Fungal Genetics and Biology 70 (2014) 33–41

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Tools and Techniques

Photo-convertible tagging for localization and dynamic analyses of low-expression proteins in filamentous fungi

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ARTICLE INFO

Article history:
Received 17 February 2014
Accepted 17 June 2014
Available online 9 July 2014

Keywords:
Filamentous fungi
Growth
Asexual development
Protein dynamics
Photo-convertible tagging
Dendra2

ABSTRACT

Photo-convertible fluorescent proteins (PCFPs) undergo a dramatic change in their excitation and emission spectra upon irradiation at specific wavelengths, thus rendering a different color. Dendra2 is a commercially available PCFP used to track the redistribution of proteins within cellular compartments, their life-time or interactions. Before photo-conversion Dendra2 exhibits green fluorescence, which becomes red after irradiation with either UV or blue lights. Multiple studies including Dendra2 as a molecular tool have been described in eukaryotes but not in filamentous fungi. Here we present a method to tag low-expression proteins from the filamentous fungus *Aspergillus nidulans* with Dendra2 and track their cellular dynamics. The regulator of asexual development FlbB was selected as control, a transcription factor that is expressed at low levels and can be used as a marker for the tip and nuclei of vegetative hyphae. This control provided us with a visual way to confirm the functionality of our genomic and plasmid constructs, since a non-functional FlbB protein renders a block in development and a characteristic aconidial phenotype. Our protocol combines standardized cloning and transformation procedures with the use of a mercury lamp microscope to convert and follow Dendra2 within cells. Hence, we present a rapid, simple and inexpensive method that makes tracking analysis of proteins that present technical difficulties to be followed feasible in filamentous fungi.

1. Introduction

The use of photo-switchable (PSFP; switch between fluorescent and dark states), photo-activatable (PAFP; fluorescence intensity increases upon irradiation) and photo-convertible (PCFP; change from one emission wavelength to another) fluorescent proteins (FP) gives unique opportunities to photo-label and track fusion proteins in a living cell (Chudakov et al., 2007a). Dendra2 is a green-to-red monomeric PCFP that has become a valuable tool due to the following benefits (Fig. 1): (1) the tagged protein can be tracked in green before photo-conversion (excitation and emission at 490 and 507 nm, respectively); (2) Dendra2 can be photo-activated using either UV (405 nm) or blue (488 nm) activating lights, and (3) the activated red Dendra2 signal renders high photo-stability, with excitation and emission at 553 and 573 nm, respectively (Chudakov et al., 2007b). The commercial availability of Dendra2 and its beneficial features have improved its use in several studies, which were mostly conducted in higher eukaryotes and also in yeast (Chudakov et al., 2007b; Jasik et al., 2013; Onischenko et al., 2009; Scholz et al., 2013).

In this work, we describe a simple method to tag with Dendra2 proteins from the filamentous fungus *A. nidulans* and track them within vegetative cells. These cells are non-specialized, syncytial (multinucleated) structures characterized by a continuous elongation of the tip, which occurs through the addition of new materials that are transported from distal regions (Penalva et al., 2012; Riquelme, 2013). The protocol by Pantazopoulou and Peñalva (2009) was elaborated to drive the expression of the tagged protein through the constitutive $gpdA^{mini}$ promoter. To validate our method, we focused on the tip of vegetative hyphae, which includes key factors for the signaling of development, such as the transcription factor FlbB (Etxebeste et al., 2008). The use of FlbB as control provided us, on one hand, with a visual screen method to determine the functionality of our constructs since mutations in *flbB* or misscheduled expression and localization of the protein lead to a characteristic aconidial growth pattern known as the *fluffy* phenotype (Etxebeste et al., 2008; Wieser et al., 1994). On the other hand, the analysis of FlbB localization and dynamics through Dendra2 labeling confirmed the same behavior as described in previous works (Etxebeste et al., 2008, 2009; Garzia et al., 2009). Since *flbB* expression in vegetative cells is low (Garzia et al., 2010), photo-conversion of Dendra2 was not induced using confocal microscopy and laser irradiation of a region of interest (ROI). In this

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http://dx.doi.org/10.1016/j.fgb.2014.06.006
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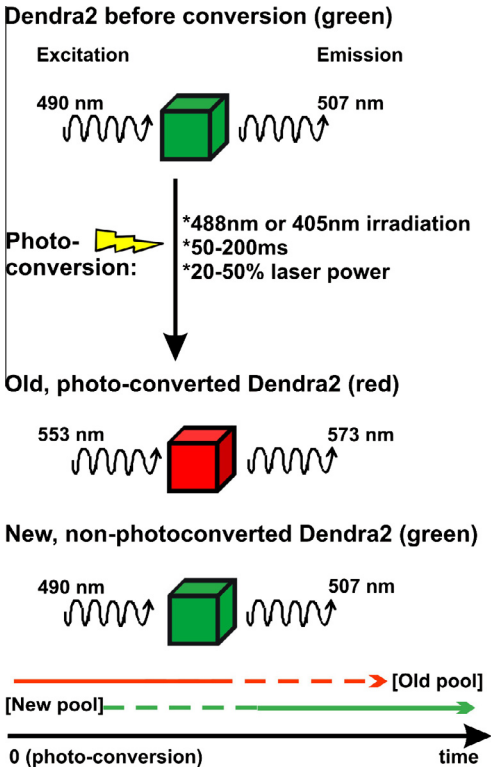

