

Kuang-Ren Chung · Marilyn Ehrenshaft  
Margaret E. Daub

## Functional expression and cellular localization of cercosporin-resistance proteins fused with the GFP in *Cercospora nicotianae*

Received: 12 December 2001 / Accepted: 12 February 2002 / Published online: 31 May 2002  
© Springer-Verlag 2002

**Abstract** The *Cercospora nicotianae* *pdx1* and *crg1* genes were previously identified as genes required for resistance to the singlet oxygen ( $^1\text{O}_2$ )-generating toxin cercosporin. The *pdx1* gene has subsequently been shown to be required for pyridoxine biosynthesis, but both the precise biochemical function of the PDX1 protein and the function of the CRG1 protein remain undefined, as both sequences lack defined enzymatic domains or cofactor-binding sites. The *gfp* gene encoding green fluorescent protein was translationally fused with *pdx1* and *crg1*. Transformation of these constructs into strains mutant in these respective genes resulted in green-fluorescent transformants complemented for the mutant phenotype. Microscopic studies revealed that in transformants transformed with *gfp* alone, fluorescence was distributed evenly throughout the cytoplasm and excluded from the vacuoles. Expression of PDX1::GFP either under the constitutive *Aspergillus nidulans* *gpdA* promoter or its own native promoter was visualized as distinct fluorescent circular structures in the cytoplasm, suggesting that PDX1::GFP was probably localized in the intracellular vesicles. Expression of CRG1 fused with GFP at either its N- or C-terminus resulted in low green fluorescence, compared with that of GFP alone or PDX1::GFP. The green fluorescence of either of the CRG1::GFP fusion proteins was barely observable in

transformants and was generally seen as a few scattered regions of fluorescence in the hyphae. Southern blot analysis indicated multiple copies of the constructs were integrated into the fungal genome. Northern analysis revealed that *pdx1::gfp* and *crg1::gfp* were each expressed as an intact transcriptional unit. Cell fractionation followed by immunoblotting against a GFP antibody showed that GFP alone and PDX1::GFP were detected exclusively in the cytoplasmic fraction. The two CRG1::GFP proteins were barely detected in the cytoplasmic fraction and not at all from the membrane fraction, a result inconsistent with microscopic observation and computer sequence analysis, which suggests that CRG1 is a membrane protein.

**Keywords** Photosensitizer · Singlet oxygen · Fungi · Pyridoxine

### Introduction

The green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea victoria*, is a small protein of 238 amino acid residues. GFP absorbs blue light at wavelengths of 395 nm and 470 nm and emits green light with a maximum wavelength of 507 nm (Cody et al. 1993). The protein is stable and has been successfully expressed in many heterologous organisms, including bacteria, fungi, plants, and animal cells (Baulcombe et al. 1995; Chalfie et al. 1994; Cormack 1998; Cubitt et al. 1995; Haseloff and Amos 1995; Margolin 2000; Sheen et al. 1995; Wang and Hazelrigg 1994). GFP produces a strong green fluorescence under aerobic conditions and, unlike other reporter gene products, such as  $\beta$ -glucuronidase, chloramphenicol acetyl transferase, and luciferase, can be used without destructive or invasive techniques and without addition of substrates. GFP fused to cellular polypeptides can be used to investigate the subcellular location of the target proteins and to analyze protein trafficking and dynamic subcellular processes over time (Walker et al. 1999). These key

Communicated by B.G. Turgeon

M. Ehrenshaft · M.E. Daub (✉)  
Department of Botany, North Carolina State University,  
Raleigh, NC 27695-7616, USA  
E-mail: margaret\_daub@ncsu.edu

K.-R. Chung  
Department of Plant Pathology,  
North Carolina State University,  
Raleigh, NC 27695-7616, USA

*Current address:* K.-R. Chung  
University of Florida,  
Citrus Research and Education Center,  
7 00 Experiment Station Road,  
Lake Alfred, FL 33850-2299, USA

features make GFP uniquely valuable as a reporter to study gene expression and protein localization in living cells (Chalfie et al. 1994).

*Cercospora* species are a group of plant-pathogenic fungi that cause disease on a multitude of plants. Their production of cercosporin, a light-activated perylenequinone toxin, is considered to be a significant contributor to the success of this genus as phytopathogens (Daub and Ehrenshaft 2000). Cercosporin is a photosensitizing compound that can absorb light energy and then react with oxygen to produce oxygen radicals, such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical (OH), as well as the non-radical but highly destructive singlet oxygen ( $^1O_2$ ). Cercosporin plays a substantial role in fungal infection and symptom development and is considered to be an important fungal pathogenicity factor (Upchurch et al. 1991). Cercosporin is highly toxic to cellular components, including nucleic acids, proteins, and membranes, and damage to these components, especially cell membranes, can result in cell death. Few organisms can tolerate  $^1O_2$  and little is known about cellular resistance mechanisms against it. In contrast, *Cercospora* fungi can not only tolerate the high concentrations of cercosporin they produce, but are also resistant to other potent  $^1O_2$ -generating photosensitizers, making this group of fungi uniquely suitable for studies of the cellular mechanisms for  $^1O_2$  resistance. Several potential resistance mechanisms, such as cellular antioxidants, antioxidant enzymes, and  $^1O_2$ -quenching compounds (e.g. carotenoids), were tested and ruled out (Ehrenshaft et al. 1995; Jenns et al. 1995; Sollod et al. 1992). A transient reduction and detoxification of the cercosporin molecule by *Cercospora* correlated strongly with resistance (Daub et al. 1992; Leisman and Daub 1992; Sollod et al. 1992), but was not confirmed genetically.

Functional complementation of two groups of *C. nicotianae* mutants sensitive to cercosporin and other photosensitizers (Jenns and Daub 1995; Jenns et al. 1995) led us to the isolation of two distinct genes involved in cercosporin resistance (Chung et al. 1999; Ehrenshaft et al. 1998, 1999a, b). The gene originally called *sor1* (singlet oxygen resistance) was identified by genetic complementation of a group of *C. nicotianae* mutants completely sensitive to both cercosporin and a number of other  $^1O_2$ -generating photosensitizers. This gene was found to be extremely conserved throughout a wide diversity of organisms, a surprising result as photosensitizer resistance is relatively rare. Further studies led to the discovery that *sor1* was involved in pyridoxine (vitamin B6) biosynthesis in eukaryotes, eubacteria, and archaeobacteria (Ehrenshaft et al. 1999a) and we therefore changed the name of this gene to *pdx1*. We also uncovered evidence that *pdx1* represents a divergence from the well characterized pyridoxine pathway of *Escherichia coli*; and we showed for the first time that pyridoxine is a highly effective quencher of  $^1O_2$ . As *pdx1* is involved in part of a previously undescribed pathway for pyridoxine synthesis, both the precise

biochemical function of PDX1 and that of other genes/enzymes in the pathway remain undefined.

