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IDC1, a Pezizomycotina-specific gene that belongs to the PaMpk1 MAP kinase transduction cascade of the filamentous fungus *Podospora anserina*

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Abstract

Components involved in the activation of the MAPK cascades in filamentous fungi are not well known. Here, we provide evidence that *IDC1*, a pezizomycotina-specific gene is involved along with the PaNox1 NADPH oxidase in the nuclear localization of the PaMpk1 MAP kinase, a prerequisite for MAPK activity. Mutants of *IDC1* display the same phenotypes as mutants in *PaNox1* and *PaMpk1*, i.e., lack of pigment and of aerial hyphae, female sterility, impairment in hyphal interference and inability to develop Crippled Growth cell degeneration. As observed for the *PaNox1* mutant, *IDC1* mutants are hypostatic to *PaMpk1* mutants. *IDC1* seems to play a key role in sexual reproduction. Indeed, fertility is diminished in strains with lower level of IDC1. In strains over-expressing *IDC1*, protoperithecia reach a later stage of development towards perithecia without fertilization; however, upon fertilization maturation of fertile perithecia is diminished and delayed. In addition, heterokaryon construction shows that *IDC1* is necessary together with *PaNox1* in the perithecial envelope but not in the dikaryon resulting from fertilization.

Index Descriptors and Abbreviations: MAP kinase; NADPH oxidase; Sexual reproduction; Cell degeneration; *Podospora anserina*; Differentiation; Signal transduction; Molecular genetics; Mutagenesis; Mutations; Ascomycetes; Filamentous fungi; Inheritance; Gene sequencing; Mycelium; Perithecia; Sexual reproduction

1. Introduction

MAP kinases are proteins that function in succession to transmit various cellular signals (Widmann et al., 1999). The MAP kinase kinase kinase (MAPKKK) is at the top of the cascade and activates by phosphorylation the MAP kinase kinase (MAPKK), which in turn activates the MAP kinase (MAPK). These MAPK cascades are present in all eukaryotes and are involved in many different physiological responses ranging from differentiation, cell proliferation and cell survival to programmed cell death. The upstream and downstream effectors are usually species-specific or at least specific for a defined phylogenetic group. In Pezizomycotina ascomycetes, three MAP kinase modules are now recognized (Xu, 2000). They are homologous to the *Saccharomyces cerevisiae* HOG1, FUS3 and MPK1. Their roles seem very diverse and little is known about the upstream components involved in activating the MAP kinase modules.

This is especially true for the MPK1-like cascade, which is the least studied of the three cascades. In *Podospora anserina*, we recently described the identification and role of the MPK1-like cascade (Kicka et al., 2006; Kicka and Silar, 2004). The MAPK module is composed of the PaASK1 MAPKKK, the PaMKK1 MAPKK and the PaMpk1 MAPK. We have identified the PaNox1 NADPH oxidase as a component of the cascade (Malagnac et al., 2004). NADPH oxidases are plasma membrane enzymes that generate superoxide from NADPH and are involved in

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differentiation and defence mechanisms (Bokoch and Knaus, 2003). Epistasis of the MAP kinase module mutants over the PaNox1 mutant (Malagnac et al., 2004) is explained by the requirement of PaNox1 for a regulated nuclear localization of PaMpk1, as it is not required for phosphorylation (Kicka et al., 2006). We propose a model in which the PaNox1 enzyme generates superoxide ions, which act through protein modifications to transmit the signal ensuring nuclear localization of PaMpk1.

The cellular role of the cascade was defined by careful analysis of null alleles of the *PaNox1*, *PaASK1*, *PaMKK1* and *PaMpk1* genes. The mutants carrying those alleles display the same phenotypes. First, the mutants are not able to present Crippled Growth (CG) cell degeneration (Haedens et al., 2005; Silar et al., 1999). This cell degeneration results in slow and spindly growth as well as in female sterility. Second, several stationary phase differentiations are lacking in the mutant strains (Kicka and Silar, 2004; Malagnac et al., 2004). This includes the lack of aerial hyphae and pigments as well as inability to differentiate the fruiting bodies (protoperithecia and later on perithecia), although gametes (ascogonia and microconidia) formation is unaltered. Finally, when *P. anserina* meets a fungal competitor, it displays hyphal interference (Silar, 2005), a defence reaction that includes the accumulation of peroxide and death of either the competitor fungus (when confronted with *Penicillium chrysogenum*) or of *P. anserina* (when confronted with *Coprinopsis cinereus*). The *PaNox1* and *PaASK1* mutants are impaired in this defence mechanism (Silar, 2005), as are the *PaMKK1* and *PaMpk1* mutants (see Section 3). Seemingly, whereas stationary phase differentiation and hyphal interference results from the normal activity of the cascade, CG stems from the abnormal activation of the MAPK cascade through an uncontrolled positive feedback loop (Kicka et al., 2006).

