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Expression of Green Fluorescent Protein in *Aureobasidium pullulans* and Quantification of the Fungus on Leaf Surfaces

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ABSTRACT

A red-shifted, mutated form of the jellyfish green fluorescent protein (GFP) under control of a TEF promoter was expressed at high levels in the filamentous fungus *Aureobasidium pullulans*. In the three transformants studied, all morphotypes of the fungus, including pigmented chlamydozoospores, expressed GFP and fluoresced brightly. Confocal microscopy showed that the intracellular distribution of GFP was nonuniform. When applied to leaf surfaces, the transformants were readily visible and amenable to quantification by image analysis. Thus, GFP expression, together with quantitative image analysis, may provide a powerful method for ecological studies of plant-microbe relationships in nature.

INTRODUCTION

Within the past three years, green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been developed as a reporter for gene expression, a marker of subcellular protein localization, a tracer of cell lineage and a label to follow development of pathogens within their plant hosts (5,10,17,22,24). GFP is a 238-amino acid polypeptide involved in bioluminescence of aquatic invertebrates in the phylum Cnidaria (10,20). These animals emit green light ($\lambda_{\max} = 509 \text{ nm}$) from the GFP, which accepts excitation energy from luciferases or photoproteins, depending on the species (20).

In 1994, Chalfie et al. (5) showed that a jellyfish cDNA encoding GFP could be expressed in *E. coli* and *Caenorhabditis elegans*. Expression of wild-type or enhanced, mutated forms of GFP has since been demonstrated in numerous organisms, including plants, insects, mammals, yeasts (6,8,10,22)

and, recently, in a filamentous fungus (24). As a marker system, especially for living cells, GFP has several advantages over existing reporters such as β -glucuronidase (GUS), chloramphenicol acetyltransferase (CAT), β -galactosidase (LacZ) or luciferase (LUC) (22). These include: (i) its stability (little or no photobleaching); (ii) ability to form fluorescent, functional products with other proteins; and (iii) simplicity and versatility for in vivo use (10,22). For example, it is amenable to fluorescence-activated cell sorting and requires only UV or blue light and oxygen to fluoresce. Co-factors, substrates or antibodies are not needed for the detection system to function, thus facilitating protocols and avoiding problems related to cell permeabilization and uptake of substrate or leakiness of product (10,17,22).

We report expression of GFP in *Aureobasidium pullulans* (de Bary) Arnaud (Deuteromycetes: Moniliales), a filamentous fungus that grows in diverse habitats (7), including the phyllosphere (leaf surfaces). We also show that GFP-labeled cells applied to leaf surfaces can be detected and enumerated by quantitative image analysis. This system provides a method to rapidly and accurately assess colonization or dispersal of marked populations released to nature.

MATERIALS AND METHODS

Fungal Strain, GFP Vector and Gene Expression

A. pullulans (ATCC Catalog No. 90393; Rockville, MD, USA) (15) was maintained in 15% glycerol at -80°C until needed. Working cultures used routinely were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, USA) at $25^\circ\text{--}28^\circ\text{C}$ and transferred at 1-2-wk intervals. Transformants were grown generally on Holiday's complete medium (13) amended with $50 \mu\text{g/mL}$ hygromycin B (Sigma Chemical, St. Louis, MO, USA). Liquid media (unamended) used to induce the various morphotypes (blastospores, swollen cells, chlamydozoospores, hyphae and pseudohyphae) have been previously described (1).

The strategy for expressing GFP

involved fusing a highly expressed, constitutive promoter with an improved GFP cDNA and then stably introducing the expression vector into the *A. pullulans* genome. Based on a previously reported sequence (25), approximately 800-bp, 5'-untranslated region of the translation elongation factor (*TEF*) gene was polymerase chain reaction (PCR)-amplified from *A. pullulans* strain CBS 105.22. Overlap extension (14) with high-fidelity *Pfu* DNA Polymerase (Stratagene, La Jolla, CA, USA) was used to fuse the *TEF* promoter, an engineered form (EGFP) of the *A. victoria* GFP cDNA (9), and a 200-bp terminator region derived from the *Aspergillus awamori* glucoamylase gene (12,18). The entire expression cassette was ligated into pBluescript® II KS(-), and the sequence of junctions was confirmed (Figure 1).

The pTEFEGFP expression vector was introduced into *A. pullulans* (ATCC Catalog No. 90393) by co-transformation with pDH33, a plasmid conferring hygromycin resistance (23). Transformation was as previously described (11,26) with minor modifications. Protoplasts were treated with 0.5-8.0 µg of both pDH33 and pTEFEGFP. Following polyethylene glycol-induced aggregation, protoplasts were plated on osmotically stabilized Holliday's complete medium supplemented with soluble starch (1 g/L) and hygromycin B (62.5 µg/mL). After 4 days at

room temperature, three visibly green, putative transformants were transferred to complete medium supplemented with 150 µg/mL hygromycin B.

