The gene cutA of Fusarium fujikuroi, encoding a protein of the haloacid dehalogenase family, is involved in osmotic stress and glycerol metabolism

Jorge García-Martínez, Marta Castrillo and Javier Avalos

Departamento of Genética, Facultad of Biología, Universidad de Sevilla, E-41012 Seville, Spain

Survival of micro-organisms in natural habitats depends on their ability to adapt to variations in osmotic conditions. We previously described the gene cut-1 of Neurospora crassa, encoding a protein of the haloacid dehalogenase family with an unknown function in the osmotic stress response. Here we report on the functional analysis of cutA, the orthologous gene in the phytopathogenic fungus Fusarium fujikuroi. cutA mRNA levels increased transiently after exposure to 0.68 M NaCl and were reduced upon return to normal osmotic conditions; deletion of the gene resulted in a partial reduction in tolerance to osmotic stress. ΔcutA mutants contained much lower intracellular levels of glycerol than the wild-type, and did not exhibit the increase following hyper-osmotic shock expected from the high osmolarity glycerol (HOG) response. cutA is linked and divergently transcribed with the putative glycerol dehydrogenase gene gldB, which showed the same regulation by osmotic shock. The intergenic cutAgldB regulatory region contains putative stress-response elements conserved in other fungi, and both genes shared other regulatory features, such as induction by heat shock and by illumination. Photoinduction was also observed in the HOG response gene hogA, and was lost in mutants of the white collar gene wcoA. Previous data on glycerol production in Aspergillus spp. and features of the predicted CutA protein lead us to propose that F. fujikuroi produces glycerol from dihydroxyacetone, and that CutA is the enzyme involved in the synthesis of this precursor by dephosphorylation of dihydroxyacetone-3P.

INTRODUCTION

In nature, survival of fungi relies on their ability to deal with a changing environment. Appropriate regulatory networks are necessary to overcome variations in different physical agents such as temperature, pH or osmotic pressure. Changes in water activity in fungal ecological niches determine their capacity for growth and colonization. Fungal growth requires the maintenance of appropriate turgor pressure in their hyphae (Lew, 2011), a parameter governed by an osmotic mitogen-activated protein kinase (MAPK) cascade. This regulatory mechanism controls the internal pressure through ion uptake and synthesis of osmolytes such as glycerol (Ellis et al., 1991), and is also used to adapt the fungal cells to hyper-osmotic conditions. The components of the high osmolarity glycerol (HOG) regulatory cascade have been thoroughly investigated in Saccharomyces cerevisiae (O’Rourke et al., 2002) and are widespread in filamentous fungi (Krantz et al., 2006).

Abbreviations: GPP, glycerol-3-phosphatase; HAD, haloacid dehalogenase; HOG, high osmolarity glycerol; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; RT-PCR, reverse transcription PCR.

Three supplementary figures are available with the online version of this paper.

The genes of the HOG pathway were first identified through the study of osmosensitive mutants in Neurospora crassa (Mays, 1969). Genetic analyses of a collection of 25 mutants with reduced ability to grow on 0.68 M NaCl led to the identification of six genes. Mutants of genes os-1, os-2, os-4 and os-5, which are unable to grow on 0.68 M NaCl, were found to be affected in different components of the HOG cascade: a histidine kinase, and MAP, MAPK and MAPKK kinases (Fujimura et al., 2003; Schumacher et al., 1997; Zhang et al., 2002). Two other mutants, cut and flm-2, were partially sensitive to hyper-osmotic conditions. The flm-2 mutation was later identified as an os-4 allele (Perkins et al., 2001) and the cut mutation affects a protein of the haloacid dehalogenase (HAD) family, called CUT-1 (Youssar et al., 2005), with no apparent function in the HOG regulatory cascade. Sensitivity of the cut and os mutants to hyper-osmotic conditions is due to their inability to accumulate glycerol to counteract this stress. However, the cut mutant differs from the os mutants in several aspects: (i) slant cultures of the cut strain exhibit a sharp interruption of aerial development, the origin of the mutant denomination. This unusual morphological alteration is absent in os mutants. (ii) Some dicarboximide antifungals, such as fludioxonil, trigger the synthesis of...
glycerol in the wild-type through activation of the HOG pathway in the absence of hyper-osmotic conditions. The os mutants are resistant to this antibiotic (Fujimura et al., 2003; Zhang et al., 2002), but the cut mutant is sensitive (Fujimura et al., 2000), suggesting that it does not participate in the regulatory HOG cascade.

