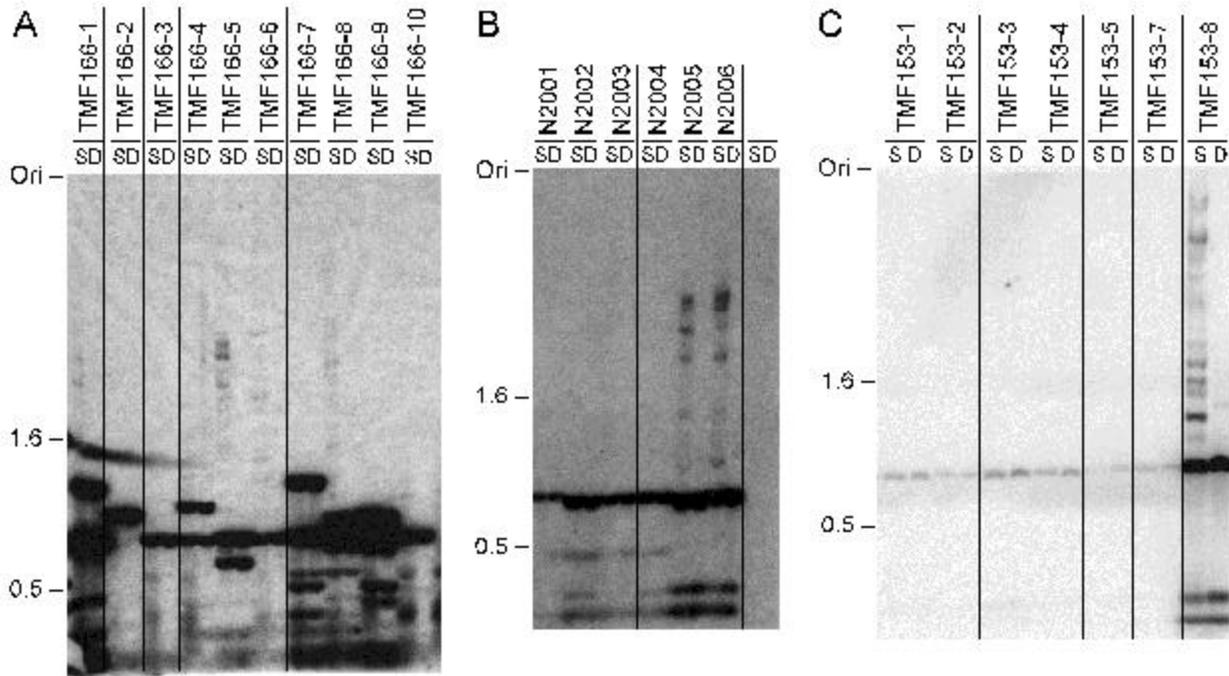
Figure 3: DNA methylation analysis of selected *sGFP⁺* transformants. (A) Southern analysis of genomic DNA isolated from transformants with randomly inserted copies of *sGFP* showed that *sGFP* remained mostly unmethylated (TMF166-1 to -10). Genomic DNA of *sGFP⁺* transformants was digested with methylation-sensitive (*Sau*3AI; S) or methylation-insensitive (*Dpn*II; D) isoschizomers, fractionated in agarose gels, blotted to nylon membranes and probed with a radioactively labeled *sGFP* fragment. (B) Single copies of *sGFP* integrated at *his-3* were always unmethylated (N2001 to N2004), whereas RIP-mutated alleles of *sGFP* were methylated (N2005 and N2006); lane H shows the non-transformed host strain (FGSC# 6103). (C) *SGFP* was unmethylated in transformants (TMF153-1 to -7) with single copies of pCT74, but methylated in a transformant (TMF153-8) with multiple copies of the plasmid. GFP was expressed at high levels in all seven strains. The position of size markers (kb) and the gel origin (Ori) are indicated.