A gene apparently unique to *Cercospora* species, *crg1* (cercosporin resistance gene), rescues a mutant that is partially sensitive to cercosporin, but is unaffected in its resistance to other  $^1O_2$ -generating photosensitizers (Chung et al. 1999). The encoded CRG1 protein contains four putative transmembrane domains and is predicted to localize in membranes. As with PDX1, the CRG1 sequence also lacks enzymatic domains and cofactor-binding sites; and we have been unable to elucidate its biochemical function or its specific role in cercosporin resistance.

Determination of the subcellular localization of proteins can facilitate the identification of their biochemical role and perhaps lead to the identification of other proteins with which they interact. In this study, we used GFP to investigate the subcellular localization of the two *C. nicotianae* proteins (PDX1, CRG1) involved in pyridoxine synthesis and in cercosporin and photosensitizer resistance. GFP fusion proteins were expressed in *C. nicotianae* *pdx1* and *crg1* mutants and their functionality was assessed by their ability to produce green fluorescence while concomitantly genetically complementing the respective *C. nicotianae* mutant strains. Localization of the fusion proteins was then assessed using a combination of fluorescence and confocal microscopy and Western blot analysis.

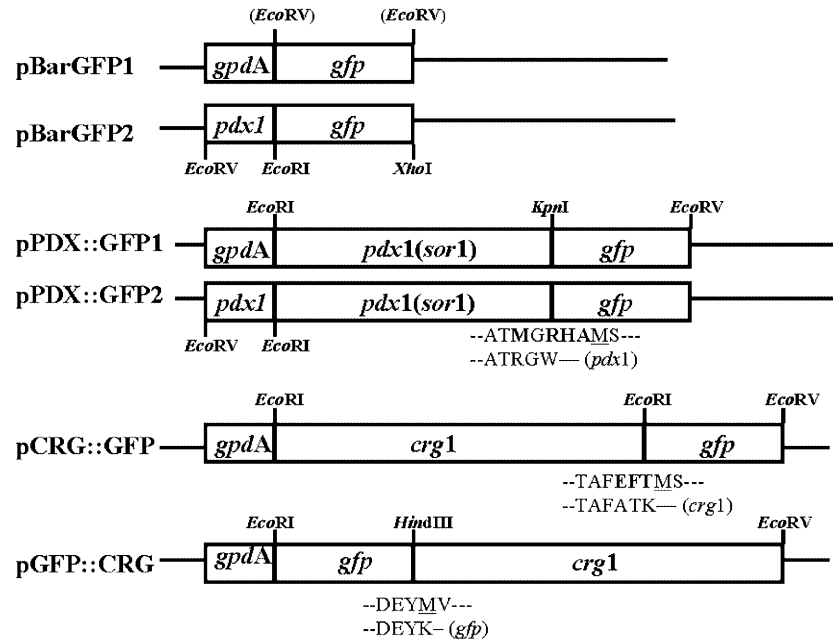
## Materials and methods

### Strains, media, growth conditions, and fungal transformation

*C. nicotianae* wild-type strain ATCC 18366 and the two previously described mutant strains, CS8 (cercosporin-sensitive mutant, pyridoxine auxotroph) and CS10 (cercosporin partially sensitive mutant; Jenns and Daub 1995; Jenns et al. 1995), were routinely maintained on malt medium as described by Jenns et al. (1989). Fungal protoplast isolation and transformation were conducted as described by Ehrenshaft et al. (1995, 1998). Fungal transformants were selected on medium containing 50  $\mu$ g bialaphos/ml as described by Chung et al. (1999) and maintained on complete medium (CM). Genetic complementation of the cercosporin-sensitive mutants CS8 and CS10 was performed as described by Chung et al. (1999), except using solid CM medium. The CS8 mutant can be rescued to prototrophy and cercosporin resistance by transformation with *pdx1*, whereas CS10 can be complemented by transformation with *crg1*. Strains used for DNA or RNA isolation were grown in liquid CM and kept shaking at room temperature for 5–7 days.

### Construction of plasmids

To construct pBarGFP1, the *NotI* fragment containing the entire protein coding region of *gfp* was excised from plasmid pGreen-lantern-1 (Life Technologies BRL, Gaithersburg, Md.) and made blunt-ended, using the Klenow fragment of DNA polymerase I. It was then ligated immediately downstream of the constitutive *gpdA* promoter from *Aspergillus nidulans* (Punt et al. 1990) in the *EcoRV* site of the bialaphos resistance-conferring fungal transformation vector pBarGPE1 (Pall and Brunelli 1993; Fig. 1). A *NotI-XhoI* fragment from a modified, "synthetic" *gfp* (SGFP) construct containing a mutation in the chromophore (65SYG→TYG; Heim et al.



**Fig. 1.** Schematic representation of *gfp* constructs cloned in the fungal expression vector pBarGPE1. Plasmid constructs were expressed under the control of the constitutive *Aspergillus nidulans* *gpdA* promoter or the *Cercospora nicotianae* *pdx1* promoter. Plasmid pBarGFP1 was constructed by cloning the *gfp* fragment under the control of the *gpdA* promoter at the *EcoRV* site. The *gfp* gene was fused to the *pdx1* promoter via an *EcoRI* site to yield pBarGFP2. *Pdx1* was translationally fused in-frame with *gfp* at a *KpnI* site to form pPDX::GFP1, with the fusion gene under the control of the *gpdA* promoter. The *pdx1::gfp* fusion was expressed under the *pdx1* promoter in pPDX::GFP2. The *crg1* gene was cloned either at the 5' end (pCRG::GFP) or the 3' end (pGFP::CRG) of *gfp*. The restriction enzyme cleavage sites incorporated from linker primers are indicated. The amino acid sequences located in the junctions of GFP and PDX1 or CRG1 are listed and the amino acids that were modified due to cloning are in bold. The initiator methionine of the downstream ORF is underlined. Detailed procedures for cloning are described in the text

1995) was also cloned behind the same promoter in the same vector. To construct plasmids with in-frame translational fusions between *pdx1* or *crg1* and *gfp*, the coding region of *gfp* was amplified from pGreenlantern-1 by Expand high fidelity DNA polymerase (Roche Molecular Biochemicals, Indianapolis, Ind.), using primers containing restriction endonuclease sites to facilitate further manipulations. The two 5', N-terminal primers used spanned the ATG initiation codon and contained either a *KpnI* site (5'-GGCCGCCATGGCCATGAGCAAGG-3') or an *EcoRI* site (5'-GGCGGCCGAATTCACCATGAGC-3'). Three 3', C-terminal primers were also used. One contained both a termination codon and an *EcoRV* site (5'-TAGAGCGATATCTCACTTGACAGCT-3'). The other C-terminal primers contained either an *EcoRI* site (5'-CACTTGTAGAATTCGTCCAT-3') or a *HindIII* site (5'-AGCGGCCGAAGCTTGTACAGC-3'), but both lacked a termination codon.

