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MINIREVIEW

Green Fluorescent Protein Is Lighting Up Fungal Biology

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Prasher (42) cloned a cDNA for the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* in 1992. Shortly thereafter, to the amazement of many investigators, this gene or derivatives thereof were successfully expressed and conferred fluorescence to bacteria and *Caenorhabditis elegans* cells in culture (10, 31), followed by yeast (24, 39), mammals (40), *Drosophila* (66), *Dictyostelium* (23, 30), plants (28, 49), and filamentous fungi (54). The tremendous success of GFP as a reporter can be attributed to unique qualities of this 238-amino-acid, 27-kDa protein which absorbs light at maxima of 395 and 475 nm and emits light at a maximum of 508 nm. The fluorescence of GFP requires only UV or blue light and oxygen, and therefore, unlike the case with other reporters (β-glucuronidase, β-galacturonidase, chloramphenicol acetyltransferase, and firefly luciferase) that rely on cofactors or substrates for activity, in vivo observation of *gfp* expression is possible with individual cells, with cell populations, or in whole organisms interacting with symbionts or environments in real time. Complications caused by destructive sampling, cell permeablization for substrates, or leakage of products do not occur. Furthermore, the GFP protein is extremely stable in vivo and has been fused to the C or N terminus of many cellular and extracellular proteins without a loss of activity, thereby permitting the tagging of proteins for gene regulation analysis, protein localization, or specific organelle labeling. The mature protein resists many proteases and is stable up to 65°C and at pH 5 to 11, in 1% sodium dodecyl sulfate or 6 M guanidinium chloride (reviewed in references 17 and 67), and in tissue fixed with formaldehyde, methanol, or glutaraldehyde. However, GFP loses fluorescence in methanol-acetic acid (3:1) and can be masked by autofluorescent aldehyde groups in tissue fixed with glutaraldehyde. Fluorescence is optimal at pH 7.2 to 8.0 (67).

Limitations on GFP as a reporter for some applications are its low turnover rate, 2-h lag time for autoactivation of its chromophore, improper folding at high temperatures (37°C), which results in nonfluorescent and insoluble forms of the protein, and requirement for oxygen, which is not present in equal concentrations in all subcellular locations or cell types (reviewed in references 17 and 67). These characteristics of GFP, however, have not posed a problem for many applications, and mutant forms of GFP that have an ability to fold properly at high temperatures, increased solubility and fluorescence, reduced photobleaching (16, 17, 51), and reduced half-lives (1) have been developed. Coupled with fluorescence-activated cell sorting, confocal microscopy or quantitative image analysis techniques, GFP technology can be used to isolate transformed cells or specific cell types from populations of cells (14), to quantify gene expression of individual cells within whole organisms (8), or to assess the dispersal and biomass of organisms in complex environments, such as in animal or plant hosts (38, 59), in biofilms (55), in fermentors (41), on leaf surfaces (53, 61), or in soils (2).

The vast majority of studies utilizing GFP expression in fungi have been with yeast (reviewed in reference 13). *Ustilago maydis* was the first filamentous fungus for which successful expression of *gfp* was reported (54), followed closely by *Aspergillus nidulans* (22, 57) and *Aureobasidium pullulans* (61). Presently, *gfp* expression has been reported for 16 species comprising 12 genera of filamentous fungi, including *Colletotrichum* (21, 44), *Mycosphaerella* (52), *Magnaporthe* (32, 35), *Cochliobolus* (38), *Trichoderma* (2, 70), *Podospora* (5), *Sclerotinia* (63), *Schizophyllum* (37), *Aspergillus* (20, 47, 50) and *Phytophthora* (7, 62). In this review we draw on published reports, with the goal of providing an overview of GFP technology as it applies to the biology of filamentous fungi. These reports are not exhaustive of potential applications of GFP technology, as examples of genomic approaches to utilizing GFP in bacterial and yeast systems attest (4, 46, 60, 65).

