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A codon-optimized green fluorescent protein for live cell imaging in *Zymoseptoria tritici* ☆

S. Kilaru [a], M. Schuster [a], D. Studholme [a], D. Soanes [a], C. Lin [b], N.J. Talbot [a], G. Steinberg [a,*]

[a] *Biosciences, University of Exeter, Exeter EX4 4QD, UK*
[b] *Mathematics, University of Exeter, Exeter EX4 3QF, UK*

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ABSTRACT

Fluorescent proteins (FPs) are powerful tools to investigate intracellular dynamics and protein localization. Cytoplasmic expression of FPs in fungal pathogens allows greater insight into invasion strategies and the host-pathogen interaction. Detection of their fluorescent signal depends on the right combination of microscopic setup and signal brightness. Slow rates of photo-bleaching are pivotal for *in vivo* observation of FPs over longer periods of time. Here, we test green-fluorescent proteins, including *Aequorea coerulescens* GFP (AcGFP), enhanced GFP (eGFP) from *Aequorea victoria* and a novel *Zymoseptoria tritici* codon-optimized eGFP (ZtGFP), for their usage in conventional and laser-enhanced epi-fluorescence, and confocal laser-scanning microscopy. We show that eGFP, expressed cytoplasmically in *Z. tritici*, is significantly brighter and more photo-stable than AcGFP. The codon-optimized ZtGFP performed even better than eGFP, showing significantly slower bleaching and a 20–30% further increase in signal intensity. Heterologous expression of all GFP variants did not affect pathogenicity of *Z. tritici*. Our data establish ZtGFP as the GFP of choice to investigate intracellular protein dynamics in *Z. tritici*, but also infection stages of this wheat pathogen inside host tissue.

1. Introduction

Live cell imaging has greatly facilitated our understanding of the invasion strategies and cell biology of pathogenic fungi. For example, the establishment of fluorescent proteins in the rice blast fungus *Magnaporthe oryzae* demonstrated that autophagy is pivotal for infection (Kershaw and Talbot, 2009) and septins scaffold penetration peg organization during early invasion of the host plant (Dagdas et al., 2012). In the corn smut fungus *Ustilago maydis*, green-fluorescent protein, fused to a putative receptor involved in membrane fusions, revealed the existence of highly mobile endosomes in fungi (Wedlich-Soldner et al., 2000). Interestingly, *in planta* observation of these organelles and fluorescent effector proteins revealed that endosome motility is crucial for effector secretion and, consequently, for virulence in *U. maydis* (Bielska et al., 2014). Thus, visualization of the dynamic behavior of fluorescent fusion proteins allows unique insight into the ways in which pathogenic fungi invade their hosts.

A fluorescent protein for live cell imaging must meet certain criteria. The protein needs to be bright enough to allow signal perception over the auto-fluorescent background. It should also be photo-stable to allow long-term observation. Finally, the protein needs to be non-toxic when expressed in cells. The green fluorescent protein (GFP) meets these requirements. This FP was first identified in the jellyfish *Aequorea victoria*, where it works in concert with the calcium-binding blue fluorescent protein aequorin (Shimomura et al., 1962). The gene encoding GFP was cloned in 1992 (Prasher et al., 1992), and the break-through for live cell imaging of GFP came when the FP was stably expressed in prokaryotic and eukaryotic cells, highlighting its potential as a reporter of protein localization and expression (Chalfie et al., 1994; Inouye and Tsuji, 1994). Since then, GFP has been used in numerous organisms in a very wide range of applications, including the study of protein localization and cellular dynamics, protein expression analysis, protein–protein interactions studies and biosensors (e.g. Garamszegi et al., 1997; Kahana and Silver, 2001; Voss et al., 2013). In filamentous fungi, GFP from *A. victoria* was first used in

Abbreviations: FPs, fluorescent proteins; eGFP, enhanced green fluorescent protein; AcGFP, *Aequorea coerulescens* green fluorescent protein; ZtGFP, *Z. tritici* codon-optimized green fluorescent protein; GFP, green fluorescent protein; Val, valine; Arg, arginine; Ser, serine; Cys, cysteine; Ile, isoleucine; Tyr, tyrosine; Leu, leucine; His, histidine; *tub2*, α tubulin; *sdi1*, succinate dehydrogenase 1; RB and LB, right and left border; dpi, days post infection; ROI, region of interest; *n*, sample size.