The *pdx1* fragment was amplified from either wild-type genomic DNA or a plasmid clone (Ehrenshaft et al. 1998, 1999b) using a N-terminal primer containing an ATG initiation codon and an *EcoRI* recognition site (5'-ACCAGCGAATTCATGGCCTCTAA-3') and a C-terminal primer containing a *KpnI* restriction site (5'-CGTGCCCATGGTCCTGCAGCA-3'). For ligation to *pdx1*, *gfp* was amplified using the N-terminal, *KpnI*-recognition site-containing primer and the C-terminal *EcoRV*-containing primer. The *pdx1* and *gfp* DNA fragments were purified using the

Wizard DNA clean up system (Promega, Madison, Wis.), cleaved with *KpnI*, and ligated together. This fused DNA fragment was digested with *EcoRI* and *EcoRV* and then cloned into pBarGPE1, to produce pPDX::GFP1, with the fusion construct under the control of the *gpdA* promoter (Fig. 1). To express *gfp* alone under the *pdx1* promoter, the *gfp*-coding region was excised with enzymes *EcoRI* and *XhoI* from pBarGFP1 and then fused with the *pdx1* promoter amplified from a *pdx1* clone, using a N-terminal primer containing an *EcoRV* recognition site (5'-TGGGAGGGA-TATCTGTTTTCCGGTG-3') and a C-terminal primer containing an *EcoRI* recognition site (5'-GCCATGAATTCGCTGGTGCTGGA-3'). The fused fragment was further digested with *EcoRV* and *XhoI* and cloned into pBarKS1 (Pall and Brunelli 1993), to yield pBarGPE2. The plasmid pPDX::GFP2 was constructed by ligation of the *pdx1* promoter with the *EcoRI/EcoRV* fragment from pPDX::GFP1 and then cloned into pBarKS1 after *EcoRV* digestion. Sequence analysis revealed that the construction of this plasmid resulted in the alteration of two amino acids (R→M, W→R) at the C-terminus of PDX1 and the removal of the *pdx1* termination codon. Two extra amino acids (H, A) were added immediately preceding the *gfp* initiation codon.

To create a translational fusion with CRG1 at the N-terminus of GFP, *crg1* was amplified using two primers with *EcoRI* recognition sites (5'-CCCAGAATTCATGGTAAGCGC-3', 5'-CACTTG AATTCAAACGCAGT-3'), which were used to amplify the entire *crg1*-coding region without a termination codon (Chung et al. 1999). The *gfp* fragment, amplified with primers containing an *EcoRI* recognition site at the N-terminal end and an *EcoRV* recognition site at the C-terminal end, was cloned first into pBarGPE1 to yield pKRC55. The *EcoRI*-digested *crg1* fragment was then cloned into pKRC55 to produce pCRG::GFP (Fig. 1). The resulting construct comprised the entire *crg1* sequence minus its termination codon with the three C-terminal amino acids alternated as shown (A→E, T→F, K→T; Fig. 1).

Another plasmid containing GFP at the N-terminus of CRG1 (pGFP::CRG) was also constructed. *crg1* was amplified using a N-terminal *HindIII* recognition site-containing primer (5'-CACCTACACCAAGCCTTATGGT-3') and a C-terminal *EcoRV*-recognition site and termination codon-containing primer (5'-CCGCGATATCGATCACTTCGT-3'). The resulting *crg1* fragment was ligated to a *gfp* fragment amplified with a N-terminal *EcoRI* site-containing primer and a C-terminal *HindIII* site-containing primer (see above). The fused fragment was digested with *EcoRI/EcoRV* and cloned into pBarGPE1, to produce pGFP::CRG (Fig. 1). In this construct, the terminal amino acid

residue of GFP was replaced with the *arg1* initiation codon. All primers used in this study were synthesized by Genosys Biotechnologies (Woodlands, Tex.) or Integrated DNA Technologies (Coralville, Iowa). Sequence analysis of the fusion junction of each construct (at the Molecular Genetics Facility, University of Georgia, Athens, Ga.) using *pdx1*- or *arg1*-specific primers confirmed the modified sequences. The amino acids constituting the fusion borders and the original amino acid sequences are shown in Fig. 1.

*Escherichia coli* DH-5 $\alpha$  competent cells were purchased from Life Technologies BRL and bacterial transformations were conducted by standard methods.

#### DNA/RNA manipulation

Fungal total DNA was isolated as described by Woloshuk et al. (1989). Standard procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern blot analysis. Fungal RNA was purified with a RNA isolator kit (Genosys Biotechnologies). Denaturing electrophoresis of RNA in a formaldehyde-containing agarose gel was performed as described by Chung et al. (1999). DNA or RNA was blotted onto a Magnagraph membrane (Osmomics, Westbrough, Mass.), using standard protocols. DNA probes used for Southern and Northern blot analyses were PCR fragments labeled by incorporation of digoxigenin-dUTP (Roch Molecular Biochemicals) as described by Chung et al. (1996). Hybridization was performed in aqueous solution ( $5 \times$  SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% nonfat dried milk) at 65 °C overnight. After hybridization, the membranes were washed in  $0.1 \times$  SSC, 0.1% SDS at 65 °C for 1 h. Hybridization probes were detected immunochemically (Ausubel et al. 1994) using CSPD ready-to-use chemiluminescent substrate (Roch Molecular Biotechnologies), as recommended by the manufacturer.

#### Microscopy

The green fluorescent signal was monitored in fungal colonies in culture, using a hand-held long wavelength UV lamp (UVP, San Gabriel, Calif.). Microscopic analyses were performed using a Zeiss Axiophot phase-contrast microscope. To produce hyphae for microscopic analysis, mycelial fragments (produced by grinding mycelium from agar cultures) were transferred to liquid minimal medium (Jenns et al. 1989) or minimal medium + 1 mg pyridoxine/ml (for CS8 transformed with *gfp* alone) and incubated on a shaker for 24 h. For the observation of GFP and GFP fusions, fungal hyphae from the liquid cultures were placed onto glass slides, covered with a cover slip, and observed using a 450–490 nm excitation filter and a 520 nm barrier filter. For nuclear staining, fungal mycelium was fixed to a glass slide at 70 °C, treated with a few drops of 4'-6-diamidino-2-phenylindole (DAPI) solution (1  $\mu$ g DAPI/ml, 1 mg *p*-phenylenediamine/ml, 50% glycerol), covered with a coverslip, and viewed using a 365 nm excitation/420 nm barrier filter combination. Confocal microscopy was done in the Cellular and Molecular Imaging Facility (Department of Botany, North Carolina State University, Raleigh), using a Leica confocal microscope, equipped with a  $40 \times$  PL Fluotar objective. Single images (1,024  $\times$  1,024 pixels) were collected using line-averaging 64 times. Images were exported as TIF files and were processed for printing on a Tektronix phaser 440, using Adobe Photoshop.