Cell-free extracts from 24-72-h-old cultures of the three EGFP transformants and the pDH33 transformant (control) were produced by washing cells once in Tris-phosphate buffer (10 mM, pH 8.0), followed by disruption with glass beads in a Mini-Bead-Beater® (BioSpec Products, Bartlesville, OK, USA). Extracts were clarified by brief centrifugation at 16000× g. Absorption spectra of the extracts was determined between 350-550 nm with a Model DMS 100S UV/Visible Spectrophotometer (Varian, San Fernando, CA, USA). Purified recombinant rGFP-S65T protein (CLONTECH Laboratories, Palo Alto, CA, USA) was used as a reference for comparison.

Microscopy and Image Analysis

The transformants grown in liquid media described above were concentrated by centrifugation, washed in distilled water (DW) and examined directly or fixed in 3% paraformaldehyde (PF) in phosphate buffer (50 mM, pH 7.4) or used to spray-inoculate the adaxial (upper) surface of detached leaves from apple (*Malus × domestica* Borkh.) seedlings or orchard trees (1). Following incubation for 24-30 h, disks (13-mm diameter) carrying the zone of inoculum were punched from

the leaves and fixed as above. For examination, the leaf disks were washed and mounted in VECTASHIELD® (Vector Laboratories, Burlingame, CA, USA) on slides.

Specimens were examined with an Olympus BX-60 Microscope (Olympus America, Lake Success, NY, USA) equipped for epifluorescence with a HBO 100-W mercury arc lamp (16). Filters used were either a NIB Narrow Excitation FITC Set (Olympus) or the dual-filter set for fluorescein isothiocyanate (FITC)/Texas Red® in the U83000 FISH Filter Set (Olympus). The GFP transformants fluoresced so brightly that it was necessary to install a neutral density filter (Model U-ND6, 6% transmission; Olympus) in the light path before the exciter filters. Images were recorded with a cooled charged-coupled device (CCD) video camera (Model DEI-470; Optronics Engineering, Goleta, CA, USA) and converted from an analog to a digital format (digitized) with a Precision/Chroma-P Frame Grabber (Imagraph, Chelmsford, MA, USA) controlled by Optimas 6 Processing Software (Optimas, Bothell, WA, USA) running on a Pentium®/166 MHz PC. The digitized images were stored as 24bit, red-green-blue (RGB) tagged image format

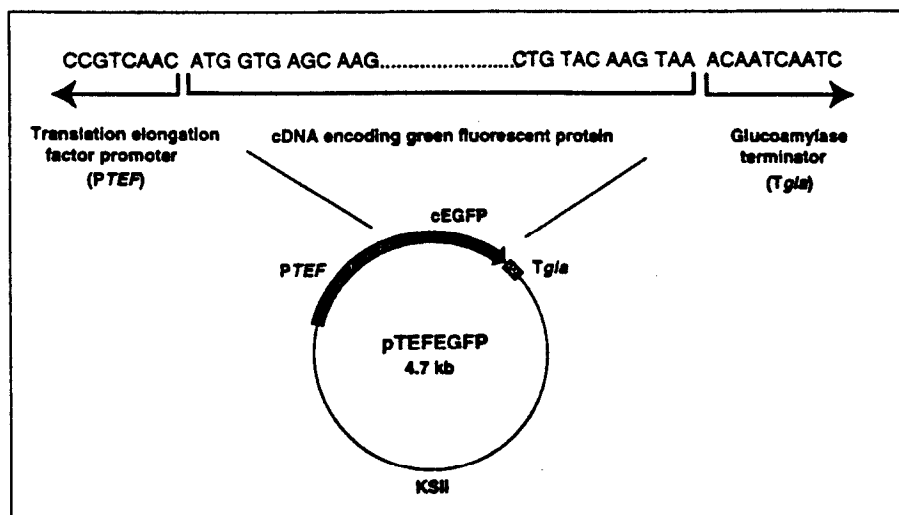


Figure 1. Expression vector pTEFEGFP. A red-shifted, mutant GFP cDNA (pEGFP-1; CLONTECH) was fused to the *A. pullulans* *TEF* promoter and to the *A. awamori* glucoamylase gene terminator. Nucleotide sequence surrounding PCR junctions were experimentally confirmed by dideoxy sequencing (21).

