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Fluorescent markers of the endocytic pathway in *Zymoseptoria tritici* ☆

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ABSTRACT

Hyphal growth in filamentous fungi is supported by the uptake (endocytosis) and recycling of membranes and associated proteins at the growing tip. An increasing body of published evidence in various fungi demonstrates that this process is of essential importance for fungal growth and pathogenicity. Here, we introduce fluorescent markers to visualize the endocytic pathway in the wheat pathogen *Zymoseptoria tritici*. We fused enhanced green-fluorescent protein (eGFP) to the actin-binding protein fimbrin (ZtFim1), which is located in actin patches that are formed at the plasma membrane and are participating in endocytic uptake at the cell surface. In addition, we tagged early endosomes by eGFP-labelling a Rab5-homologue (ZtRab5) and late endosomes and vacuoles by expressing eGFP-Rab7 homologue (ZtRab7). Using fluorescent dyes and live cell imaging we confirmed the dynamic behavior and localization of these markers. This set of molecular tools enables an in-depth phenotypic analysis of *Z. tritici* mutant strains thereby supporting new strategies towards the goal of controlling wheat against *Z. tritici*.

1. Introduction

Tip growth is a hallmark of filamentous fungi, used to explore soil, exploit substrates or invade host organisms during fungal pathogenesis (Steinberg, 2007). Tip growth is characterized by polar extension of the hyphal growth region, which requires constant delivery of growth supplies, such as membranes and proteins, but also protein complexes and cell wall-forming enzymes. It is widely accepted that tip growth involves the delivery of Golgi-derived secretory vesicles, which are transported to the hyphal tip and accumulate in the Spitzenkörper (Harris et al., 2005; Riquelme and Sanchez-Leon, 2014). This process involves cytoskeleton and molecular motors, which utilize ATP to transport the membranous cargo to the tip for polarized secretion (Egan et al., 2012a; Steinberg, 2007; Xiang and Plamann, 2003). The discovery of endocytosis in the late 1990s added another important process to the mechanism of tip growth. It was shown firstly in *Ustilago maydis* that early endosomes participate in fungal morphogenesis and hyphal tip growth (Wedlich-Söldner et al., 2000). Subsequent studies showed that these endosomes participate in apical recycling of receptors at the hyphal tip (Fuchs et al., 2006). Shortly thereafter, evidence for apical endocytic recycling in fungal growth and morphology was found in filamentous ascomycetes (Higuchi et al., 2009; Lee et al., 2008; Araujo-Bazán et al., 2008; Upadhyay and Shaw, 2008), which led to the concept of an apical recycling model (Shaw et al., 2011; overview in Penalva, 2010; Steinberg, 2014). Interestingly, early endosomes move bi-directionally along microtubules (Wedlich-Söldner et al., 2000), a process driven by kinesin-3 and dynein (Wedlich-Söldner et al., 2002; Lenz et al., 2006; Abenza et al., 2009; Zekert and Fischer, 2009; Zhang et al., 2010; Egan et al., 2012b; overview in Steinberg, 2014). Recent work in the corn smut fungus *U. maydis* has shed light on the function of this motility. Surprisingly, it demonstrates that this motility distributes the protein translation machinery, including mRNA (Baumann et al., 2012) and ribosomes (Higuchi et al., 2014), which is required for extended hyphal growth. In addition, long-range motility of early endosomes mediates communication between the invading hyphal tip and the nucleus (Bielska et al., 2014). This long-range signaling is required for production of effector proteins and, therefore, is essential for virulence of *U. maydis* (overview in Higuchi and Steinberg, 2015).

Early endosomes are part of the endocytic pathway. This begins with the uptake of membranes and fluid at the plasma membrane (Fig. 1A). Endocytosis in yeasts and filamentous fungi involve polar-localized actin patches (Warren et al., 2002;

Abbreviations: Tub2, α-tubulin; Enhanced green-fluorescent protein, eGFP; Zt, *Zymoseptoria tritici*; *sdi1*, succinate dehydrogenase 1; RB and LB, right and left border; mCherry, monomeric cherry; *hph*, hygromycin phosphotransferase; *nptII*, neomycin phosphotransferase; *bar*, phosphinothricin acetyltransferase.

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

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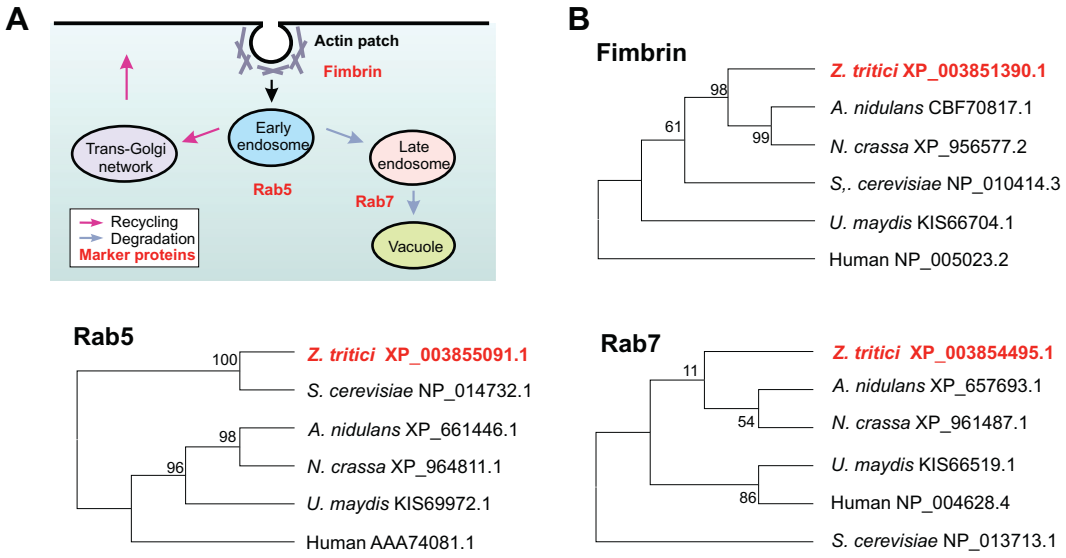