Expression of *gfp* in filamentous fungi requires a *gfp* variant that is efficiently translated in fungi, a transformation system, and a fungal promoter that satisfies the requirements of a given experimental objective. Transformation of fungi has recently been reviewed by Gold et al. (26). Robinson and Sharon (44) suggest that GFP can actually be used to optimize transformation protocols. In addition to reporting the construction of a new fungal transformation vector that expresses *SGFP* under the control of the *ToxA* gene promoter from *Pyrenophora tritici-repentis* (12) and demonstrating its use in plant pathogens belonging to eight different genera of filamentous fungi (*Fusarium, Botrytis, Pyrenophora, Alternaria, Cochliobolus, Sclerotinia, Colletotrichum*, and *Verticillium*), in this review we also

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enumerate and describe a comprehensive list of vectors for expressing GFP in fungi.

WILD-TYPE AND ENGINEERED *GFP* GENES

The wild-type *A. victoria gfp* gene does not confer appreciable fluorescence to many fungi, primarily because it is not efficiently translated (15, 22, 38, 54). Modified forms of *gfp* with optimized codon usage for yeast (15), plants (11), or mammals (27), and whose proteins have increased fluorescence and solubility and decreased photobleaching (16, 17, 51), have been developed. Most of these *gfp* variants express proteins (SGFP, yEGFP, and EGFP1) that contain a serine-to-threonine substitution at amino acid 65 (S65T) that, aside from conferring the above-described benefits, causes a "red shift" from excitation maxima of 395 and 470 nm to a maximum of 488 nm. Red-shifted GFPs are not easily detected on a UV light box or with a hand-held UV light, as detection near 508 nm with blue light (488 nm) excitation requires special filters (fluorescein isothiocyanate; Lieca, Zeiss); however, they are ideally suited for fluorescence microscopy and fluorescence-activated cell sorting.

Fernández-Ábalos et al. (22) reported on the expression of four *gfp* variants driven by a common promoter in *A. nidulans* and concluded that *SGFP* (11) conferred the highest GFP concentration and level of fluorescence to transformants. *SGFP* contains the S65T mutation as well as plant-optimized codon usage that also deletes a cryptic intron splice site reported to reduce GFP expression in *Arabidopsis* (29). *SGFP* (Blue-*SGFP-TYG*) has been the *gfp* gene most often used for transformation of filamentous fungi. *EGFP1* (Clonetech, Inc., Palo Alto, Calif.) is similar to *SGFP* in that it contains the S65T mutation and 190 silent base mutations corresponding to human codon usage preferences (68). *EGFP1* has been successfully expressed to high levels in *Aspergillus flavus, A. pullulans, Magnaporthe grisea*, and *Podospora anserina* (5, 20, 35, 62). y*EGFP* contains the S65T mutation and codon usage optimized specifically for *Candida albicans* (15). *SGFP* and *EGFP1* both confer high levels of fluorescence in fungi, but a direct quantitative analysis comparing the expression of these genes driven by a common fungal promoter has not yet been reported.

The choice of *gfp* gene ultimately depends on codon or other preferences of the organism to be transformed, the strength of the promoter driving *gfp*, and the method of fluorescence detection. For example, Lugones et al. (37) found that the fluorescence of transgenic *Schizophyllum commune* required an *S. commune* intron inserted after the stop codon of *SGFP*, thus confirming their hypothesis that introns are required for proper transcript processing in this fungus. In plants, wild-type *gfp* was transiently expressed to high levels with either a maize universal transcription enhancer or a tobacco mosaic virus translational enhancer and thus conferred fluorescence. In subsequent plant transformations with less-robust expression systems, however, *SGFP* was required to produce detectable levels of fluorescence (11, 49). *EGFP1* fused to a nuclear localization signal and driven by the human cytomegalovirus (*cmv*) promoter produced fluorescence sufficient for detection of *A. flavus* transformants on a UV light box with no additional filters. In the absence of such a strong promoter, excitation at the optimal 488 nm for EGFP1 and the appropriate fluorescein isothiocyanate filters would have been necessary to visualize fluorescence (20). *EBFP* (Clonetech) is a *gfp* variant that expresses a blue fluorescent protein. Although its use has not yet been reported in fungi, EBFP can be differentiated from GFP when both proteins are simultaneously expressed and thus affords opportunities for double-labeling studies.