The gene cut-1 is implicated in a different mutant phenotype, called Ovc from overaccumulation of carotenoids (Harding et al., 1984). Induction of carotenogenesis by light is a widespread phenotypic trait in filamentous fungi, including Neurospora spp. (Avalos & Corrochano, 2013). The ovc mutant was identified because of its higher accumulation of carotenoids in response to light, although it is similar to the wild-type in the dark (Harding et al., 1984). The only ovc mutant described so far contains a large DNA deletion, covering cut-1 and 20 additional genes (Youssar & Avalos, 2007); cut-1 plays a central role in the Ovc phenotype, since reintroduction of a wild-type cut-1 allele restores the normal carotenoid photoresponse. However, the single cut mutant exhibits normal carotenogenesis, indicating that the Ovc phenotype is due to the simultaneous loss of cut-1 and at least a second unidentified gene (Youssar & Avalos, 2007). Intriguingly, cut-1 gene expression is repressed by light and is controlled by proteins involved in light regulation in N. crassa (Youssar & Avalos, 2006), suggesting a possible link with the light-dependent phenotype of the ovc mutant.

The phytopathogenic fungus Fusarium fujikuroi is a model for the production of secondary metabolites, such as gibberellins, carotenoids, bikaverin and fusarins (Avalos et al., 2007). The biosynthesis of these compounds is subject to complex regulation involving different environmental signals, such as nitrogen availability, pH or light (Avalos & Estrada, 2010; Díaz-Sánchez et al., 2012; Limón et al., 2010; Tudzynski, 2005). These regulatory networks are frequently interconnected and may share common elements with stress-control mechanisms, such as those involving the key regulatory signal cAMP (García-Martínez et al., 2012; Studt et al., 2013). Because of the downregulation of N. crassa cut-1 expression by light and the possible regulatory connections of this gene with carotenoid biosynthesis, we analysed the function of the cut-1 orthologue in F. fujikuroi, here called cutA. Deletion of this gene resulted in normal morphology and pigmentation but reduced tolerance to osmotic stress. cutA mRNA levels correlated with osmotic conditions and, in contrast to cut-1, were induced by light. ΔcutA mutants did not show the increase in intracellular glycerol levels exhibited by the wild-type under hyper-osmotic conditions expected from the HOG response. Interestingly, cutA is linked and co-regulated with gldB, which encodes a putative glycerol dehydrogenase, suggesting that both genes participate in enzymic steps of glycerol metabolism.

**METHODS**

**Strains and culture conditions.** The wild-type strain of Fusarium fujikuroi, FKMC1995 (Gibberella fujikuroi mating population C) was kindly provided by J. F. Leslie (Kansas State University Collection, Manhattan, KS, USA). The ΔwcoA mutants SF225 and SF226 and the ΔcryD mutants SF236 and SF237 were described previously: ΔwcoA (Estrada & Avalos, 2008); ΔcryD (Castillo et al., 2013).

To obtain conidia, the strains were grown on CG agar [10 g d-+(-)-glucose 1 M, 0.1 g NH4NO3 1 M, 1 g KH2PO4 1 M, 0.5 g MgSO4.7H2O 1 M and 16 g agar 1 M] for 1 week under continuous illumination in a 22 °C chamber. Conidia were collected from the surface of the cultures in water, filtered through sterile Whatman paper and counted with a haemocytometer.

For reverse-transcription PCR (RT-PCR) analyses, 15 cm diameter Petri dishes with 80 ml of liquid DGasn medium [DG minimal medium (Avalos et al., 1985) with 3 g asparagine 1 M instead of NaNO3] were inoculated with 10⁶ fresh conidia of each strain and incubated in the dark at 30 °C for 3 days. After this time, the mycelia were collected from the Petri dishes before or after exposure to illumination, heat shock, or hyper-osmotic shock. For illumination, the Petri dishes were exposed to 7 W white light m⁻² from four fluorescent lamps (TL-D 18W/840; Philips) for 15, 30, 60, 120 and 240 min. For heat shock, Petri dishes were laid on a water bath set to 42 °C and incubated for 30, 60 and 120 min. For hyper-osmotic shock, 12.4 ml 30 % NaCl was added to each Petri dish (0.68 M final concentration) and incubated for 15, 30, 60, 120 and 240 min. In a complementary experiment, the effect of hypo-osmotic shock was investigated by using Petri dishes with 80 ml liquid DGASN supplemented with 0.68 M NaCl. After 3 days, the medium was removed and replaced by the same volume of fresh DGASN (without NaCl) and the mycelia were recovered immediately or after incubation for 15, 30, 60, 120 and 240 min under the new culture conditions.

