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# Red fluorescent proteins for imaging *Zymoseptoria tritici* during invasion of wheat ☆

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ABSTRACT

The use of fluorescent proteins (FPs) in plant pathogenic fungi provides valuable insight into their intracellular dynamics, cell organization and invasion mechanisms. Compared with green-fluorescent proteins, their red-fluorescent "cousins" show generally lower fluorescent signal intensity and increased photo-bleaching. However, the combined usage of red and green fluorescent proteins allows powerful insight in co-localization studies. Efficient signal detection requires a bright red-fluorescent protein (RFP), combined with a suitable corresponding filter set. We provide a set of four vectors, suitable for yeast recombination-based cloning that carries mRFP, TagRFP, mCherry and tdTomato. These vectors confer carboxin resistance after targeted single-copy integration into the *sdi1* locus of *Zymoseptoria tritici*. Expression of the RFPs does not affect virulence of this wheat pathogen. We tested all four RFPs in combination with four epi-fluorescence filter sets and in confocal laser scanning microscopy, both *in* and *ex planta*. Our data reveal that mCherry is the RFP of choice for investigation in *Z. tritici*, showing highest signal intensity in epi-fluorescence, when used with a Cy3 filter set, and laser scanning confocal microscopy. However, mCherry bleached significantly faster than mRFP, which favors this red tag in long-term observation experiments. Finally, we used dual-color imaging of eGFP and mCherry expressing wild-type strains *in planta* and show that pycnidia are formed by single strains. This demonstrates the strength of this method in tracking the course of *Z. tritici* infection in wheat.



## 1. Introduction

The identification of red-fluorescent proteins (RFPs), with red-shifted wavelength spectra, opened a new dimension in live cell imaging. Here, the simultaneous visualization of green-fluorescent protein (GFP) and RFP allows us to track two different organelles or proteins in the same living cell (Su et al., 2004), or in different populations of particular species in a defined environment (Bloemberg et al., 2000). The first RFP described was derived from a red colored *Discosoma* coral species (Matz et al., 1999). While its excitation and emission maxima, at 558 nm and 583 nm, made it suitable for dual color imaging with GFP, DsRed oligomerizes in living cells (Baird et al., 2000). This feature limits its use as a genetic fusion tag to study protein dynamics. This caveat was overcome by genetic modification which led to a monomeric red fluorescent protein, named mRFP (Campbell et al., 2002). Further improvement of mRFP resulted in tdTomato and mCherry, which showed slightly different excitation and emission maxima, increased brightness and photo-stability (Shaner et al., 2004). The repertoire of these molecular tools was recently extended with a novel red fluorescent protein, TagRFP, from the sea anemone *Entacmaea quadricolor*, which is reported to be 3-times brighter than mCherry (Merzlyak et al., 2007).

All red fluorescent proteins described are established molecular tools with which fungi can be studied. For example, mCherry allowed expression studies in *Magnaporthe oryzae* (Saitoh et al., 2014), localization of metabolic and other enzymes in *Fusarium fujikuroi* (Albermann et al., 2013) and *Candida guilliermondii* (Courdavault et al., 2011) and effector protein secretion in host

*Abbreviations:* FPs, fluorescent proteins; RFP, red fluorescent protein; mCherry, monomeric cherry; TagRFP, monomeric red (orange) fluorescent protein; tub2, α tubulin; mRFP, monomeric red fluorescent protein; eGFP, enhanced green fluorescent protein; tdTomato, tandem dimeric red fluorescent protein; sdi1, succinate dehydrogenase 1; dpi, days post infection; ROI, region of interest; *n*, sample size.