THE UTILITY OF GFP AS A REPORTER GENE

GFP particularly shines as a reporter because it affords detection of gene expression and protein localization that is continuous in time and development within a single living specimen. These properties are in marked contrast to the destructive sampling required by other reporters that limit observations to a moment in time, each data point being derived from a different individual. The simplicity of in vivo detection that does not require additional substrates or fixing of tissue makes GFP an excellent tool for teaching transformation and gene expression in a classroom setting. Furthermore, *gfp* gene expression is easily quantified in whole cultures via fluorimetry (22, 54) or in individual cells or subcellular compartments with confocal microscopy (35, 53). For example, confocal observation of *M. grisea* expressing a fusion of *EGFP1* and a calmodulin gene required for appressorium formation (*CAMmg*) revealed that expression of *CAMmg* requires surface attachment of conidia and is inhibited by self-inhibitors of germination. Furthermore, *CAMmg* inhibition could be reversed by the addition of plant waxes (35). Also with *M. grisea*, Kershaw et al. (32) investigated the functional relationship of fungal hydrophobins by using the *mpg1* gene promoter fused to *SGFP*. Fluorescence present in conidia and in appressoria did not migrate into infection pegs or hyphae, indicating that *mgp1* expression is restricted to conidial development and early infection events prior to plant penetration. In *U. maydis, SGFP* was used to delineate the promoter of *Crgl*, a gene regulated by carbon sources. Relative fluorescence of arabinose-induced transformants in this system did not correlate with Northern blot analysis of *Crg1*, which led Bottin et al. to conclude that SGFP was too stable for quantitative determination of *Crg1* expression in some individuals and may have aggregated, thus reducing fluorescence in other transformants (6). Expression of an endopolygalacturonase gene from *Colletotrichum lindemuthianum* (*clpg2*) transcriptionally fused to *SGFP*, on the other hand, was visualized with epifluorescence microscopy at early stages of conidial germination and appressorium formation, and an identical pattern of gene expression was confirmed by reverse transcription-PCR (21).

gfp translational fusions for evaluating gene expression and protein localization also have been successful in fungi. A fusion of the *U. maydis* motor protein (KIN2) to SGFP produced a functional fusion protein that localized to the cytoplasm or submicroscopic vesicles in a specialized growth zone at the hyphal tip under normal circumstances but is associated with microtubules when oxidative phosphorylation is uncoupled (34). The fact that both GFP and other protein domains can function simultaneously in fusion proteins allows precise investigation of cellular proteins under diverse physiological circumstances, as Lehmler et al. have clearly demonstrated. GFP

protein fusions also have been used extensively to study eukaryotic cellular dynamics.

INVESTIGATING CELL DYNAMICS WITH GFP

In vivo detection over time, the hallmark of GFP, is perhaps best appreciated when considering the cellular biology of filamentous fungi. Due to their simple anatomy and rapid growth rates, filamentous fungi are ideally suited for such investigations. When viewed by video epifluorescence microscopy, GFP-transformed fungi can provide a heretofore unprecedented perspective and appreciation of fungal cell dynamics. This capability has been eloquently demonstrated with *A. nidulans* (56, 57), a model system for investigating the molecular basis of eukaryotic, cellular morphogenesis (reviewed in references 3 and 19). Nucleus-targeted GFP in *A. nidulans* allowed real-time visualization of nuclear migration and mitosis, detailing for the first time the behavior of specific nuclei at various developmental stages (22, 57). Optically sectioned nuclei in the early stages of mitosis revealed previously undescribed chromosomal behaviors (22), and epifluorescence video microscopy facilitated identification of new mutant phenotypes and, ultimately, the genes affecting nuclear migration (see the video at http://www.blacksci.co.uk/products/journals/molextra.htm.) (57, 58).