#### Cell fractionation and immunoblotting

Cell fractionation extracts were prepared from 5-day mycelial cultures, according to the methods of Lindstrom et al. (1993) with some modification. Fungal mycelium grown in liquid CM was harvested, washed three times with water, and then ground in 125 mM Tris-HCl buffer (pH 8.0) with glass beads. The sample was filtered through Miracloth (22–25  $\mu$ m; Calbiochem, La Jolla, Calif.) and centrifuged at 3,000 *g* for 10 min to remove cell walls and unbroken cells. This supernatant fraction was then spun at

100,000 *g* for 1 h to pellet membranes. The proteins remaining in the cytoplasmic/supernatant fraction were precipitated with 65% ammonium sulfate. The membrane pellet was solubilized in 125 mM Tris-HCl (pH 8.0) with 4.6% SDS and then centrifuged for 10 min in a microcentrifuge (13,000 *g*). The resulting supernatant was saved as the crude membrane fraction. Proteins were dialyzed against 125 mM Tris-HCl (pH 8.0) buffer and concentrated in Microsep microconcentrators (Pall Gelman, Northborough, Mass.). Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, Calif.) and 10- $\mu$ g samples were used for gel analysis. The protein samples were denatured in  $2 \times$  SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4.6% SDS,  $\beta$ -mercaptoethanol, 20% glycerol, 0.002% bromophenol blue; Laemmli 1970) by heating at 95 °C for 5 min, fractionated on a denaturing 10% SDS-polyacrylamide gel and either stained with Coomassie brilliant blue or electroblotted onto a nitrocellulose membrane. For immunodetection of proteins, a rabbit anti-GFP antibody (Clontech, Palo Alto, Calif.) at a 1:500 dilution and a goat anti-rabbit-IgG alkaline phosphate (AP)-conjugate antiserum at a 1:7,500 dilution were used as primary and secondary antibodies, respectively. The anti-cercosporin facilitator protein (CFP) antibody (1:400 dilution) was kindly provided by Dr. R.G. Upchurch (North Carolina State University, Raleigh, N.C.). Detection of the AP-coupled immune complex using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega) was performed as described by Sambrook et al. (1989).

## Results

### Transformation of *gfp* and *gfp* fusions into *C. nicotianae*

In previous work, we described the cloning and characterization of two genes, *pdx1* (*sor1*) and *arg1*, that rescue the cercosporin-sensitive *C. nicotianae* mutants, CS8 and CS10, respectively. In order to localize these two proteins in *C. nicotianae*, the genes were each fused to the gene encoding GFP. Figure 1 shows the strategies used for creation of the fusion constructs. Two *arg1* fusions were made because the original one exhibited very low green fluorescence. We originally used a SGFP, modified to express more efficiently in plants and fungi, but found that SGFP produced only very low levels of green fluorescence in our organism (data not shown). A wild-type version of *gfp* was therefore used throughout these experiments. A high fidelity DNA polymerase was used to amplify fragments for constructing the *gfp* fusions. In each construct, the fused gene cassette was driven under the constitutive *A. nidulans gpdA* promoter or the *C. nicotianae pdx1* promoter (Fig. 1).

To ascertain that none of the nucleic acid modifications used to facilitate cloning had resulted in unexpected effects on protein activity, we first tested our constructs for complementation of mutants CS8 (mutant in *pdx1*) and CS10 (mutant in *arg1*). Approximately 40% of CS8 transformants containing either *gfp* alone or *pdx1::gfp* fluoresced green when illuminated by a hand-held UV lamp. Approximately 40% of the *pdx1::gfp* transformants were also complemented to wild-type levels of pyridoxine prototrophy and cercosporin resistance by that construct. Interestingly, not all of the *pdx1::gfp* transformants expressing green fluo-

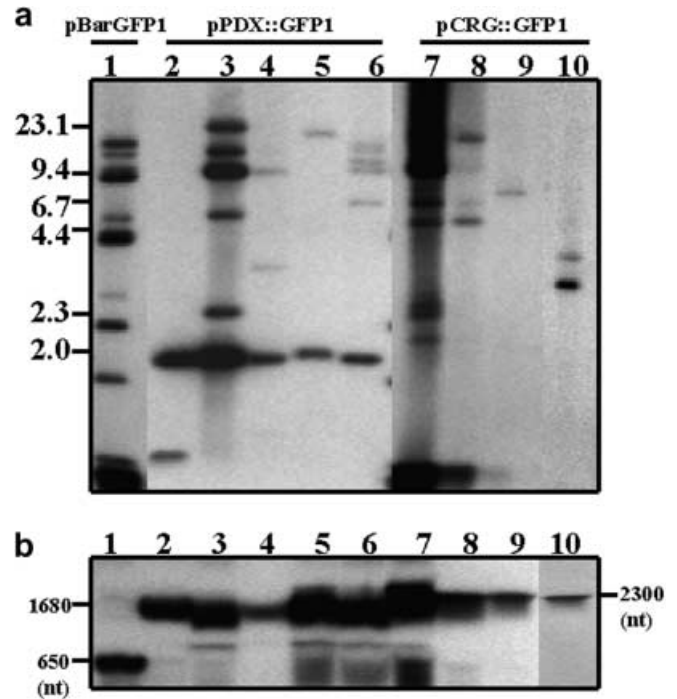
rescence were complemented for cercosporin resistance/pyridoxine prototrophy; and some complemented transformants did not fluoresce. Only those strains expressing both phenotypes were chosen for microscopic analysis.

CS10 transformants containing constructs encoding CRG fused at either the N- or C-terminus of GFP produced only a very low intensity of green fluorescence, which was usually undetectable using a hand-held UV lamp. Genetic complementation of the CS10 mutation was therefore used as the primary screen. Fewer than 10% of bialaphos-resistant fungal colonies transformed with either *crg1::gfp* (9.2%) or *gfp::crg1* (5.8%) were able to complement CS10 to wild-type levels of cercosporin resistance. Among these transformants, only ca. 5% displayed green fluorescence. Again, only transformants exhibiting both phenotypes were subjected to microscopic study. Expression of GFP through transformation with any of the constructs did not cause deleterious effects to *C. nicotianae*, as transformants expressing this gene exhibited normal growth (data not shown).

The presence and structure of the fusion constructs within the *C. nicotianae* transformants was analyzed by Southern blot hybridization, using a full-length *gfp* probe (Fig. 2). Transformation of pBarGFP1 into either CS8 or CS10 resulted in multiple copies of *gfp* in the *C. nicotianae* genome, an example of which is shown in Fig. 2a (lane 1). Transformation of the pPDX::GFP1 construct also resulted in the integration of multiple copies, although a strong band (ca. 1.8 kb) was detected in all transformants (Fig. 2a, lanes 2–6). Multiple insertions were also observed in transformants harboring the pCRG::GFP construct (Fig. 2a, lanes 7–10). To ensure the fusion constructs were expressed correctly as a chimeric unit and to examine the expression level of constructs, Northern blot analysis was performed (Fig. 2b). As expected, a 650-nucleotide (nt) transcript was detected in the transformant expressing pBarGFP1 alone (Fig. 2b, lane 1). Expression of pPDX::GFP1 and pCRG::GFP constructs resulted in the accumulation of 1,680-nt (lanes 2–6) and 2,300-nt (lanes 7–10) transcripts, respectively, indicating the constructs did express the chimeric gene as expected.