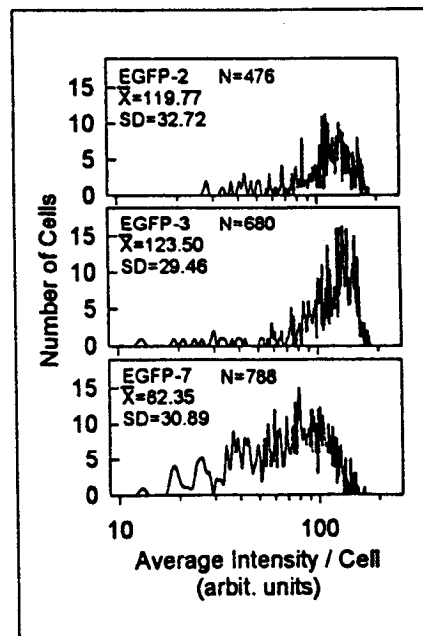


Figure 2. Fluorescence intensity distributions of the three GFP transformants: EGFP-2, EGFP-3 and EGFP-7.

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files (TIFFs) on 230-MByte magneto-optical disks. The population distributions of fluorescing cells were determined by preparing histograms of average fluorescence per cell vs. cell number by use of Excel™ Version 7.0 software (Microsoft, Redmond, WA, USA) and plotted on a log axis with SigmaPlot® Version 4.0 (SPSS, Chicago, IL, USA). Scanning confocal laser microscopy (SCLM) was conducted with an MRC-600 System (Bio-Rad, Hercules, CA, USA) equipped with an argon ion laser (excitation wavelength, 488 nm) and FITC filters. The confocal image sets on optical media were merged and pseudocolored with the Bio-Rad software supplied with the microscope or the shareware program Confocal Assistant™ Version 3.10 (3). Final image adjustment for color printing was done with Adobe® Photoshop® Version 4.0 (Adobe Systems, Mountain View, CA, USA).

Image analysis of GFP-labeled *A.*

pullulans populations on leaves was conducted as follows (16). Images in the RGB color format collected as above were converted to 8-bit (256 gray level) form and a region of interest (ROI) was selected to encompass an area in clear focus. This ROI was copied to form a new image frame, and thresholds were set to include only the fluorescing cells. Joined cells were separated by sequential erosion and non-merging dilation (19). Cells were outlined, filled, converted to a 1-bit (B/W) image and quantified as specified (19). Areas occupied by fungal biomass, and the total area of the selected ROI, were determined from stored calibration parameters. From these values, the area of cell coverage was determined as a percentage of the ROI.

RESULTS AND DISCUSSION

Southern blots with genomic DNA derived from the three GFP transfor-

mants (termed EGFP-2, EGFP-3 and EGFP-7), the parental strain and a pDH33 transformant, confirmed integration of pTEFEGFP (data not shown). Absorption spectra of cell-free extracts of all three EGFP transformants and of the purified protein rGFP-S65T (CLONTECH) (but not those from the pDH33 transformant alone) peaked at $\lambda_{\max} = 480\text{--}489\text{ nm}$, which is consistent with absorption at $\lambda_{\max} = 489\text{ nm}$ for the red-shifted mutant GFP-S65T (10) (data not shown). The means and variation of the cell population distributions of fluorescence differed only marginally ($P = 0.047$) for transformant Nos. 2 and 3, though EGFP-7 was markedly different from the former two ($P < 0.001$) (Figure 2). This difference presumably reflects variation in copy number and/or integration context of pTEFEGFP, a common feature of fungal transformation systems.