GFP also has been targeted to the endoplasmic reticulum (ER) and mitochondria of *A. nidulans*. Optical sectioning through hyphae transformed with ER-targeted *gfp* illuminated a branching tubular network that extends throughout the cell to within 1 to 2 μm from the tip, surrounds the nucleus, and contains variously shaped, bright nodes (22, 56). GFP-tagged mitochondria created in *A. nidulans* by fusing the N terminus of a citrate synthase gene to *SGFP* (56) allowed novel descriptions of dynamic behavior in the mitochondrial reticular network; frequent fragmentation and then fusion to restore the network occurred along with "snakelike elongation and retraction of the tubular network." Mitochondria migrated into the tips of growing hyphae and moved bidirectionally at velocities as high as 15 μm/min (see the video at http://www.uni-marburg.de/mpi/movies/mitochondria/mitochondria.html) (56). These investigators also demonstrated that a functional actin cytoskeleton is required for mitochondrial morphology and dynamic behavior of the mitochondrial network, but *MyoA*, a myosin motor protein involved in vesicle migration, is not responsible for mitochondrial movement. Specific tagging of organelles with GFP localized to the nucleus, mitochondria, ER, peroxisome, vacuole, and plasma membrane also has been accomplished in yeast (reviewed in reference 13) and will undoubtedly provide research strategies for future work with filamentous fungi. Thus, by providing a real-time tool for visualizing organelles and subcellular structures, GFP is facilitating the discovery of new genes and gene products that participate in specific cellular functions associated with the tagged structures.

VISUALIZING FUNGI IN THEIR ENVIRONMENTS: HOST-PATHOGEN INTERACTIONS, MYCOPARASITISM, AND THE PHYLLOSPHERE

Important biological questions in fungal systems often consider fungi in their natural and sometimes not-so-natural environments, such as in soil, decaying organic matter, or fermentors and in living plant, animal, or fungal hosts. In fact, filamentous fungi that have been transformed with *gfp* thus far are all plant pathogens or residents of plant surfaces, and genes of interest in these systems are those that play roles in host-fungus interactions. The *C. lindemuthianum clpg2* gene and the *M. grisea camMg* and *mgpl* genes discussed above, for example, are expressed during fungal developmental stages that are crucial to successful infection of a plant host. Similarly, the extracellular enzyme genes *nagl* and *ech42* from the mycoparasite *Trichoderma harzianum* were shown to be induced by the host *Rhizoctonia solani* (70). The merits of GFP in elucidating regulation of such genes and the cellular location of their protein products in situ have been addressed above. Tracking fungal strains that carry mutations for or that overexpress such genes in planta is also of great interest.

To facilitate the tracking of fungi in planta, to monitor their distribution, and to estimate their biomass, whole fungi have been labeled with GFP. A discussion of this topic is inextricably tied to methods for visualizing and quantifying GFP. Spear et al. (53) used *A. pullulans*, a fungus that inhabits the phylloplane, to develop an excellent collection of GFP detection methods which should be consulted by any investigator considering purchasing optical equipment or software for this purpose. These authors review protocols for laser scanning confocal microscopy and contrast these to conventional epifluorescence microscopy using a computer-controlled, z-stepper motor (Ludl Electronics Products, Ltd., Hawthorne, N.Y.) (Volume Scan PC software; Vaytech, Fairfield, Iowa) and filters in conjunction with a cooled charged-coupled device video camera (DEI-470; Opronices Engineering, Goleta, Calif.) and digital deconvolution software (Vaytech, for personal computers [PC] or Macintosh computers). Data gathered by either method can be analyzed with image analysis software (Optimas v6.2 for PC; Media Cybernetics, Del Mar, Calif.). Similarly, Maor et al. (38) followed phytopathogenic development of the fungus *Cochliobolus heterostrophus* inside maize leaves and were able to correlate fluorescence with mycelial mass and disease levels by using a charged-coupled device camera and digital analysis by PMIS image analysis software (for PC; Photometrics, Tucson, Ariz.).

Labeling whole fungi with GFP generally requires strong constitutive expression of *gfp*, which typically results in a cytoplasmically located protein occurring in all fungal morphotypes (hyphae, spores, appressoria, etc.) with no obvious effects on fungal growth or pathogenicity (38, 54, 62; this work). In our experience with GFP tagging of plant pathogenic fungi, SGFP was easily detected with epifluorescence microscopy (Leica DMRB and Endow GFP filter cube, exciter HQ470/40, emitter HQ525/50 with beamsplitter Q495LP) (Fig. 1). Transformants generated from uninucleate protoplasts fluoresced more or less uniformly (*Colletotrichum magna, Verticillium dahliae, Alternaria alternata, Cochliobolus sativus, Fusarium sambucinum*), whereas transformants generated from multinucleate protoplasts (*Botrytis cinerea, P. tritici-repentis*, and *Sclerotinia sclerotiorum*) often exhibited fluorescence only in some fungal cells that continued to sector for the fluorescent phenotype and thus required further purification by subculturing of hyphal tips and serial transfers on selective media. Visualizing pathogens that uniformly express GFP in planta can facilitate a diversity of