☆ All material and protocols described here are available upon request.

* Corresponding author. Tel.: +44 1392 723476; fax: +44 1392 723434.
E-mail address: G.Steinberg@exeter.ac.uk (G. Steinberg).

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the corn smut fungus *U. maydis* (Spellig et al., 1996) and *Aureobasidium pullulans* (Vanden Wymelenberg et al., 1997) to visualize these inside the plant. Subsequently, GFP was used in a broad range of fungi (overview in Lorang et al., 2001); including *Mycosphaerella graminicola* (=*Zymoseptoria tritici*, Rohel et al., 2001). However, the GFP-variants used showed relatively low brightness, due to low expression and slow protein folding, and relatively poor photo-stability. These limitations were overcome in two ways. Firstly, individual amino acid residues were mutated, and secondly, the gene for *gfp* was codon-optimized to increase expression levels. An example of such a synthetically optimized protein is "enhanced" GFP (eGFP), which is improved in fluorescent brightness due to two point mutations (S65T and F64L). In addition, the codon-optimized gene carries 190 silent mutations to adapt for codon usage in humans, which increased mRNA translation rates (Haas et al., 1996; Yang et al., 1996). In fungi, codon-optimized eGFP was used in *Botrytis cinerea.* Adapting the gene for GFP to the codon usage in this fungus led to an increase in fluorescent brightness by ~12-fold (Leroch et al., 2011). In this report, we adapt the gene for eGFP to the codon usage in *Z. tritici*. We express this ZtGFP, eGFP and a GFP from the jellyfish *Aequorea coerulescens* (AcGFP), previously used to visualize hydrophobins in *Fusarium verticillioides* (Fuchs et al., 2004), in yeast-like cells of *Z. tritici*. We then compare photo-bleaching and the brightness of the three GFPs in conventional and laser-based epi-fluorescence and confocal laser scanning microscopy. Our results show that ZtGFP, expressed from codon-optimized *egfp*, performs better than either eGFP or AcGFP for analysis of *Z. tritici*.

2. Materials and methods

2.1. Bacterial and fungal strains and growth conditions

Escherichia coli strain DH5α was used for the maintenance of plasmids. *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) was used for maintenance of plasmids and subsequently for *A. tumefaciens*-mediated transformation of *Z. tritici*. *E. coli* and *A. tumefaciens* were grown in DYT media (tryptone, 16 g/l; yeast extract, 10 g/l; NaCl, 5 g/l; with 20 g/l agar added for preparing the plates) at 37 °C and 28 °C respectively. The fully sequenced *Z. tritici* wild-type isolate IPO323 was used as recipient strain for the genetic transformation experiments. Cells were maintained as glycerol stocks (NSY glycerol; nutrient broth, 8 g/l; yeast extract, 1 g/l; sucrose, 5 g/l; glycerol, 700 ml/l), and cultures were grown on YPD agar (yeast extract, 10 g/l; peptone, 20 g/l; glucose, 20 g/l; agar, 20 g/l) at 18 °C for 4–5 days.

2.2. Molecular cloning

Plasmid pJ244-ZtGFP carries codon-optimized *ztgfp* gene and was obtained from DNA 2.0 (Menlo Park, CA, USA). All other vectors in this study were generated by *in vivo* recombination in the yeast *Saccharomyces cerevisiae* DS94 (MATα, *ura3-52*, *trp1-1*, *leu2-3*, *his3-111*, and *lys2-801* (Tang et al., 1996) following published procedures (Raymond et al., 1999; Kilaru and Steinberg, 2015). For all the recombination events, the fragments were amplified with 30 bp homologous sequences to the upstream and downstream of the fragments to be cloned (see Table 1 for primer details). PCR reactions and other molecular techniques followed standard protocols (Sambrook and Russell, 2001). The DNA fragments of interest were excised from the agarose gel and purified by using silica glass suspension as described previously (Boyle and Lew, 1995). Plasmid DNA was isolated from the positive yeast colonies as described previously (Hoffman and Winston, 1987). All restriction enzymes and reagents were obtained from New England Biolabs Inc (NEB, Herts, UK).

