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The pH regulatory factor Pac1 regulates *Tri* gene expression and trichothecene production in *Fusarium graminearum*

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ABSTRACT

Fungi manage the adaptation to extra-cellular pH through the PacC transcription factor, a key component of the pH regulatory system. PacC regulates the production of various secondary metabolites in filamentous fungi. In the important cereal pathogen *Fusarium graminearum,* the production of trichothecene is induced only under acidic pH conditions. Here, we examined the role of the PacC homologue from *F. graminearum*, *FgPac1*, on the regulation of trichothecene production. An *FgΔPac1* deletion mutant was constructed in *F. graminearum* which showed a reduced development under neutral and alkaline pH, increased sensitivity to H_2O_2 and an earlier *Tri* gene induction and toxin accumulation at acidic pH. A strain expressing the *FgPac1*^{c} constitutively active form of Pac1 exhibited a strongly repressed *Tri* gene expression and reduced toxin accumulation at acidic pH. These results demonstrate that Pac1 negatively regulates *Tri* gene expression and toxin production in *F. graminearum*.

1. Introduction

Trichothecenes are toxic secondary metabolites produced by several pathogenic fungi including a number of *Fusarium* species (Desjardins, 2006). Among the *Fusarium* trichothecene producers, *Fusarium graminearum* and *Fusarium culmorum* are major pathogens that cause a devastating disease in cereal crops known as Fusarium head blight (FHB). In addition to the economic losses, these pathogens have serious impacts on human and animal health by contaminating maize, wheat and barley with trichothecenes (Goswami and Kistler, 2004; Bennett and Klich, 2003). *F. graminearum* (teleomorph: *Gibberella zeae* schwabe) can produce three different classes of B-type trichothecene: Some strains produce deoxynivalenol and either its 3-acetyled derivative (DON/3ADON chemotype) or its 15-acetyled derivative (DON/15ADON chemotype) and other strains produce nivalenol and its 4-acetyled derivative (NIV/4ANIV chemotype) (Alexander et al., 2009). The biosynthetic pathway of trichothecenes initiates from farnesyl pyrophosphate (FPP), and the different steps and intermediates are mostly known (Kimura et al., 2007). The genes encoding the enzymes implicated in these different steps were identified and referred to *Tri* genes. In *F. graminearum*, 15 *Tri* genes are functional and reside at three unlinked loci: 12 are clustered in the core "*Tri* cluster" locus, two genes form the *Tri1–Tri16* locus and the last one is a locus with a single gene *Tri101* (Kimura et al., 2003; Brown et al., 2004; Gale et al., 2005; Alexander et al., 2009). Among the genes located in the core "*Tri* cluster", *Tri5* encodes the trichodiene synthase involved in the first step of the biosynthetic pathway (Hohn and Beremand, 1989), which catabolizes the cyclization of FPP leading to trichodiene formation. *Tri4* encodes a multifunctional oxygenase catabolizing the next four steps in the biopathway (Hohn et al., 1995). *Tri6* and *Tri10* encode two transcription factors playing a positive regulatory role in trichothecene biosynthesis (Proctor et al., 1995; Tag et al., 2001). *Tri6* activates the expression of other *Tri* genes and negatively regulates *Tri10 in planta* (Seong et al., 2009). *Tri101* is located at a single gene locus and encodes a 3-O-acetyltransferase which catalyses the acetylation of trichothecenes. Its role in self-protection against the toxin has been demonstrated (Kimura et al., 1998).

Many studies aiming at investigating the regulation of trichothecene biosynthesis in *F. graminearum* have been conducted. Most of these studies show an influence of environmental or extra-cellular factors on toxin production but only few have elucidated a molecular regulation of *Tri* genes expression by highlighting the role of *Tri6* and *Tri10*. Among the factors modulating trichothecenes regulation, a profound effect has been demonstrated in liquid cultures for treatments with hydrogen peroxide (Ponts et al., 2007) or fungicides (Covarelli et al., 2004), temperature (Shmidt-heydth et al., 2008), carbon and nitrogen sources (Miller and Greenhalgh, 1985; Jiao et al., 2008) magnesium (Pinson-Gadais et al., 2008) and phenolic acids (Boutigny et al., 2009). A variety of amines were also identified as potent inducers of DON production (Gardiner et al., 2009a). In addition to those factors, it has been recently

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reported that low extra-cellular pH both promotes and is required for toxin production in *F. graminearum* (Gardiner et al., 2009b; Merhej et al., 2010). However, the mechanism by which pH regulates trichothecenes remains unknown.