For DNA extractions, 250 ml Erlenmeyer flasks with 100 ml liquid DGasn medium were inoculated with 10⁶ fresh conidia and incubated in the dark at 30 °C for 3 days.

**Growth assays and glycerol determination.** Growth and conidiation assays were performed in 250 ml Erlenmeyer flasks containing 100 ml DGasn, inoculated with 10⁶ fresh conidia and incubated for 7 days at 30 °C in an orbital shaker at 150 r.p.m. When indicated, the medium was supplemented with either 0.68 M NaCl or 1.2 M sorbitol. After incubation, 1 ml samples were taken from each flask and conidia were counted in a haemocytometer. For growth determination, the whole cultures were filtered through sterile Whatman paper, and the mycelia were frozen in liquid nitrogen and freeze-dried before weighing.

Growth experiments on solid media were carried out on 15 cm diameter Petri dishes containing 100 ml DG or DGasn medium, alone or supplemented with NaCl (final concentration 0.68 M), sorbitol (final concentration 1.2 M) or glycerol (final concentration 0.036 M). The strains were inoculated with a sterile toothpick and incubated for 7 days at 30 °C in darkness.

An additional growth assay was achieved in glass race tubes (17 cm length, 1.6 cm inner width) filled with 15 ml DGasn agar supplemented with 0.68 M NaCl. Approximately 1 mm mycelial pieces were set at the extremes of the tubes, and cultured at 100 % relative humidity in a 30 °C chamber. Linear growth was measured after 31 days.

For determination of glycerol content, 10⁷ fresh conidia were inoculated on 100 ml liquid DGasn medium in a 250 ml Erlenmeyer flask and incubated for 3 days at 30 °C on an orbital shaker at 150 r.p.m. After 3 days (time 0), mycelia from 15 ml culture samples were filtered, frozen in liquid nitrogen and stored. Then 33.3 ml of 30 % NaCl was added (final concentration 0.68 M) and mycelia from 15 ml aliquots were filtered 30 and 60 min after
osmotic shock. Mycelia were freeze-dried and 10 mg was ground in a mortar to obtain a powder, which was dissolved in 1 ml double-distilled water. Glycerol content was determined using the Glycerol Enzymic BioAnalysis kit (Boehringer Mannheim/R-Biopharm AG); following the manufacturer’s instructions, the samples were heated at 96 °C for 5 min prior to glycerol determination.

**Transformation and molecular biology techniques.** To obtain ΔcutA mutants, a plasmid was constructed with a hygromycin resistance cassette (HygR) surrounded by two internal cutA segments. For this purpose, a 3.7 kb segment containing the whole cutA gene was obtained by PCR with primers 1 (5’-ATGGTATGTTAGTAGGTGA-3’) and 2 (5’-CAGTTACAGGCGACATGGTGA-3’) and cloned into the pGEM-T Easy vector (Promega). The resulting plasmid was used as a template for PCR amplification with primers 3 (5’-GGCCTTCCGCAAAGATGTGC-3’) and 4 (5’-CTTAGACGACAGCGCCGG-3’), producing a DNA segment with cutA flanking sequences, lacking 1433 bp of the 1499 bp cutA ORF. This PCR product was ligated with a 4 kb HygR cassette, obtained from pAN7-1 (Punt et al., 1997), lacking 1433 bp of the 1499 bp cutA sequence (Wiemann et al., 1997), and digested with HindIII and BglII, to give pDCut. For transformation (Proctor et al., 1997), FKMCl995 protoplasts obtained from 2 x 10^6 conidia were incubated with 30 µg pDCut previously linearized with NotI.

The presence of wild-type or mutant ΔcutA alleles was checked by PCR with primers 1 and 5 (5’-TATCTCTCTGCCCTCCGAG-3’). Primers 1, 2, 3 and 4 were chosen from the cutA coding sequence, and primer 5 corresponded to an internal segment of the HygR cassette.