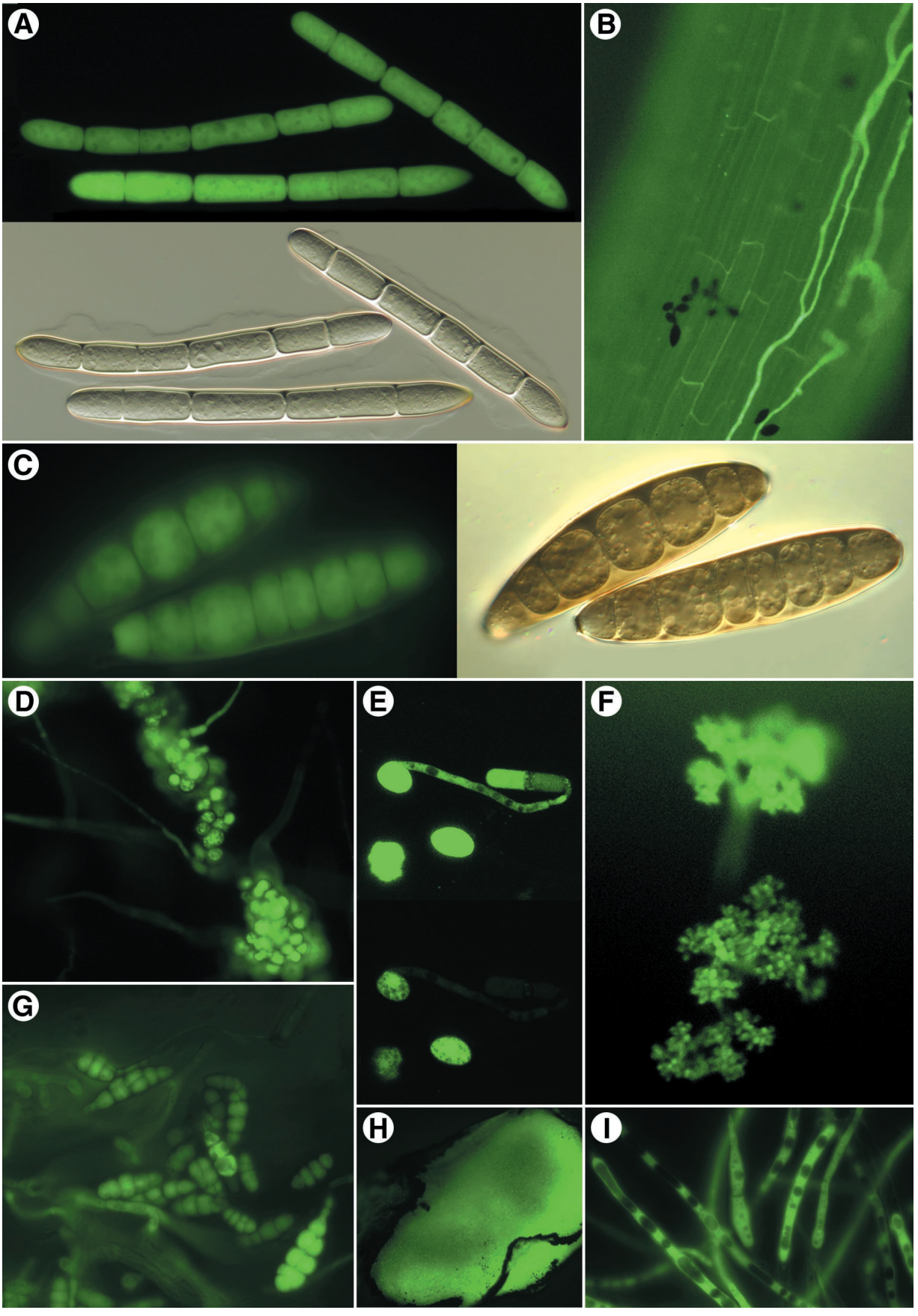
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TABLE 1. A selection of GFP expression vectors for transformation of filamentous fungi