Adaptation to extra-cellular pH in filamentous fungi and yeasts is a prerequisite for growing in extreme pH conditions. This adaptation is mediated by a mechanism that tailors genes expression to environmental pH. *Aspergillus nidulans* is the well known example in which the pH regulatory system is deeply characterized. It involves at least six components encoded by *palA*, *palB*, *palC*, *palF*, *palI* in addition to the key zinc finger transcription factor PacC (Peñalva and Arst, 2004). Briefly, under neutral to alkaline pH, the full-length closed conformation of PacC undergoes two proteolytic steps yielding in a shorter functional form (Díez et al., 2002). This final mature form activates the transcription of "alkaline-expressed genes" and represses a set of "acidic expressed genes" (Tilburn et al., 1995). The activation of these proteolytic steps is mediated by the *Pal* pathway in response to neutral or alkaline pH (Mingot et al., 2001). Mutations in *pacC* gene result in mutants unable to respond to changes in pH. Mutations leading to a loss-of-function phenotype, mimic the effect of growth under acidic conditions. For this class of mutants, the "acid-expressed genes" are positively regulated and the "alkaline-expressed genes" are not induced in all pH conditions. In contrast, *pacC*^{c} mutants, that constitutively express an active PacC factor, mimic growth under alkaline conditions. In these mutants, the "alkaline-expressed genes" are induced but not the "acidic expressed" ones (Arst and Peñalva, 2003a). An ortholog of *A. nidulans pacC* has been also characterized in several fungi including *Penicillium chrysogenum*, *Fusarium oxysporum* and *Fusarium verticillioides* (Suarez and Peñalva, 1996; Caracuel et al., 2003; Flaherty et al., 2003). Its role in positive or negative regulation of some genes involved in fungal development and virulence such as those encoding extra-cellular enzymes involved in cell wall degradation (Ramon et al., 1999; Prusky et al., 2001; Caracuel et al., 2003; Davis, 2003; Rollins, 2003; Bignell et al., 2005; You et al., 2007) has been demonstrated. In addition, PacC also regulates the biosynthesis and secretion of toxins and secondary metabolites, including sterigmatocystin, aflatoxins, cephalosporin, penicillin and fumonisins (Suarez and Penalva, 1996; Keller et al., 1997; Schmitt et al., 2001; Flaherty et al., 2003).

The goal of this study was to investigate the role of the pH regulatory factor Pac1 in trichothecene production by *F. graminearum*. Here, we confirm the effect of pH variation on *Tri* genes expression and toxin production observed previously (Merhej et al., 2010), and describe the isolation of the putative *pacC* homologue, *FgPac1*, from *F. graminearum*. We investigated its role in toxin production using a loss-of-function mutant (*FgΔPac1* strain) and a mutant expressing a constitutively active form of the protein (*FgPac1*^{c} strain). The data obtained indicate that Pac1 plays a major role in regulating trichothecenes biosynthesis by sensing extra-cellular pH in *F. graminearum*.

2. Materials and methods

2.1. Strains, media and culture conditions

Escherichia coli strain DH5α and TOP10F′® (provided with TOPO TA cloning® Kit – Invitrogen) were prepared to be electro-competent using a 10% glycerol solution and used for routine plasmid propagation.

F. graminearum strain CBS185.32 (Centraalbureau voor Shimmelcultures, The Netherlands) producing type B trichothecenes was used throughout this work. This strain produces a high amount of DON and 15ADON which differs from DON by the presence of an acetyl group on his C-15 oxygen (Kimura et al., 2007). In our culture conditions, the acetylated form of the toxin was predominantly detected with a ratio of 1/100 (Merhej et al., 2010). The wild-type strain and the mutant strains issued from this work were maintained as frozen microconidial suspension and stored at −80 °C with 25% glycerol for long term storage and propagated on Potato Dextrose Agar (PDA, Difco – France) when necessary.