Mutant in the *IDC1* gene were selected in the same screen as the *PaNox1*, *PaASK1* and *PaMKK1* mutants (Haedens et al., 2005). Here, we identify *IDC1* as a Pezizomycotina-specific gene, provide evidence that *IDC1* is a component of the *PaMpk1* cascade and that its expression level affects the making of the female organs (protoperithecia) that when fertilized give rise to the fructifications.

2. Materials and methods

2.1. Strains and culture conditions

The *P. anserina* strains used here were all derived from the *S* strain ensuring a homogenous genetic background (Rizet, 1952). The *AS4-43* strain contains an amino acid change in the translation elongation factor eEF1A (Silar and Picard, 1994) and the *AS6-5* strain contains a mutation located in a gene encoding a ribosomal protein homologous of *Escherichia coli* S12 (Dequard-Chablat and Silar, 2006). The *193* mutant has a mutation in a polyketide synthase gene involved in melanin biosynthesis and lacks colour at every stage of its life cycle (Picard, 1971; Coppin and Silar, submitted for publication). The strains mutant for *PaASK1* (IDC^{118}), *PaMKK1* (IDC^{404}), *PaMpk1* (*ΔPaMpk1*) and *PaNox1* (IDC^{343}) were thoroughly described previously (Kicka et al., 2006; Kicka and Silar, 2004; Malagnac et al., 2004). The recovery of IDC^{1}, IDC^{308} and IDC^{502} is described in Haedens et al. (2005). Standard culture conditions, media and genetic methods for this fungus have been described (Rizet and Engelmann, 1949; http://podospora:igmors.u-psud.fr). M2 medium has the following composition: KH_2PO_4 0.25 g/l, K_2HPO_4 0.3 g/l, $MgSO_4/7H_2O$ 0.25 g/l, urea 0.5 g/l, thiamine 0.05 mg/l, biotine 0.25 μg/l, citric acid 2.5 mg/l, $ZnSO_4$ 2.5 mg/l, $CuSO_4$ 0.5 mg/l, $MnSO_4$ 125 μg/l, boric acid 25 μg/l, sodium molybdate 25 μg/l, iron alum 25 μg/l, dextrine 5 g/l, agar 12.5 g/l.

Podospora anserina is pseudohomothallic. Ascospores yielding homokaryotic and heterokaryotic mycelia can be obtained after sexual reproduction. Alternatively, heterokaryons form spontaneously and readily in this species when mycelium fragments are mixed together.

2.2. Cosmids and plasmids

The genomic library (a kind gift of V. Berteaux-Lecellier) used for transformation experiments was constructed from the *s mat+* strain in the cosmid vector pMOcosX. This integrative vector carries as a dominant selective marker the bacterial hygromycin B resistance gene under the control of the *cpc1* promoter of *Neurospora crassa* (Orbach, 1994). Subcloning of the *IDC1* gene was performed with the pBC-Hygro vector (Silar, 1995).

2.3. Nucleic acids manipulations

Standard methods were used for nucleic acids manipulation (Ausubel et al., 1987). For RNA extraction, the mycelium grown on a cellophane layer was processed as described (Lecellier and Silar, 1994), except that the mycelium was broken for 50 s at speed 4.5 in a Fastprep apparatus (Qbiogen) before addition of phenol. *P. anserina* transformation was performed according to Brygoo and Debuchy (1985).

2.4. Cloning of IDC1

Protoplasts from the IDC^{1} mutant were sequentially transformed with pools of 100 cosmids from the *P. anserina* wild-type genomic bank. The female fertility of the transformants was then checked and in pool II some were fertile, albeit poorly. Two successive rounds of SIB selection (Akins and Lambowitz, 1985) made it possible to isolate the cosmid II_{69} that after transforming the IDC^{1} mutant produced hygromycin B resistant (hyg^{R}) transformants, 50% of which displayed a wild-type or near wild-type phenotype. Four of these were backcrossed with the IDC^{1} strain and co-segregation of resistance to hygromycin B and wild-type phenotypes was verified in the progeny.