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

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pathogen interaction in *Ustilago maydis* (Bielska et al., 2014; Djamei et al., 2011; Doehlemann et al., 2009). mRFP was used to investigate microtubule dynamics in *U. maydis* (Straube et al., 2006) and effector secretion in *M. oryzae* (Ribot et al., 2013). TagRFP revealed dynamics of the actin cytoskeleton in *Neurospora crassa* (Berepiki et al., 2010), while tdTomato visualized the grass pathogen *Ophiosphaerella herpotricha* in infected plant tissue (Caasi et al., 2010) and organelles and effector secretion in *M. oryzae* (Khang et al., 2010) and in the oomycete *Phytophthora infestans* (Kelley et al., 2010).

In this study, we aim to establish the optimal red fluorescent tag in the wheat pathogen *Zymoseptoria tritici*. We take into account that the type of microscope, illumination settings and filter sets to capture the fluorescence intensities impacts significantly on the signal brightness and rate of photo bleaching (Shaner et al., 2005). We constructed vectors for targeted single integration of vectors, carrying mRFP, TagRFP, mCherry and tdTomato, under the control of the *Z. tritici* α-tubulin promoter, placed into the defined *sdi1* locus. This allowed comparative and quantitative analysis of fluorescent brightness and photobleaching behavior in all RFP-expressing *Z. tritici* strains, using epi-fluorescent and confocal laser scanning microscopy. Our results demonstrate that mCherry is the optimal red fluorescent protein for studies in *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 (Goodwin et al., 2011; Kema and van Silfhout, 1997) and another wild-type isolate IPO94269 (Kema et al., 2000) were used as recipient strains 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

All 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 pCmRFP, pCTagRFP, pCmCherry, and pCtdTomato

The vector pCmRFP contains *mrfp* under the control of *Z. tritici tub2* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. 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 690 bp *mrfp* (amplified with SK-Sep-85 and SK-Sep-86; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCmRFP. The vector pCTagRFP contains *tagrfp* under the control of *Z. tritici tub2* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. A 12,704 bp fragment of pCeGFPTub2 (digested with *Zra*I), 1149 bp *tub2* promoter (amplified with SK-Sep-14 and SK-Sep-15; Table 1) and 714 bp *tagrfp* (amplified with SK-Sep-81 and SK-Sep-82; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCTagRFP. The vector pCtdTomato contains *tdtomato* under the control of *Z. tritici tub2* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. A 12,704 bp fragment of pCeGFPTub2 (digested with *Zra*I), 1149 bp *tub2* promoter (amplified with SK-Sep-14 and SK-Sep-15; Table 1) and 1431 bp *tdtomato* (amplified with SK-Sep-89 and SK-Sep-90; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCtdTomato. The vector pCmCherry contains *mCherry* under the control of *Z. tritici tub2* promoter for integration into the *sdi1* locus by using carboxin as a selectable marker. A 12,704 bp fragment of pCeGFPTub2 (digested with *Zra*I), 1149 bp *tub2* promoter (amplified with SK-Sep-14 and SK-Sep-15; Table 1) and 714 bp *mCherry* (amplified with SK-Sep-83 and SK-Sep-84; Table 1) were recombined in *S. cerevisiae* to obtain the vector pCmCherry. The vector pHeGFP contains *egfp* under the control of *Z. tritici tub2* promoter for ectopic random integration by using hygromycin as a selection agent. A 13,534 bp fragment of pCeGFPTub2 (digested with *Bam*HI and *Bgl*II), and 1523 bp hygromycin resistance cassette (amplified with SK-Sep-128 and SK-Sep-129; Table 1) were recombined in yeast *S. cerevisiae* to obtain the vector pHeGFP. Further details on vector construction and yeast recombination-based cloning is provided in Kilaru and Steinberg (2015).

### 2.4. Z. tritici transformation and molecular analysis of transformants

The vectors pCmRFP, pCTagRFP, pCmCherry, and pCtdTomato 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 into 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 pCmRFP, pCTagRFP, pCmCherry, and pCtdTomato 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 labelling 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 plant infection

Attached wheat leaf infections were performed, as described previously (Rudd et al., 2008) with slight modifications. Wheat cultivar