Fig. 1. Background of the Dendra2 photo-convertible tag. Before irradiation, the localization of the Dendra2-tagged protein can be analyzed following green fluorescence. Then, the entire cell or a ROI (region of interest) of the cell is irradiated with 405 nm or 488 nm laser light (50–200 ms; 20–50% of the laser power; optimal conditions have to be set for each system). Immediately, green Dendra2 is photo-converted to the red form. Dynamics of the old protein pool as well as its half-life may be followed in red. New protein fluoresces in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

work, the entire field of view was photo-activated using standard mercury arc illumination and an UV (DAPI; excitation at 355 nm) filter, as described previously by Vorvis et al. (2008). Photo-conversion of specific regions within vegetative hyphae was also achieved by locating them in the center of the visible field and closing the diaphragm to pinhole size (Baker et al., 2010). Then, red (old, photo-converted pool) and green (new pool) fluorescence of FlbB::Dendra2 were followed with time. Overall, this strategy opens an avenue for an easy, standardized and economical way to study protein localization and dynamics in filamentous fungi through PCFPs.

2. Materials and methods

2.1. Tools: plasmids and strains

Dendra2 sequence was obtained from Clontech as plasmid pDendra2 (http://www.clontech.com). According to the company's website, the brightness of Dendra2 was significantly lower compared to that of E-GFP (http://www.clontech.com/OA_MEDIA/xxclt_media/MainWP063553.html). In addition, we have previously described that the detection of FlbB::GFP or GFP::FlbB chimeras in vegetative hyphae required long exposure times (~1000 ms; Etxebeste et al., 2009). Taking these constraints into consideration, the use of a low expression protein as FlbB as control to analyze the viability of our Dendra2 labeling protocol might require the use of a strain expressing higher amounts of FlbB. Consequently, we generated *A. nidulans* strains in which FlbB::Dendra2 expression was driven either by the native *flbB* promoter, $flbB^{p}$, or the constitutive, attenuated (mini) version of the glyceraldehyde 3-phosphate dehydrogenase promoter, $gpdA^{mini}$ (Pantazopoulou and Peñalva, 2009). We then analyzed which strain rendered reasonable fluorescence levels (see Section 3.1).

To obtain a strain expressing FlbB::Dendra2 driven by the native promoter, p*GEM::*^{5x}*GA::dendra2::pyrG*^{Afum} plasmid, p146 at our database, was generated according to the procedure detailed in Supplementary Fig. 1A (see oligonucleotides used in Supplementary Table 1). This plasmid allows the use of the same oligonucleotides that may be used for GFP-labeling (pFN03 as template) based on the fusion-PCR technique described by Yang et al. (2004) (Supplementary Fig. 1A). The fusion-PCR *flbB::dendra2::pyrG*^{Afum} cassette was used to transform protoplasts of strain TN02A3 (Nayak et al., 2006). Homologous recombination at the native *locus* was confirmed by Southern-blot (*EcoRV* digestion; flbB-GSP3/GSP4 probe; not shown), as a 5.1 Kb band was detected in the genomic DNA of the transformants compared to the 2.4 Kb band of the parental wild-type strain. Strain BD608 (*flbB::dendra2::pyrG*^{Afum}) was stored.

To obtain a strain expressing FlbB::Dendra2 driven by the $gpdA^{mini}$ promoter (Pantazopoulou and Peñalva, 2009), plasmid p$gpdA^{mini}$*::flbB::dendra2::pyroA**, p154 at our database, was generated (see the detailed procedure in Supplementary Fig. 1B) and used to transform protoplasts of a Δ*flbB* strain (Garzia et al., 2010). Recombination at the *pyroA locus* was confirmed by Southern-blot (*BamHI* digestion, *pyroA* probe; not shown), since two bands of 10.5 and 2.0 Kb (integration of one copy of the plasmid; two or more copies generated a third band of 5.5 Kb), respectively, were detected in the transformants compared to the unique 5.8 Kb band of the parental strain. Strain BD660, which bore one copy of the plasmid, was stored at our database.

2.2. Functionality of constructs

With the aim of confirming the functionality of our constructs, we first analyzed conidia production per unit area for each strain generated and the corresponding control or parental strains. It was expected that the appropriate recombination of the constructs, which bore no mutation within *flbB* or *dendra2* sequences, had to render a conidiation phenotype with a similar conidia production compared to control wild type strains. A significantly lower conidia production would be indicative of a loss of function of FlbB. Fig. 2 confirms a non-significant variation in conidia/cm^2 production when $flbB^{p}$*::flbB::dendra2* and its parental wild type, or $gpdA^{mini}$*::flbB::dendra2* and $gpdA^{mini}$*::gfp::flbB* were compared (three replicates for each strain; $p > 0.01$ in both comparisons; non-significant change). Thus, we concluded that all strains contained functional FlbB sequences.

2.3. Techniques: fluorescence microscopy

Cellular localization of GFP- or Dendra2-tagged FlbB in vegetative hyphae was analyzed by culturing for 16 h conidiospore suspensions in 8-well plates (Ibidi, Germani; Cat. No. 80821) containing 300 μl/well of adequately supplemented Watch Minimal Medium (WMM; Penalva, 2005). Fluorescence images were obtained from these *in vivo* cultures at 37 °C using one of the following microscopes: (1) a DMI 6000B Leica inverted microscope, equipped with a 63× Plan Apo 1.4 N.A. oil immersion lens (Leica), and fitted with GFP (excitation 470 nm; emission 525 nm), Txred (excitation 562 nm; emission 624 nm) and D (UV; excitation 355 nm; emission 470 nm) filters, for GFP and Cherry-Red, or induce photo-conversion of Dendra2, respectively and (2) an Axio Observer Z1 inverted microscope, equipped with a 63× Plan-Apochromat 1.4 oil immersion Lens, and fitted with filters number