For Southern blot analysis, genomic DNA from the wild-type and four transformants was extracted from filtered samples following Weinkeve et al. (1998), and digested with EcoRV or HindIII. DNA was quantified using a Nanodrop spectrophotometer (NanoDrop Technologies), and the same amounts of digested DNA were loaded per lane on a 0.8 % agarose gel. After electrophoresis, DNA fragments were transferred to a Hybond-N+ membrane (GE Healthcare). For the Southern blot, a cutA 1.7 kb internal fragment was amplified by PCR with primers 6 (5’-CGCTATCCCTCTTACCGGACTG-3’) and 7 (5’-GGATTCTATCGCTGGTCTATGC-3’), and digested with Ncol and NcoI. The resulting 1.4 kb fragment was used as a probe for hybridization with the membrane-bound fragments of genomic DNA (Sambrook & Russell, 2001). The probe was heated for 10 min at 100 °C and incubated afterwards for 4 h at 37 °C with 2 µl hexanucleotide mix (10 X), 2 µl of a pool of nonradioactive dAT, dGT, dTP and dTP (0.5 mM), 2 µl of [α-32P]dCTP (1 X 10^6 GBq mmol^-1); Perkin Elmer), and 2 U of the Klenov fragment of Escherichia coli DNA polymerase I. The labelled probe was purified with the GFX DNA purification kit (GE Healthcare).

**Gene expression analyses.** The mRNA levels of genes cutA and gldB were determined in total RNA samples by real-time RT-PCR. Total RNA samples were obtained with the RNeasy Plant Mini kit (Qiagen) and their concentrations were estimated using a Nanodrop spectrophotometer (NanoDrop Technologies). Total cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis kit (Roche) and stored at −80 °C until use. RT-PCR analyses were performed with a LightCycler 480 Real-Time PCR Instrument (Roche) using the LightCycler 480 SYBR Green 1 Master (Roche). The primers were chosen with the software Primer Express v2.0.0 (Applied Biosystems) from exon sequences of each gene and synthesized (HPLC grade) by StabVida (Oeiras, Portugal). Primers were: cutA, 5’-CGATCAAGATCCCTACATAC-3’ and 5’-AGAGTGCCTGCGCGAGCAG-3’; gldB, 5’-CTCTTGCGCCTAGCTAAATCT-3’ and 5’-CGCTATCAAGAGAAGGATCTTCTAC-3’; hogA, 5’-GGAGGTCTGTAAGTACGCTGATT-3’ and 5’-GAGATATTGCTGCGGTATTCGAT-3’. The results for each gene were normalized to the corresponding results obtained with mRNA of the tubulin gene tubA, using the primers 5’-CGCTGTCGCAAACACTG-3’ and 5’-CGAGGACCTGTCGACAACT-3’. Each RT-PCR analysis was performed at least twice using cDNA samples obtained from at least two independent biological replicates.

**Sequence analyses.** Sequence alignments were performed with CLUSTAL_X 2.1 (Larkin et al., 2007), obtained from http://www.clustal.org/clustal2/. Potential fungal transcription factor binding sites were identified online with the MatInspector tool (Genomatix Software) (Cartharius et al., 2005).

**RESULTS**

**Identification of cutA, a gene of Fusarium orthologous to cut-1 of N. crassa**

The Fusarium orthologues for the N. crassa gene cut-1, previously associated with the osmotic stress response (Yousser et al., 2005), were identified through BLAST analyses against the Comparative Fusarium genome Database (Broad Institute). This gene, here called cutA, consists in Fusarium verticillioiides of a 1485 bp continuous ORF encoding a predicted 494 aa polypeptide (FVEG_12092), a size slightly smaller than the 526 aa CUT-1 protein. Very similar cutA genes were found in the genomes of Fusarium oxysporum (FOXG_03768) and Fusarium graminearum (FVEG_12092). The F. fujikuroi counterpart was identified afterwards in a sequence of the genome of this fungus (Wiemann et al., 2013), corresponding to the locus FJFUJ14151 in EMBl accession number HF679026.1.

CUTa of F. fujikuroi exhibits high similarity to CUT-1 of N. crassa, with 66 % coincident positions, rising to 84 % in the HAD domain (Fig. 1a). The amino extension of CUT-1, unusual for the proteins of the HAD family, contains presumed regulatory segments, such as a PEST domain, a polyglutamine tract, and two predicted nuclear localization signals (Yousser et al., 2005). Such elements were not found in this region of the Fusarium spp. CutA predicted proteins, which however did contain a 52 aa segment with 37 positions coincident with the same CUT-1 segment (Fig. 1b). This sequence is highly conserved in orthologues from other ascomycete fungi, such as Aspergillus nidulans, Trichoderma reesei, Botryotinia fuckeliana and Podospora anserina (Fig. 1c), suggesting a role in the function of this protein family.