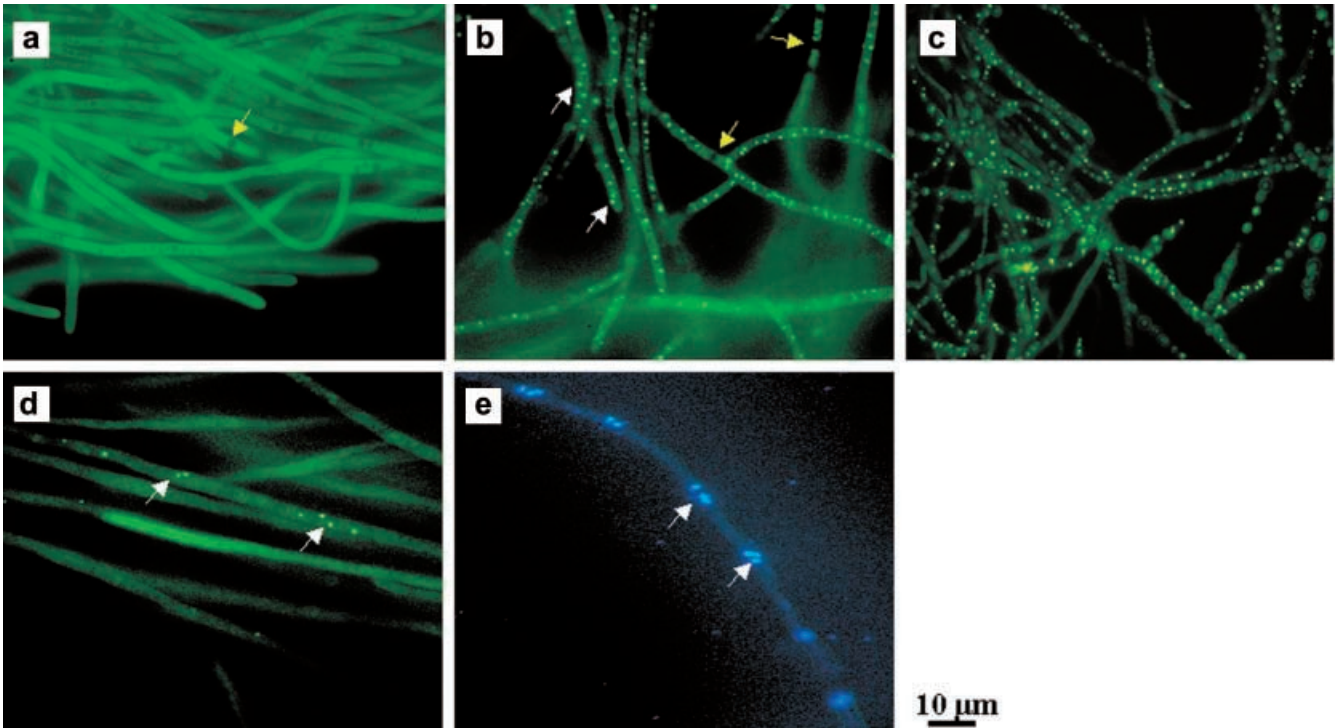
### Microscopy

Fluorescence microscopy indicated that, in *C. nicotianae*, expression of GFP alone under the constitutive *A. nidulans gpdA* promoter (Fig. 3a) or the *pdx1* promoter (data not shown) resulted in bright green fluorescence in the hyphal cytoplasm that was excluded from vacuoles. Fluorescence was visible at 24 h after inoculation of the culture (the earliest time-point tested) and did not appear to change over 7 days of culture. The GFP protein also continued to emit green fluorescence, even after the protein was purified (data not shown), indicating that



**Fig. 2a, b.** Southern and Northern blot analyses of transformants expressing *gfp* fusions. **a** Fungal DNA from *C. nicotianae* transformed with pBarGFP1 (lane 1), pPDX::GFP1 (lanes 2–6), or pCRG::GFP1 (lanes 7–10) was digested with *Eco*RI and *Bam*HI, electrophoresed, blotted, and washed at high stringency after hybridization with a *gfp* probe. The position of size markers is indicated in kilobase pairs. Transformation of all constructs resulted in the integration of multiple copies of *gfp* in the genome. **b** Total fungal RNA from *C. nicotianae* transformed with pBarGFP1 (lane 1), pPDX::GFP1 (lanes 2–6), or pCRG::GFP1 (lanes 7–10) was electrophoresed in a formaldehyde-containing, denaturing gel, blotted, and washed at high stringency after hybridization with a *gfp* probe. Sizes of hybridizing bands are indicated in nucleotides. A 650-nt transcript was identified in the pBarGFP1 transformant (**b**, lane 1). Expression of pPDX::GFP1 and pCRG::GFP constructs resulted in the accumulation of 1,680-nt (lanes 2–6) and 2,300-nt (lanes 7–10) transcripts, respectively, indicating expression of a chimeric gene. Low levels of smaller transcripts were also detected in some of the transformants with either the *pdx1::gfp* or *crg1::gfp* construct (lanes 3, 5–7). The nature of these smaller transcripts remains unknown

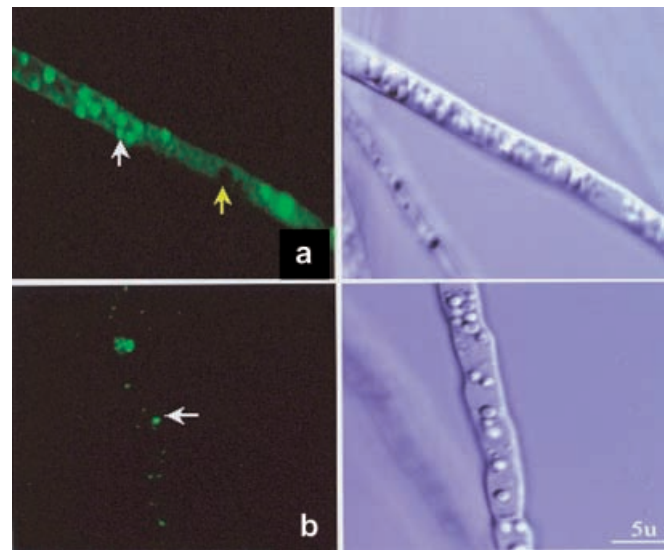
GFP expressed in *C. nicotianae* is very stable. Expression of PDX::GFP, either under the control of the *gpdA* promoter or the *pdx1* promoter was distinctly different (Fig. 3b, c). In these transformants, fluorescence appeared as distinct patches distributed along the fungal hyphae, suggesting localization within cytoplasmic vesicles. Confocal microscopy (Fig. 4a) supported the light microscopy results, showing distinct, circular regions of fluorescence, consistent with a hypothesis of vesicular localization. Expression of PDX::GFP controlled by the *gpdA* promoter (Fig. 3b) differed slightly from that controlled by the *pdx1* promoter (Fig. 3c) in that there was greater background fluorescence with *gpdA*. This background fluorescence resembled that seen with GFP alone (cytoplasmic, excluded from vacuoles), suggesting that high expression may result in some cleavage of the