Culture in CMC liquid medium (Carboxylmethyl cellulose 15%, yeast extract 1%, $MgSO_4{\cdot}7H_2O$ 0.5%, NH_4NO_3 1%, KH_2PO_4 1%) at 25 °C and 150 rpm was used to generate spores suspensions (Cappellini and Peterson, 1965). After 3 days, the culture was filtered through mira-cloth (100 μm). The filtrate was used as a spore suspension to inoculate the media with a concentration of 10^4 spores/ml throughout this study. GYEP medium (50 g/l glucose, 1 g/l yeast extract, 1 g/l peptone) was used for spores' germination to prepare protoplasts. For toxin measurements or RNA preparation, conidia obtained from CMC cultures were used to inoculate Petri plates (∅ 55 mm) containing 8 ml of one of the following liquid-culture media: (i) Minimum synthetic medium (MS medium) containing KH_2PO_4 0.5 g/l, K_2HPO_4 0.6 g/l, $MgSO_4$ 0.017 g/l, $(NH_4)_2SO_4$ 1 g/l, glucose 20 g/l and 0.1 ml/l of Vogel's trace elements stock solution (Vogel, 1956). This medium already used in previous works (Merhej et al., 2010) is low buffered. The initial pH close to 6.5, drops quickly during fungal development. (ii) Minimum synthetic medium buffered to pH3 (BMS3) is made with MS medium buffered with citric acid 8.4 g/l and Na_2HPO_4 2.83 g/l. (iii) Minimum synthetic medium buffered to pH6.5 (BMS6.5) is made with MS medium buffered with MOPS 10.66 g/l and MES 10.46 g/l. (iv) Minimum synthetic medium buffered to pH8 (BMS8) containing also the same components as MS medium except that the concentration of the phosphate source was modified to 5.3×10^{-3} g/l of KH_2PO_4 and 0.113 g/l of K_2HPO_4 to maintain pH8. For growth experiments, cultures were conducted in the same minimal media solidified with 1.5% agar. For medium neutralization, MS cultures were supplemented with 800 μl of a solution of MOPS and MES to give the same effective buffering capacity as the BMS6.5 medium. Incubation was performed at 25 °C in darkness. Cultures were collected by centrifugation in sterile conditions. The liquid medium was stored at −20 °C until toxin extraction and the mycelium was used to perform total RNA extraction or lyophilized for fungal biomass quantification after storage at −80 °C.

2.2. Identification and cloning of FgPac1 gene

Conserved regions of amino acids within the coding regions of PacC homologues from *F. verticillioides* (GenBank Accession No. AY216461), *F. oxysporum* (GenBank Accession No. AY125958) and *A. nidulans* (GenBank Accession No. Z47081) were used to identify the *FgPac1* in *F. graminearum* genome database (MIPS: Munich information centre for protein sequences) by alignment using the Basic Local Alignment Search Tool (BLAST) (Zhang et al., 2000). Two highly conserved partial sequences corresponding to the N-terminal and the C-terminal of *Pac1* consensus sequence were identified at the end of the contigs 1.267 and 1.268 respectively, suggesting that *Pac1* gene overlaps these two contigs. Primers Pac1debF and Pac1finR were designed to amplify the missing *Pac1* coding sequence from genomic DNA of strain PH-1 NRRL31084. The resulting amplicon was sequenced (Cogenics – Genome express Grenoble-France) and found to include the missing *Pac1* predicted coding sequence. The nucleic acid sequence and predicted amino acid sequence were submitted to GenBank (Accession No. **HM745929**).

Primers Pac1stF and Pac1endR were used to amplify a 4 kb fragment encompassing the full-length *Pac1* gene using genomic DNA from *F. graminearum* strain CBS185.32. PCR reaction was performed using the high fidelity Pfu polymerase (Promega) as follows: an initial denaturation step at 95 °C for 5 min, followed by