**Targeted ΔcutA mutants are partially sensitive to osmotic stress**

The cut-1 mutation results in enhanced sensitivity to hyper-osmotic conditions (Yousser et al., 2005). To analyse the possible biological role of cuta in Fusarium spp., we obtained targeted knockout mutants for this gene by transformation with a plasmid in which a HygR cassette replaced the cutA coding sequence (Fig. S1a, available in Microbiology Online). After incubation of protoplasts of the wild-type strain FKMCl995 with this plasmid, 26 transformants were obtained and subcultured from uninucleate microconidia to ensure homokaryosis. Culture in medium supplemented with 0.68 M NaCl showed retarded growth of some of the transformants. Three of them (T1, T2 and
(a) Schematic representation of the CLUSTAL alignment between the CutA polypeptides from *F. graminearum* (Fg, FGSG_07907), those from *F. verticillioides* (FVEG_12092), *F. oxysporum* (FOXG_03768) and *F. fujikuroi* (Ff; identical patterns, only Ff shown), and the CUT-1 polypeptide of *N. crassa* (Nc). The HAD domain is marked in grey. Breaks between the boxes represent gaps introduced by the CLUSTAL program to facilitate alignment. Residues conserved in the five proteins are indicated below using bars. (b) Detailed representation of the alignment of the amino segment preceding the HAD domain in *F. graminearum* (Fg), *F. oxysporum* (Fo), *F. verticillioides* (Fv), *F. fujikuroi* (Ff) and *N. crassa* (Nc). The putative PEST (HTPPESPTETQI) and polyglutamine (QQQEQPQQ) domains of CUT-1 are indicated; a highly conserved 52 aa segment is boxed. (c) CLUSTAL comparison of the highly conserved segment highlighted above and the corresponding segments in the predicted CutA orthologues from *Trichoderma reesei* (Tr, EGR47034), *Podospora anserina* (Pa, XP_001909058), *Aspergillus nidulans* (An, XP_663168) and *Botryotinia fuckeliana* (Bf, XP_00155677).

Fig. 1. Sequence features of CutA. (a) Schematic representation of the CLUSTAL alignment between the CutA polypeptides from *F. graminearum* (Fg, FGSG_07907), those from *F. verticillioides* (FVEG_12092), *F. oxysporum* (FOXG_03768) and *F. fujikuroi* (Ff; identical patterns, only Ff shown), and the CUT-1 polypeptide of *N. crassa* (Nc). The HAD domain is marked in grey. Breaks between the boxes represent gaps introduced by the CLUSTAL program to facilitate alignment. Residues conserved in the five proteins are indicated below using bars. (b) Detailed representation of the alignment of the amino segment preceding the HAD domain in *F. graminearum* (Fg), *F. oxysporum* (Fo), *F. verticillioides* (Fv), *F. fujikuroi* (Ff) and *N. crassa* (Nc). The putative PEST (HTPPESPTETQI) and polyglutamine (QQQEQPQQ) domains of CUT-1 are indicated; a highly conserved 52 aa segment is boxed. (c) CLUSTAL comparison of the highly conserved segment highlighted above and the corresponding segments in the predicted CutA orthologues from *Trichoderma reesei* (Tr, EGR47034), *Podospora anserina* (Pa, XP_001909058), *Aspergillus nidulans* (An, XP_663168) and *Botryotinia fuckeliana* (Bf, XP_00155677).

T3), and one with wild-type growth, T4, were chosen for detailed molecular and phenotypic analysis. Genomic DNA samples from the wild-type and the four transformants were digested with *Eco*RV or *Hin*dIII and hybridized in a Southern blot with a *cutA* probe (Fig. S1b). The wild-type and the T4 samples showed a hybridizing band of the expected size but no bands could be detected in the T1, T2 and T3 samples. To confirm the replacement of *cutA* with the HygR cassette, PCR assays were carried out combining a primer from the *cutA* DNA region not affected by the gene replacement and a primer from an internal sequence of the HygR cassette. Amplification products of the expected size were obtained only from T1, T2 and T3, indicating the occurrence of the expected *cutA* deletion in these transformants. No amplification was detected with T4 DNA, suggesting that this transformant resulted from integration of the vector through ectopic recombination.