**Fig. 3a–e.** Fluorescence microscopy of *C. nicotiana* expressing *gfp* or *gfp*-fusion constructs. Mycelial fragments of transformants were inoculated into liquid minimal medium and incubated with shaking for 24 h. **a** *C. nicotiana* transformed with pBarGFP1: note uniform green fluorescence throughout the cytoplasm, but excluded from vacuoles (yellow arrow). **b** *C. nicotiana* transformed with pPDX::GFP1 (*gpdA* promoter construct): fluorescence is visible as distinct patches within the cytoplasm (white arrows), with a background of general cytoplasmic fluorescence, excluded from vacuoles (yellow arrows). **c** *C. nicotiana* transformed with pPDX::GFP2 (*pdx1* promoter construct): fluorescence is visible as distinct patches within the cytoplasm and lacks the more pronounced background cytoplasmic fluorescence seen with pPDX1::GFP1. **d** *C. nicotiana* transformed with pGFP::CRG: very weak cytoplasmic fluorescence with some distinct spots indicated by arrows. **(e)** 4'-6-Diamidino-2-phenylindole fluorescence showing distribution of nuclei (arrows). Bar 10 µm

fusion protein, resulting in free GFP. Occasionally, a proportion of colonies in a culture did not fluoresce and the fluorescence intensity varied from colony to colony (data not shown).

In contrast to the strong and early fluorescence seen in the GFP and PDX::GFP transformants, transformants carrying either the pCRG::GFP or pGFP::CRG construct displayed very weak emission signals, even after 4 days. To facilitate enhanced detection of the green fluorescence from these transformants, either older cultures or increased excitation time were used. Both light and confocal microscopy of transformants carrying either pCRG::GFP or pGFP::CRG (Figs. 3d, 4b) demonstrated the presence of very few foci of fluorescence, localized in scattered hyphae in regions away from the hyphal tip. Overall background fluorescence was very faint and only detectable with long exposure times (Fig. 3d).



**Fig. 4a, b.** Confocal microscopy of *C. nicotiana* strains expressing *gfp*-fusion constructs. *C. nicotiana* was transformed with: **a** pPDX1::GFP1, or **b** pGFP::CRG. Images were visualized with fluorescence (left panels) or via transmitted light (right panels). **a** PDX1::GFP fusion shows distinct circular regions of fluorescence in the cytoplasm (white arrow), consistent with localization in vesicles. A vacuole (yellow arrow) is also visible. **b** GFP::CRG fusion shows very scattered points of fluorescence. Excitation time was increased 10-fold over that used for PDX1::GFP, in order to visualize the fluorescence

Comparison of the fluorescence from GFP fusions with that from the DAPI-stained nuclear DNA (Fig. 3e) revealed that the distribution pattern of nuclei was not coincident with that of the green fluorescent spots, suggesting neither the PDX1::GFP nor the CRG1::GFP fusion protein was localized in nuclei.

## Western blot analysis

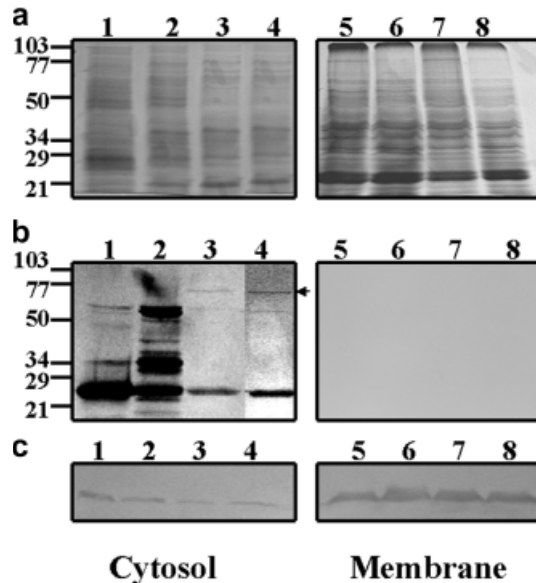
The expression and intracellular distribution of GFP fused with PDX1 and CRG1 was further investigated by Western blot analysis (Fig. 5). Homogenization of the cells followed by crude separation into particulate and supernatant fractions, resolution by SDS-PAGE (panel A), and immunoblotting with an antibody to GFP (panel B) resulted in the detection of a 26-kDa, strongly cross-reacting band in the supernatant/cytosolic fraction from transformants with GFP alone (Fig. 5b, lane 1). The weak, higher molecular weight signals were probably due to overflow of the lane 2 sample. In all cases, GFP and the GFP-fusion proteins were found entirely in the soluble fraction (Fig. 5b, lanes 1–4) and were not detectable in the non-soluble membrane fraction (Fig. 5b, lanes 5–8). In the PDX1::GFP sample (Fig. 5b, lane 2), the GFP antibody detected several cross-reacting bands in addition to the expected 62-kDa band, suggesting the occurrence of either multiple translation starts or post-translational modifications. In contrast, only a small amount of CRG1::GFP or GFP::CRG1 protein of the predicted mass of 70 kDa was detected in the soluble fraction (Fig. 5b, lanes 3–4, indicated by arrow). A more strongly cross-reacting band that co-migrated with GFP alone was detected in transformants with either pCRG1::GFP (Fig. 5b, lane 3) or

pGFP::CRG1 (Fig. 5b, lane 4), suggesting that both fusion proteins are unstable.

As a control, we also included the distribution of CFP, a protein from *C. kikuchii* that has strong homology to the membrane facilitator superfamily of integral membrane transporter proteins (Callahan et al. 1999). The CFP protein was detected, to a large extent, in the 100,000 g non-soluble fraction (Fig. 5c, lanes 5–8), indicating that this fraction contained membrane proteins. A relatively small amount of CFP was also detected in the soluble cytoplasmic fraction (Fig. 5c, lanes 1–4), demonstrating some contamination of our cytoplasmic fraction with proteins from the non-soluble membrane fraction.

## Computer analysis of protein localization

Data from the microscopy studies above suggested that PDX1::GFP localizes in vesicles within the cytoplasm. In order to provide additional evidence for our hypothesis, we conducted sequence analysis using the PSORT program (prediction of protein localization sites; <http://psort.nibb.ac.jp/>). Using the PSORT mode for yeast and animal proteins, *C. nicotianae* PDX1 is predicted to be a cytoplasmic protein with a 60.9% certainty. Analysis of the *C. nicotianae* PDX1 using the PSORT mode for plant proteins, however, predicts its localization in the chloroplast stroma with a certainty of 86.9%. Similarly, ChloroP, a program that predicts chloroplast transit peptides (<http://www.cbs.dtu.dk/services/ChloroP/>), also predicts the *C. nicotianae* protein to localize in the chloroplast and to contain a N-terminal chloroplast transit peptide that is cleaved between amino acid residues 81 and 82. Although not definitive, as our sequence is of fungal and not plant origin, these results suggest that PDX1 may have an organelle-targeting sequence, consistent with our conclusion of localization within cytoplasmic vesicles.