Construct (reference)	*gfp* gene	Promoter driving *gfp*, origin	Expression, localization features	Fungal species transformed
pOTEF-SG (54)	*SGFP*	*Otef,* modified *tef, U. maydis*	Constitutive, no localization	*U. maydis*
pMFA1-SG (54)	*SGFP*	*mfa1, U. maydis*	*Mfa1* phermone-inducible, no localization	*U. maydis*
pCRGFP2 (6)	*SGFP*	*crg1, U. maydis*	L-Arabinose-inducible, D-glucose and D-xylose repressible, no localization	*U. maydis*
pNUC'EM2 (20)	*EGFP*	*cmv,* human cytomegalovirus	Constitutive, SV40 nuclear localization signal	*A. flavus*
pCT74 (this review)	*SGFP*	*ToxA, P. tritici-repentis*	Constitutive, no localization	*C. magna, P. tritici-repentis, S. sclerotiorum, Colletotrichum trifolii, V. dahliae, A. alternata, B. cinerea, C. sativus, F. sambucinum*
pCMB32 (22)	*SGFP*	*alcA, A. nidulans*	Alcohol-inducible, no localization	*A. nidulans*
pCMB41 (22)	*SGFP*	*alcA, A. nidulans*	Alcohol-inducible, plant chitinase (*mgfp5*) signal peptide, ER retention signal (His-Asp-Glu-Leu)	*A. nidulans*
pCMB42 (22)	*SGFP*	*alcA, A. nidulans*	Alcohol-inducible, GAL4BD fusion for nuclear localization	*A. nidulans*
pRS31 (57)	*SGFP*	*gpd, A. niger*	Constitutive, *StuA* fusion for nuclear localization	*A. nidulans*
pRS54 (56)	*SGFP*	*gpd, A. niger*	Constitutive, citrate synthase fusion for mitochondrial localization	*A. nidulans*
gGFP (38, 44)	*SFGP*	*gpd, A. nidulans*	Constitutive, no localization	*C. heterostrophus, A. nidulans*
tGFP (38)	*SGFP*	*trpC, A. nidulans*	Constitutive, no localization	*C. heterostrophus*
pTEFEGFP (2, 61)	*EGFP*	*tef, A. pullulans*	Constitutive, no localization	*A. pullulans, T. harzianum*
pVW2 (62)	*EGFP*	*ham 34, Bremia lactucae*	Constitutive, no localization	*Phytophthora palmivora*
pAHB6 (6)	*SGFP*	*hsp70, B. lactucae*	Basal level constitutive, heat inducible, no localization	*Phytophthora parasitica* var. *nicotianae*

studies, including, for example, evaluations of susceptibility and resistance to *S. sclerotiorum* in plants transgenic for cell death pathway genes (Rollins and Dickman, unpublished data), distribution and migration of *F. sambucinum* on hops (Cynthia Ocamb, personal communication), colonization of plants by *Colletotrichum* mutants that express commensalistic rather than pathogenic lifestyles (43; Redman and Rodriguez, unpublished data), microsclerotial density determinations of *V. dahliae* from soil (48), and efficacy of biocontrol agents for control of *B. cinerea* on fruit and nursery crops (Walt Mahaffee, personal communication).

Targeting of GFP to the nucleus for tracking *A. flavus* in planta also has been successful and has resulted in strong fluorescence of infected corn kernels that could be visualized with a standard UV light (20). Du et al. suggest using this technique to screen corn genotypes for resistance to *A. flavus*.

Although necrotic cells in some plants contain phenolics that autofluoresce in the same range as GFP, chlorophyll, anthocyanins, and other plant products do not generally interfere with detection of SGFP (54; personal observations). Autofluorescence, however, varies among plant species and tissue types, and thus the choice of filters for viewing GFP-labeled fungi in planta should be empirically determined. Chloroplasts autofluoresce red when viewed with long-pass filters, providing a useful counter-fluor to GFP that aids in distinguishing fungal and plant tissues. The optical sectioning capability of confocal microscopy is also advantageous in that it affords clear visualization of GFP despite the autofluorescence and light-scattering properties of plant cell walls (28).