The wild-type and the four transformants exhibited similar pigmentation in the light or in the dark on DG agar, indicating that the ΔcutA mutation does not affect the regulation of carotenogenesis. The strains then were grown on DG agar with two different nitrogen sources and checked for the effect of hyper-osmotic conditions (Fig. 2a). The colonies were slightly larger with nitrate than with asparagine in control plates, and the diameter of the ΔcutA mutants (T1, T2 and T3) was similar to that of the *cutA* + strains (wild-type and T4) on each medium, indicating that the ΔcutA mutation does not alter the growth of the fungus under normal culture conditions. Supplementation of the medium with 0.68 M NaCl resulted in visible diameter reductions of the ΔcutA mutant colonies compared to those of the control strains. This growth reduction was compensated totally (in DG medium) or partially (in DGasn medium) by the presence of 36 mM glycerol, suggesting that the mutants are affected in their mechanism of glycerol production. The sensitivity of the ΔcutA mutants to NaCl is due to osmotic stress and not to ionic stress, as indicated by similar
The phenotype of the ΔcutA mutants indicates the participation of cutA in the hyper-osmotic resistance mechanism of *F. fujikuroi*. We investigated the effect of osmotic changes on the regulation of cutA using real-time RT-PCR. Addition of 0.68 M NaCl resulted in a rapid increase in cutA mRNA levels, by over tenfold 30 min after the onset of hyper-osmotic shock (Fig. 3a), followed by a subsequent return to initial levels 2 h later. To investigate the effect of hypo-osmotic stress, the fungus was grown in medium supplemented with 0.68 M NaCl and transferred to standard non-supplemented medium. Following the transfer, a rapid fivefold decrease of cutA mRNA levels was produced in the first hour; these low levels were maintained for at least 4 h. These results show that cutA mRNA levels are regulated in response to external osmotic conditions.

The recovery of normal growth by the ΔcutA mutants in 0.68 M NaCl when the medium was supplemented with 36 mM glycerol suggests a role for this gene in glycerol accumulation in *F. fujikuroi*. Therefore, we investigated the effect of adding 0.68 M NaCl to the culture on glycerol levels in the mycelia of the wild-type, T4 and the ΔcutA mutants (Fig. 3b). The cutA+ strains contained about 3–4 μg glycerol (g dry mass)−1 under standard growth conditions. This concentration increased to approximately 6 μg after onset of hyper-osmotic shock. By contrast, glycerol content was very low in the ΔcutA mutants [below 1 μg (g dry mass)−1], and no change was detected after addition of 0.68 M NaCl, indicating that CutA is needed for glycerol synthesis in *F. fujikuroi*.

cutA is linked and co-regulated with a gene encoding a putative glycerol dehydrogenase

The analysis of the sequences neighbouring cutA in the *Fusarium* spp. genomes revealed a divergently transcribed
orthologues are similarly organized in some ascomycetes, such as N. crassa (cut-1 and gld-1, separated by 2100 bp) or Magnaporthe grisea (MGG_00099 and MGG_00097, separated by 2113 bp), but not in others (see, e.g. the cutA orthologues of Botrytis cinerea BC1G_04790, or Puccinia graminis PG1G_17701). The genes surrounding cutA and gldB in the Fusarium spp. genomes have no predictable relationship with glycerol metabolism.

The divergent transcription of gldB with cutA and their possible functional relationship led us to investigate the effect of osmotic changes on gldB regulation. The same RNA samples used for the analysis of the effect of hyper-osmotic and hypo-osmotic shock on cutA mRNA were used to investigate the content of gldB mRNA. The results (Fig. 4b) showed the same regulatory pattern found for cutA (Fig. 3a), i.e. a rapid and transient induction in response to hyper-osmotic conditions, and a fivefold reduction in response to hypo-osmotic conditions. This result suggests coordinated transcription of both genes mediated by common upstream regulatory elements. A search for conserved transcription factor binding sites carried out with the online MatInspector tool (Cartharius et al., 2005) identified numerous candidate sequences for multistress response elements in the cutA/gldB intergenic region (Fig. 4a). Those corresponding to the F$YSSTR multistress response matrix family exhibited the maximum similarity. Similar putative regulatory elements were also found in the equivalent intergenic sequences in F. verticillioides (1745 bp) and F. oxysporum 4287 (1646 bp).