**Fig. 5a–c.** Western blot detection of GFP protein from cytosolic and membrane fractions from *C. nicotianae*. Fungal proteins from transformants harboring pBarGFP1 (lanes 1, 5), pPDX1::GFP1 (lanes 2, 6), pCRG1::GFP (lanes 3, 7), or pGFP::CRG1 (lanes 4, 8) were separated into cytoplasmic (lanes 1–4) and membrane fractions (lanes 5–8) by centrifugation, as described in the Materials and Methods. Extracts were fractionated on a denaturing 10% SDS-polyacrylamide gel and either stained with Coomassie brilliant blue (a) or electroblotted onto a nitrocellulose membrane and then probed with GFP polyclonal antibody (b) or cercosporin facilitator protein antibody (c). Size markers were low range polypeptides (Bio-Rad) and their sizes are indicated in kilodaltons

## Discussion

We have taken advantage of the GFP, recently employed successfully in many organisms, to localize two novel proteins in the fungus *C. nicotianae*. Interestingly, SGFP modified to enhance expression in plants and other fungi produced insufficient fluorescence in *C. nicotianae*. In contrast, native GFP produced fluorescence visible with long-range UV light or blue light. Similar variation between two versions of GFP has been observed in other organisms, due to the extreme codon bias among organisms (Cormack 1998). In this study, wild-type GFP was successfully expressed under the *A. nidulans gpdA* promoter, where it produced bright green fluorescence easily visualized using a hand-held UV light, facilitating the screening of hundreds of transformants. It was also successfully expressed under the *C. nicotianae pdx1* promoter. It was occasionally

observed that a proportion of cells in a culture did not fluoresce and that the fluorescence intensity varied from cell to cell. We do not know whether this variation was due to an impaired ability of some cells to express *gfp* and the fusion constructs, or simply to plasmid loss. Heterogeneity of GFP expression has also been observed in yeast (Kruckeberg et al. 1999).

To localize the two novel proteins in *C. nicotianae*, the target genes were fused translationally in-frame at either the N- or C-terminus of GFP. To ensure that the *Cercospora* proteins remained functional and were localized correctly, we transformed the constructs into strains mutant for *pdx1* or *crg1* and screened the transformants for restoration of wild-type phenotype and for expression of GFP. Southern blot analysis of construct-containing transformants indicated that multiple copies of the chimeric constructs were integrated into the fungal genome. Northern and Western blot analyses detected the expression of *gfp* with either *pdx1* or *crg1* as a chimeric unit.

*PDX1* is a gene involved in a newly discovered pathway for pyridoxine biosynthesis that occurs in eukaryotes, archaeobacteria, and selected eubacteria (Ehrenshaft et al. 1999a). This gene shows no homology to any of the pyridoxine biosynthesis genes characterized in *E. coli*, or to any gene in the completely sequenced *E. coli* genome. The precise biochemical function of the PDX1 protein is not yet known and the amino acid sequence provides no definitive clues as to the protein's function. The work presented here strongly suggests that the protein, and thus pyridoxine synthesis, occurs within vesicles in the cytoplasm of the fungal hyphae. The microscopic studies revealed that, while GFP alone produced a uniform green cytoplasmic fluorescence, expression of PDX1::GFP formed a fluorescence that was dappled and localized to circular structures within the cytoplasm (Figs. 3, 4). The dappled nature of the PDX1::GFP signal suggests that this fusion protein is localized within cytoplasmic vesicles. Western blot analysis of cell fractions detected PDX1::GFP only in the soluble, but not in the membrane fraction (Fig. 5), thus PDX1 does not appear to be localized within membranes. These results are supported by our PSORT sequence analysis data, which indicate that PDX1 is a cytoplasmic protein and may contain a transit peptide sequence that targets to an organellar domain.

Interestingly, the putative organelle targeting of the *C. nicotianae* PDX1 may be unique to *Cercospora* and may provide clues as to this protein's possible role in resistance to the *Cercospora* photoactivated toxin, cercosporin. As indicated, *pdx1* was originally identified as a gene required for *Cercospora* species to grow in the presence of cercosporin and other  $^1\text{O}_2$ -generating photosensitizing compounds (Ehrenshaft et al. 1998, 1999b). Consistent with this phenotype, we recently demonstrated that pyridoxine and the other vitameric forms of vitamin B6 are very effective chemical quenchers of  $^1\text{O}_2$  (Bilski et al. 2000; Ehrenshaft et al. 1999a). The precise role of pyridoxine in photosensitizer

resistance is not clear, however, as this is a vitamin synthesized by many and required by all organisms. The chloroplast-targeting transit peptide sequence found in the *C. nicotianae* PDX1 is found in a ca. 50-amino-acid residue N-terminal leader sequence that is found only in the *C. nicotianae* gene and is not found in any of the approximately two dozen homologues from other fungi, plants, eubacteria, and archaeobacteria. PSORT and ChloroP analysis of plant homologues to *pdx2* (the second identified gene in the pathway; Ehrenshaft and Daub 2001), interestingly, predicts them to be cytoplasmic with a certainty of 65% and shows no predicted chloroplast targeting, consistent with the absence of the N-terminal leader sequence. Thus, pyridoxine synthesis in *C. nicotianae* appears to have a unique subcellular localization; and this localization may play a role in cercosporin resistance by a mechanism that at this point remains unclear.

In contrast to our results with PDX1, this study did not provide definitive evidence for a localization of CRG1. Hydropathy analysis of the CRG1 protein identified four putative transmembrane domains and grouped it as an integral membrane protein (Chung et al. 1996). However, neither microscopic examination nor Western analysis was able to confirm a membrane locale for CRG1. The CRG1::GFP chimeric proteins, although barely detectable in the cytosolic fraction, were completely undetectable in the membrane fraction in Western blot analysis (Fig. 5). Fluorescence microscopy localized both CRG1::GFP and GFP::CRG1 to discrete cytoplasmic areas in most transformants. Similarly, localization of a putative membrane protein fusion with GFP is not completely conclusive in *Magnaporthe grisea* (DeZwaan et al. 1999).

Our inability to directly localize CRG1 appears to be due to an instability of both of the CRG1::GFP chimeric proteins. The presence of a 26-kDa GFP cross-reacting band in the transformants suggested that GFP was cleaved from both the GFP::CRG1 and CRG1::GFP fusion proteins in the fungal cells. This instability could be due to the fact that CRG1 contains a PEST region (Chung et al. 1999), which can significantly reduce the half-life of proteins (Rogers et al. 1986). The cleavage event apparently did not release an active GFP molecule, however, as the transformants lacked typical green fluorescence seen in fungi expressing GFP alone. Some chimeric protein was detected in transformants and the dappled fluorescence seen in the confocal microscopy was presumably due to this chimeric protein. However, the amount was insufficient to definitively draw conclusions regarding localization. Although transformants were complemented for the CRG1 phenotype, we cannot definitively conclude that complementation is due to proper localization of the chimeric protein, as a functional CRG1 may be released during the cleavage event. Due to ambiguity concerning CRG1 localization and its instability, the function of CRG1 against cercosporin remains unclear.