Fig. 1. Markers for the endocytic pathway in *Z. tritici*. (A) Schematic overview of the main endocytic membrane trafficking pathways in eukaryotes. Marker proteins for endocytic organelles are indicated in red. Note that the diagram is a highly simplified. (B) Phylogenetic trees comparing the predicted full-length amino acid sequence of homologues of the actin-binding protein fimbrin, and the endocytic small GTPases Rab5 and Rab7 in fungi and humans. The *Z. tritici* orthologues, used in this study, are indicated in bold and red. NCBI accession numbers are given (http://www.ncbi.nlm.nih.gov/pubmed). Maximum likelihood trees were generated using MEGA5.1 (Tamura et al., 2011). Bootstrap values are indicated at branching points.

Araujo-Bazán et al., 2008; Basu et al., 2014). The actin-binding protein fimbrin localizes to these actin patches (Wu et al., 2001; Castillo-Lluva et al., 2007; Delgado-Alvarez et al., 2010; Upadhyay and Shaw, 2008) and performs essential roles in the formation of endocytic vesicles at the plasma membrane (Shaw et al., 2011; Skau and Kovar, 2010). Endocytic vesicles deliver their cargo to early endosomes, which in animals and fungi carry the small GTPase Rab5 (Fig. 1A; Fuchs et al., 2006; Abenza et al., 2009; Chavrier et al., 1990; Seidel et al., 2013; Zerial and McBride, 2001). Rab5-positive early endosomes mature to late endosomes, which in animals and fungi carry the small GTPase Rab7 (Abenza et al., 2012; Chavrier et al., 1990; Higuchi et al., 2014). This compartment is an intermediate before endocytosed material is delivered to the vacuole for degradation.

In this study, we introduce fluorescent marker proteins for visualization of the endocytic pathway in the ascomycete *Zymoseptoria tritici*. This fungus is a major pathogen on wheat, causing significant economic damage in the European Union (Gurr and Fones, 2015) and, consequently, is considered amongst the most devastating plant pathogenic fungi (Dean et al., 2012). We confirm the specific localization of all markers using dual-color live cell imaging, pharmacological experiments and *in vivo* analysis of their cellular dynamics. We also describe 6 vectors, carrying 2 different resistance cassettes, to enable phenotypic analyses of morphological *Z. tritici* mutants or in-depth mode of action studies on novel anti-fungal chemistries.

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) was used as recipient strain for the genetic transformation experiments. The isolate was inoculated from stocks stored in glycerol (NSY glycerol; nutrient broth, 8 g/l; yeast extract, 1 g/l; sucrose, 5 g/l; glycerol, 700 ml/l) at −80 °C onto solid YPD agar (yeast extract, 10 g/l; peptone, 20 g/l; glucose, 20 g/l; agar, 20 g/l) and grown at 18 °C for 4–5 days.

2.2. Identification of Z. tritici homologues and bioinformatics

To identify homologues of the chosen marker proteins, we screened the published sequence of *Z. tritici* strain IPO323 (http://genome.jgi.doe.gov/Mycgr3/Mycgr3.home.html), using the provided BLASP function and the *U. maydis* proteins sequences of Fim1 (NCBI accession number: XP_760915.1), Rab5a (NCBI accession number: XP_757052.1) and Rab7 (NCBI accession number: 761658.1). Sequences were obtained from the NCBI server (http://www.ncbi.nlm.nih.gov/pubmed) and comparison was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) and domain structures were analyzed in PFAM (http://pfam.xfam.org/search/sequence). Finally, phylogenetic trees were generated in MEGA5.2, using a Maximum likelihood algorithm, followed by 1000 bootstrap cycles (http://www.megasoftware.net/; (Tamura et al., 2011).

2.3. Molecular cloning

All the vectors used 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). PCR reactions and other molecular techniques followed standard protocols (Sambrook and Russell, 2001). All restriction enzymes and reagents were obtained from New England Biolabs Inc (NEB, Herts, UK).

Vector pHFim1eGFP contains *egfp* fused to the full-length *ztfim1* under the control of constitutive *zttub2* promoter and terminator sequences for random ectopic integration into the genome of *Z. tritici* using hygromycin B as selection agent. A 13,159 bp fragment of pHeGFPTub2 (see Schuster et al., 2015; digested with *Bsr*GI),