**cutA** and **gldB** are upregulated by heat shock and light

The MatInspector tool identified candidate regulatory elements for heat shock (Fig. 4a). This finding, and the expected correlation between temperature and osmotic stress in natural habitats, led us to analyse the effect of heat shock on cutA and gldB mRNA levels (Fig. 5a). A sudden temperature shift from 30°C to 42°C resulted in a transient increase in the mRNA content of both genes, with a maximum level reached after 30 min (Fig. 5a).

Expression of the gene **cut-1** in N. crassa is downregulated by light and upregulated in the dark in mutants of the white collar (WC) complex (Youssar & Avalos, 2006). To investigate a possible regulatory effect of light on cutA and gldB mRNAs, dark-grown wild-type cultures were transferred to light and the effect of illumination time was investigated. cutA and gldB mRNA levels augmented rapidly upon illumination, reached the highest values after 1 h exposure, following which they decreased, returning to close to initial levels 4 h later (Fig. 5b). Significant photoinduction was also observed in mutants of the gene **cryD**, which encodes a DASH cryptochrome photoreceptor involved in the regulation of secondary metabolism of this fungus (Castrillo et al., 2013). However, photoinduction was not apparent in mutants of the WC-1 gene of F. fujikuroi, wcoA (Estrada & Avalos, 2008), indicating that

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**Fig. 3.** Relation between cutA and osmotic stress. (a) Effect of hyper-osmotic or hypo-osmotic shock on cutA mRNA levels in wild-type F. fujikuroi. The data show results of RT-PCR of total RNA samples from mycelia grown for 3 days in normal culture conditions (time zero) and after adding 0.68 M NaCl (top graph) or from mycelia grown for 3 days in 0.68 M NaCl-supplemented medium at time zero followed by transfer to fresh non-supplemented medium (bottom graph). Values are expressed relative to the values for each gene at time zero. The data show means ± SD from three independent biological replicates. (b) Internal glycerol content in the cutA+ wild-type strains (●), cutA+ transformant T4 (■) and ΔcutA transformants [T1 （△）, T2 （△） and T3 （▽）] grown for 3 days in normal culture conditions (time zero) and after adding 0.68 M NaCl. Values are means ± SD from two independent biological replicates.

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gene, coding for a putative NADP-dependent glycerol-2-dehydrogenase. A similar genomic organization was found in A. nidulans, where the cutA orthologue ANID_05564 is linked and divergently transcribed with the NADP-dependent glycerol-2-dehydrogenase gene gldB (Fig. 4a) previously described in this species (De Vries et al., 2003). For coherent terminology, we retain this name for the F. fujikuroi counterpart (locus FFU1_14512 in the F. fujikuroi database; Wiemann et al., 2013). The cutA and gldB
this photoreceptor mediates cutA and gldB photoinduction. In contrast to the regulation previously found for cut-1 in N. crassa, the cutA mRNA levels in the dark were not raised in the DwcoA mutants compared to the wild-type, but were reduced about tenfold (data not shown).

The HOG pathway gene hogA is regulated by light

The HAD enzyme encoded by the cutA gene is not a component of the regulatory HOG cascade, but could be subject to regulation by this mechanism. The available Fusarium spp. genomes contain orthologous genes for all members of the HOG MAPK pathway. In A.nidulans, the HOG cascade consists of the two-component signalling proteins TcsB, YpdA and SskA, the MAPKK SskB, the MAPKK PbsB and the MAPK HogA (Furukawa et al., 2005). The Fusarium spp. genomes contain conserved orthologues for these proteins. The orthologues and their approximate identity percentages in F. fujikuroi are FFUJ_07404 (51% with TcsB), FFUJ_09828 (42% with YpdA), FFUJ_00926 (51% with SskB), FFUJ_05618 (37% with SskA), FFUJ_06204 (49% with PbsB), and FFUJ_04484 (79% with HogA). As a first insight on a possible regulation by light of the HOG cascade, we investigated the effect of light on HogA (FFUJ_06204) mRNA levels. The results showed a rapid increase of hogA mRNA in response to light, reaching a maximum after 30 min illumination and decaying afterwards (Fig. 6). As found for cutA and gldB, similar photoinduction was also present in the ΔcryD mutants, but not in the ΔwcoA mutants, again suggesting the participation of WcoA as a key photoreceptor in this photoreceptor.
et al. F. fujikuroi (broken lines) mRNA levels in...