The transformants exhibited strong green fluorescence apparent at both the

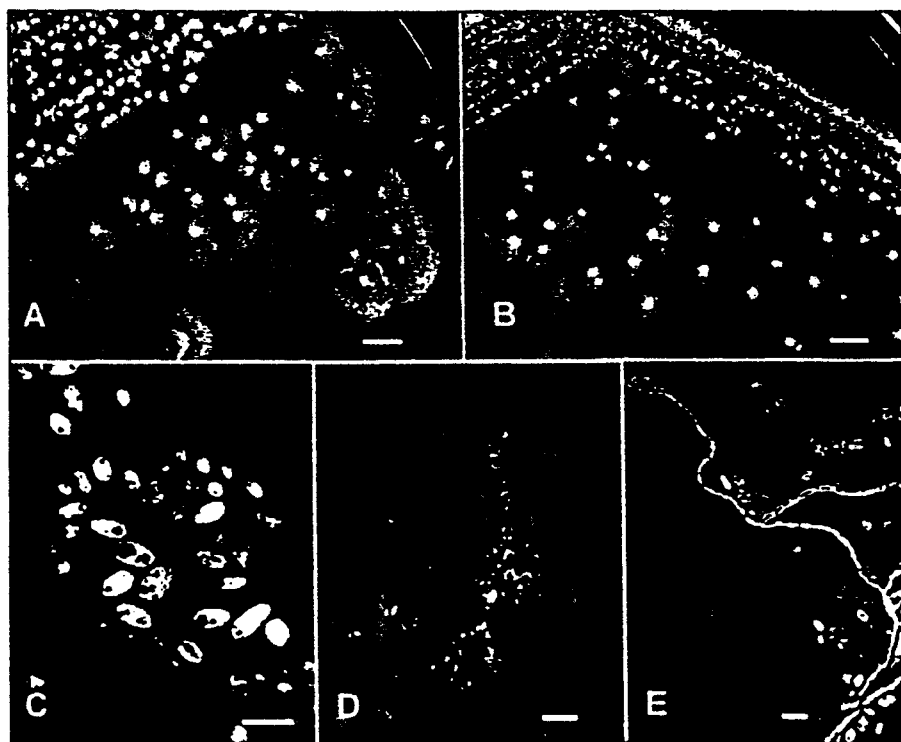


Figure 3. GFP expression in transformed *A. pullulans*. The control culture transformed with pDH33 only (A) and transformant EGFP-3 (B) 6-days-old growing on Holliday's complete medium. Culture plates illuminated with daylight photoflood lamps through a blue acetate filter and photographed through a K-8 yellow filter. (C) A merged, pseudocolored SCLM of two optical sections, 1 μm each, collected in the green (FTTC) channel showing intracellular variation in fluorescence intensity due to subcellular localization of GFP. (D) GFP fluorescence detectable in various morphotypes: blastospores (small hyaline cells); swollen cells; chlamydozoospores (pigmented, center of field); and pseudohyphae. (E) Hyphae, blastospores and swollen cells on the adaxial surface of an apple leaf. Scale bars for A and B = 2 mm; for C, D and E = 10 μm .

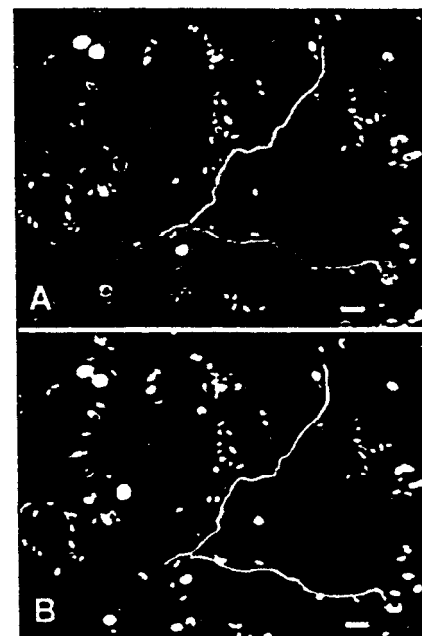


Figure 4. Adaxial surface of an apple seedling with applied transformant EGFP-3 as prepared for quantitative image analysis. (A) Typical RGB image of the ROI showing epiphytic population at low magnification. (B) The ROI as a 1-bit, black/white image after thresholds set to include the fluorescent cells only. Surface area of spores and hyphae is 1614 μm^2 within the ROI of 33496 μm^2 , giving a coverage of 4.82%. Total propagule number (where the two hyphae are tallied as two units) is 103. Scale bars = 10 μm .

level of the colony and the individual cell (Figure 3). SCLM (Figure 3C) showed that the GFP was not uniformly distributed in the cytoplasm. Figure 3C also suggests that there was considerable intercellular variation in intensity (cf. Figure 2), but this may be because the plane of the merged optical sections (two sections at 1 μ m each) did not pass through locally high concentrations of GFP. GFP was expressed in all morphotypes of *A. pullulans* (Figure 3, D and E) and the fluorescence was sufficiently bright to be readily visible with the neutral density filter in place, even in darkly pigmented chlamydospores. The transformants were readily detectable on the phylloplane of apple seedlings from growth chambers or orchard trees (Figure 3E).

The GFP-labeled transformants were amenable to quantification by image-analysis procedures (Figure 4) described in detail elsewhere (4). The data can be depicted various ways, e.g., cell numbers per unit area, area coverage or percent coverage (Figure 4). When both hyphae and spores are present, the most meaningful expression is as percent coverage of substratum.

In conclusion, we are exploring some of the many research questions presented by the GFP transformants. Among these are the long-term stability of the marker, the competitiveness of marked strains relative to the wild-type, the mechanism(s) of plasmid integration and the use of inducible rather than constitutive promoters. At this point, it is clear that the accuracy and versatility offered by quantitative image analysis, together with brightly fluorescent labels such as GFP, provide an ideal means to study populations of interest in situ in nature. For example, it will now be possible to apply GFP-tagged strains of *A. pullulans* to the phylloplane to investigate population processes such as colonization patterns, relationships (4), and the demographic factors birth, death, immigration and emigration (2).

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