2.3. Construction of vectors pCAcGFP and pCZtGFP

Vector pCeGFP was described in Kilaru et al. (2015a). Vector pCAcGFP contains *acgfp* under the control of *Z. tritici tub2* promoter for integration in to the *sdi1* locus by using carboxin as selection agent. A 12,704 bp fragment of pCeGFPTub2 (digested with *Zra*I; Schuster et al., 2015), 1149 bp *tub2* promoter (amplified with SK-Sep-14 and SK-Sep-15; Table 1) and 720 bp *acgfp* (amplified with SK-Sep-79 and SK-Sep-80; Table 1) were recombined in *S. cerevisiae* to obtain the vector PCAcGFP (AcGFP was kindly provided by Syngenta, Basel, Switzerland). Vector pCZtGFP contains *ztgfp*, amplified from pJ244-ZtGFP (see above), under the control of *Z. tritici tub2* promoter for integration in to the *sdi1* locus by using carboxin as selection agent. A 12704 bp fragment of pCeGFP-Tub2 (digested with *Zra*I), 1149 bp *tub2* promoter (amplified with SK-Sep-14 and SK-Sep-15; Table 1) 720 bp *ztgfp* (amplified with SK-Sep-101 and SK-Sep-102; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCZtGFP. Further details on vector construction and yeast recombination-based cloning are provided in Kilaru and Steinberg (2015).

2.4. Z. tritici transformation and molecular analysis of transformants

The vectors pCAcGFP, pCeGFP and pCZtGFP were transformed into *A. tumefaciens* strain EHA105 by heat shock method (Holsters et al., 1978) and *A. tumefaciens*-mediated transformation *of Z. tritici* was performed as described previously by Zwiers and De Waard (2001) with the slight modifications. Further details on this method are provided in Kilaru et al. (2015a). To confirm the integration of vector in to the *sdi1* locus and also to determine the copy number, Southern blot hybridizations were performed by using standard procedures (Sambrook and Russell, 2001). Approximately 3 μg of genomic DNA of IPO323 and transformants obtained with vectors pCAcGFP, pCeGFP and pCZtGFP were digested with *Bgl*II and separated on a 1.0% agarose gel and capillary transferred to a Hybond-N^+ membrane (GE healthcare, Little Chalfont, United Kingdom). 1014 bp *sdi1* probe (3′ end of the *sdi1* gene and *sdi1* terminator) was generated by using DIG labeling PCR mix (Life Science Technologies, Paisley, UK) with primers SK-Sep-10 and SK-Sep-13 (Table 1). Hybridizations were performed at 62 °C for overnight autoradiographs were developed after an appropriate time period.

2.5. Fungal infection of plants

Attached wheat leaf infections were performed, as described previously (Rudd et al., 2008) with few modifications. Wheat cultivar Galaxie (Fenaco, Bern, Switzerland) was used for all the plant infections and further details are provided in Kilaru et al. (2015a).

2.6. Epi-fluorescent microscopy

Fluorescence microscopy was performed, as described previously (Kilaru et al., 2015b). In brief, the fungal cells were grown in YG media at 18 °C with 200 rpm for 24 h and placed onto a 2% agar cushion and directly observed using a motorized inverted microscope (IX81; Olympus, Hamburg, Germany), equipped with a PlanApo 100x/1.45 Oil TIRF (Olympus, Hamburg, Germany) and a eGFP ET filter-set (470/40 Et Bandpass filter, Beamsplitter T 495 LPXR and 525/50 ET Bandpass filter (Chroma Technology GmbH, Olching, Germany)). The fluorescent tags were excited using a standard mercury burner or a VS-LMS4 Laser Merge System with a 488 nm solid-state laser (75 mW; Visitron Systems, Puchheim, Germany) and imaged in the stream acquisition mode. Average intensity and bleaching behavior of the