VECTORS FOR GFP TAGGING OF FILAMENTOUS FUNGI

GFP expression vectors have been developed for all major classes of filamentous fungi: basidiomycetes, ascomycetes, and oomycetes (Table 1). Which vector one chooses for a given application, obviously, will depend on the fungus to be transformed and the application. Vectors for targeting GFP to specific organelles, for expressing GFP constitutively, or for inducing and repressing GFP expression are available, but the utility of any given vector for widespread use is not known because the function of most vectors has been demonstrated in only a few fungi. Using GFP to investigate complex processes in fungi requires promoters that function in a given fungus for expressing not only GFP but also selectable markers and other proteins of interest. The simultaneous need for several promoters can be problematic in that limited numbers of fungal promoters have been characterized, and the strength of pro-

FIG. 1. Micrographs of fungi transformed with pCT74. Epifluorescence (A through I), Nomarski (A and C, second panel), or confocal (E) microscopy of *P. tritici-repentis* conidia (magnification, ×400) (A), a mint root infected with *V. dahliae* (magnification, ×200) (B), *C. sativus* conidia (magnification, ×1,000) (C), microsclerotia of *V. dahliae* (magnification, ×400) (D), two serial sections of a *C. magna* germinated conidia with germ tube and appressoria (E), *B. cinerea* conidiophores (magnification, ×100) (F), *A. alternata* hyphae and conidia (magnification, ×400) (G), cross-section of an *S. sclerotiorum* sclerotium (magnification, ×200) (H), and *F. sambucinum* hyphae (magnification, ×400) (I).

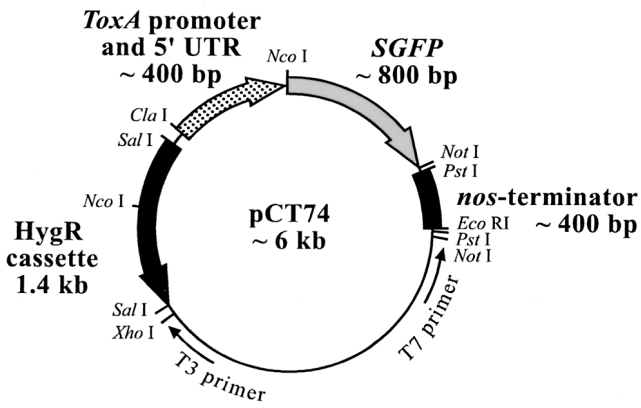

FIG. 2. A GFP expression vector for filamentous fungi, pCT74. pCT74 is based on pBlue-SGFP-TYG-nos-KS (from Jen Sheen, Department of Molecular Biology, Massachusetts General Hospital, Harvard University), which contains SGFP-TYG on an *Nco*I/*Not*I DNA fragment inserted into the *Eco*RV site and the *nos* terminator on a *Pst*I/*Eco*RI DNA fragment inserted into the *Eco*RI site of pBluescript KS (Stratagene, La Jolla, Calif.). An *Nco*I site containing the start codon of *SGFP* and a *Not*I site replaced *Eco*RV in this process. A 417-bp PCR product containing ~325 bp of the promoter region and ~90 bp of the 5′ untranslated region of the *ToxA* gene (12) was generated by PCR amplification from a genomic template with primers 28 (5′-TAGTGGA**CTGAT**TGGAATGCATGGAGGAGT-3′) and 29 (5′-GATAGAAC**CCATGG**CCTATATTCATTCAAT-3′). Engineered *Cla*I and *Nco*I sites, respectively, are in bold, and naturally occurring *Bam*HI and *Nco*I sites within the *ToxA* promoter region were destroyed in this process. This 417-bp fragment was ligated into the *Nco*I/*Cla*I sites of pBlue-SGFP-TYG-nos-KS, resulting in pCT73. A 1.4-kb *Sal*I fragment containing the modified hygromycin resistance gene *hph* under the control of the *trpC* promoter (9) was ligated into the *Sal*I site of pCT73, resulting in pCT74. Note that *Not*I and *Nco*I are not unique restriction sites in pCT74, and not all unique sites in the pBluescript KS polylinker are shown.

moters in heterologous fungi can vary (reviewed in reference 26) (personal observations).