**Acknowledgements** We thank Dr. R.G. Upchurch (Dept. of Plant Pathology, N.C. State University) for providing CFP antibody and Dr. N.S. Allen and Mr. Dana Moxley (Cellular and Molecular Imaging Facility, Department of Botany, N.C. State University) for assistance with confocal microscopy. We also thank Dr. A.E. Jenns and Mrs. D.K. Wetzel for their able assistance. This work was supported by grants MCB-9631375 from the National Science Foundation (M.E.D., M.E.) and 980886 from the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (M.E.D., M.E., K.R.C.).

## References

- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1994) Current protocols in molecular biology. Wiley, New York
- Baulcombe DC, Chapman S, Santa Cruz S (1995) Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J* 7:1045–1053
- Bilski P, Li M, Ehrenshaft M, Daub M, Chignell C (2000) Vitamin B6 (pyridoxine) and its derivatives are efficient singlet oxygen quenchers and potential fungal antioxidants. *Photochem Photobiol* 71:129–134
- Callahan T, Rose M, Meade M, Ehrenshaft M, Upchurch R (1999) *CFP*, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. *Mol Plant-Microbe Interact* 12:901–910
- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. *Science* 263:802–805
- Chung K-R, Leuchtmann A, Schardl CL (1996) Inheritance of mitochondrial DNA and plasmids in the ascomycetous fungus, *Epichloe typhina*. *Genetics* 142:259–265
- Chung K-R, Jenns AE, Ehrenshaft M, Daub ME (1999) A novel gene required for cercosporin toxin resistance in the fungus *Cercospora nicotianae*. *Mol Gen Genet* 262:382–389
- Cody CW, Prasher DC, Westler WM, Prendergast FG, Ward WW (1993) Chemical structure of the hexapeptide chromophore of the *Aequorea* green fluorescent protein. *Biochemistry* 32:121–128
- Cormack B (1998) Green fluorescent protein as a reporter of transcription and protein localization in fungi. *Curr Opin Microbiol* 1:406–410
- Cubitt AB, Adams SR, Boyd AE, Gross LA, Tsien RY (1995) Understanding, improving and using green fluorescent proteins. *Trends Biochem Sci* 20:448–455
- Daub ME, Ehrenshaft M (2000) The photoactivated *Cercospora* toxin cercosporin: contributions to plant disease and fundamental biology. *Annu Rev Phytopathol* 38:461–490
- Daub ME, Leisman GB, Clark RA, Bowden EF (1992) Reductive detoxification as a mechanism of fungal resistance to singlet-oxygen-generating photosensitizers. *Proc Natl Acad Sci USA* 89:9588–9592
- DeZwaan TM, Carroll AM, Valent B, Sweigard JA (1999) *Magnaporthe grisea* pth11p is a novel plasma membrane protein that mediates appressorium differentiation in response to inductive substrate cues. *Plant Cell* 11:2013–2030
- Ehrenshaft M, Daub ME (2001) Isolation of *PDX2*, a second novel gene in the pyridoxine biosynthesis pathway of eukaryotes, archaeobacteria, and a subset of eubacteria. *J Bacteriol* 183:3383–3390
- Ehrenshaft M, Jenns AE, Daub ME (1995) Targeted gene disruption of carotenoid biosynthesis in *Cercospora nicotianae* reveals no role for carotenoids in photosensitizer resistance. *Mol Plant-Microbe Interact* 8:569–575
- Ehrenshaft M, Jenns AE, Chung KR, Daub ME (1998) *SORI*, a gene required for photosensitizer and singlet oxygen resistance in *Cercospora* fungi is highly conserved in divergent organisms. *Mol Cell* 1:603–609
- Ehrenshaft M, Bilski P, Li M, Chignell CF, Daub ME (1999a) A highly conserved sequence is a novel gene involved in de novo vitamin B6 biosynthesis. *Proc Natl Acad Sci USA* 96:9374–9378
- Ehrenshaft M, Chung KR, Jenns AE, Daub ME (1999b) Functional characterization of *SORI*, a gene required for resistance to photosensitizing toxins in the fungus *Cercospora nicotianae*. *Curr Genet* 34:478–485
- Haseloff J, Amos B (1995) GFP in plants. *Trends Genet* 11:328–329
- Heim R, Cubitt AB, Tsien RY (1995) Improved green fluorescence. *Nature* 373:663–664
- Jenns AE, Daub ME (1995) Characterization of mutants of *Cercospora nicotianae* sensitive to the toxin cercosporin. *Phytopathology* 85:906–912
- Jenns AE, Daub ME, Upchurch RG (1989) Regulation of cercosporin accumulation in culture by medium and temperature manipulation. *Phytopathology* 79:213–219
- Jenns AE, Scott DL, Bowden EF, Daub ME (1995) Isolation of mutants of the fungus *Cercospora nicotianae* altered in their response to singlet-oxygen-generating photosensitizers. *Photochem Photobiol* 61:488–493
- Kruckeberg AL, Ye L, Berden JA, Dam K van (1999) Functional expression, quantification and cellular localization of the Hxt2 hexose transporter of *Saccharomyces cerevisiae* tagged with the green fluorescent protein. *Biochem J* 339:299–307
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Leisman GB, Daub ME (1992) Singlet oxygen yields, optical properties, and phototoxicity of reduced derivatives of the photosensitizer cercosporin. *Photochem Photobiol* 55:373–379
- Lindstrom JT, Sun S, Belanger FC (1993) A novel fungal protease expressed in endophytic infection of *Poa* species. *Plant Physiol* 102:645–650
- Margolin W (2000) Green fluorescent protein as a reporter for macromolecular localization in bacterial cells. *Methods* 20:62–72
- Pall ML, Brunelli JP (1993) A series of six compact fungal transformation vectors containing polylinkers with multiple unique restriction sites. *Fungal Genet Newsl* 40:59–62
- Punt PJ, Dingemans MA, Kuyvenhoven A, Soede RD, Pouwels PH, Hondel CA van den (1990) Functional elements in the promoter region of the *Aspergillus nidulans* *gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase. *Gene* 93:101–109
- Rogers S, Wells R, Rechsteiner M (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234:364–368
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Sheen J, Hwang S, Niwa Y, Kobayashi H, Galbraith DW (1995) Green fluorescent protein as a new vital marker in plant cells. *Plant J* 8:777–784
- Sollod CC, Jenns AE, Daub ME (1992) Cell surface redox potential as a mechanism of defense against photosensitizers in fungi. *Appl Environ Microbiol* 58:444–449
- Upchurch RG, Walker DC, Rollins JA, Ehrenshaft M, Daub ME (1991) Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. *Appl Environ Microbiol* 57:2940–2945
- Walker D, Hunt H, Hager GL (1999) Using inducible vectors to study intracellular trafficking of GFP-tagged steroid/nuclear receptors in living cells. *Methods* 19:386–393
- Wang SX, Hazelrigg T (1994) Implications for *bcd* mRNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. *Nature* 369:400–403
- Woloshuk CP, Seip ER, Payne GA (1989) Genetic transformation system for the aflatoxin producing fungus *Aspergillus flavus*. *Appl Env Microbiol* 55:86–90