We examined a number of GFP expression vectors, including those harboring *Aspergillus, Neurospora*, or *Colletotrichum* promoters, all of which resulted in unacceptably low levels of GFP expression for in planta visualization of fungi belonging to several genera. We were able to achieve strong constitutive expression of SGFP in all of eight genera of plant pathogenic fungi of interest to our laboratories (Fig. 1) using a transformation vector we constructed (pCT74), which expresses SGFP from the *ToxA* promoter of *P. tritici-repentis* (Fig. 2). Features of the *ToxA* promoter that confer versatile, high-level, constitutive gene expression have not yet been elucidated; nonetheless, pCT74 proved useful for expressing GFP in the fungi tested thus far, all of which are members of the Ascomycota. A complete selection of GFP expression vectors and features that distinguish these constructs with respect to GFP expression, protein localization, and fungal species in which fluorescence was conferred are outlined in Table 1.

GFP AND THE FUTURE OF FUNGAL BIOLOGY

Recently the complete sequence of the *S. cerevisiae* genome was determined, and genome projects for several filamentous fungi, including *A. nidulans, Neurospora crassa* (http://gene.genetics.uga.edu/), *C. heterostrophus, U. maydis, Phytophthora* spp., and *M. grisea* are well under way (36, 45, 69). The elucidation of promoter sequences for every gene in all of these organisms is inevitable. Microarray, digital imaging, and bioinformatics technologies have rapidly become more sophisticated, resulting in high-throughput systems that detect, store, and display complex fluorescent images of subcellular components, individual cells, whole organisms, or simple microarrays. How will GFP technology function in conjunction with these developments to impact the future of fungal biology?

Current microarray technologies can analyze the expression of hundreds of genes simultaneously in response to a given environmental regimen; still, they require RNA extraction and cDNA synthesis from organisms or specific tissues at various time points. An analysis of function for any gene involves defining not only which environmental stimuli induce its expression but also in which tissues and time frames expression occurs, the subcellular locations of its protein products, and the phenotypes of organisms having mutant alleles of the gene. *gfp*-based reporter transposons (46), promoter bank libraries (4), and PCR-targeting modules (65) that accomplish all these criteria have been developed for yeast and bacteria (60). Fungal genomics coupled with improved transformation technology (i.e., electroporation, particle bombardment, and *Agrobacterium*-mediated transformation (reviewed by reference 26), higher homologous recombination efficiencies (25), possibly targeted PCR (65), and characterization of fungal transposons (18) should make development of genomic approaches that have been successful in bacterial and yeast systems a reality for filamentous fungi. These developments would allow elucidation of fungal gene functions on a massive scale. For many filamentous fungi, a low frequency of transformation is the limiting step to such approaches.

Developing high-throughput screens for monitoring, in complex environments, the large numbers of transformed fungi that would be generated by genomic approaches is an as yet unmet challenge. In bacterial systems, on the other hand, Sternberg et al. (55) devised a method to determine the distributions and growth of bacteria in flow-chamber biofilms by fusing genes encoding unstable GFP proteins to rRNA and thus allowed on-line monitoring of growth and distribution in this complex community. Such innovative approaches also can be developed for fungal systems.

Many researchers look to GFP-tagged fungi for tracking fungal distributions in natural systems. Recreating the complex environments in which fungi naturally occur, in a lab or a contained greenhouse, will present other challenges. However, because many filamentous fungi produce copious amounts of easily dispersible spores that accumulate to very high concentrations in the air, contained environments for working with these engineered fungi should be used, and the ecological implications of introducing these organisms into the environment, especially those harboring selectable genes, must be seriously considered. Techniques for eliminating selectable markers from transformed organisms have been developed (33), but research that addresses the fitness, epidemiology, and possible ecological implications of releasing transformed fungi into ecosystems is lacking. The U.S. Department of Agriculture has granted permits for the release of genetically modified fungi, and information regarding these permits and their asso-

ciated environmental assessment reports can be found at a related website (http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm).

In conclusion, recent advances in GFP molecule engineering, fluorescence detection, and imaging analysis are occurring at a time in which we are poised to gain considerable information about fungal genomes. A clever synthesis of these technologies and information is likely to lead to a bright future for fungal biology.

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