Fig. 5. Effect of heat shock and light on cutA (solid lines) and gldB (broken lines) mRNA levels in F. fujikuroi. (a) Results from RT-PCR of total RNA samples from the wild-type grown for 3 days in the dark at 30 °C then exposed for 30 min, 1 h or 2 h to 42 °C. (b) Results from RT-PCR of total RNA samples from the wild-type (upper graph) and ΔwcoA and ΔcryD mutants (lower graph) grown for 3 days in the dark then exposed for 15 min, 30 min, 1 h, 2 h or 4 h to 7 W white light m⁻². Data show means ± SD from three independent biological replicates for the wild-type, and from two independent biological replicates for two mutants (ΔwcoA: SF225 y SF226; ΔcryD: SF236 and SF237). Values are expressed relative to the value obtained with mycelia from the same strain collected before heat-shock or before illumination (time zero).

and high molecular mass substrates, with numerous examples in all taxonomic groups, from E. coli (Kuznetsova et al., 2006) to humans (Seifried et al., 2013). In addition to their highly conserved HAD-like catalytic domain, CutA and CUT-1 exhibit an amino extension usually absent in other proteins from the HAD family (Youssar et al., 2005). A similar extension is found in Cut-like proteins encoded in the genomes of other ascomycetes, but not in representative zygomycete and basidiomycete species, such as Mucor circinelloides and Ustilago maydis (closest CutA relatives 87690 and UM04682 in the respective annotated genomes). The widespread occurrence of CUT-1/CutA orthologues in ascomycetes indicates a conserved functional role for this HAD subfamily in this fungal group.

The regulation of the genes cut-1 and cutA of N. crassa and F. fujikuroi by heat shock and hyper-osmotic shock and the phenotype of the respective mutants (Youssar et al., 2005) indicate a similar role of both genes in the osmotic stress response. This is further supported by the rapid reduction of cutA mRNA content after hypo-osmotic shock, and its transient induction by illumination. However, photoinduction of cutA mRNA differs strikingly from the down-regulation of cut-1 mRNA levels by light in N. crassa (Youssar & Avalos, 2006). Moreover, cut-1 mRNA content increases notably in the dark in different regulatory mutants involved in the light response, including those of the WC complex, an effect independent of the down-regulation by light. By contrast, mutants of the orthologous wc-1 gene of F. fujikuroi, wcoA (Estrada & Avalos, 2008), contain lower cutA mRNA levels in the dark than the wild-type, and have apparently lost their photoinduction. Thus, our results show different light-dependent regulations for cut-1 in N. crassa and cutA in F. fujikuroi, probably reflecting different survival requirements in their respective natural habitats.

The HOG response, mediated by a signalling transduction pathway through a MAPK cascade, triggers the accumulation of glycerol inside the cell in response to external hyper-osmotic conditions. This regulatory mechanism has been investigated in detail in S. cerevisiae (Nevoigt & Stahl, 1997), and seems to be ubiquitous in fungi (Krantz et al., 2006), including Fusarium species, as indicated by the occurrence of the expected HOG pathway genes in their genomes. Accordingly, exposure to 0.68 M NaCl results in a rapid increase of glycerol content in wild-type F. fujikuroi mycelia. However, the increase was modest compared to the proportionally higher glycerol accumulation in response to hyper-osmotic stress found in S. cerevisiae (Reed et al., 1987), suggesting that glycerol is not the major osmolyte in Fusarium spp. The ΔcutA mutants do not exhibit this response, and their glycerol content is very low even without osmotic stress, pointing to an enzymic role of CutA in the synthesis of glycerol. A similar reduction in glycerol content was previously found in the cut mutant of N. crassa (Lew & Levina, 2007). The incapacity of these mutants to accumulate glycerol, in either N. crassa or F. fujikuroi, had no apparent consequences on their ability to grow under standard laboratory conditions, probably because ion uptake mechanisms are sufficient to maintain appropriate turgor values (Lew & Levina, 2007).

The ability of the ΔcutA mutants to grow in hyper-osmotic conditions suggests that F. fujikuroi possess other mechanisms to counteract hyper-osmotic stress, such as ion uptake (Lew, 2011) or production of alternative osmolytes. In A. nidulans and Aspergillus oryzae, growth under high osmotic...
pressure results in the accumulation of arabitol and erythritol in addition to glycerol (De Vries et al., 2003; Ruijter et al., 2004). The Fusarium spp. genomes contain orthologous genes for the enzymes responsible for the last enzymic steps in the synthesis of arabitol and erythritol (Fig. 7): FFUJ_05626, FVEG_0255, FGSG_04036 and FOXG_03675 (protein codes in the F. fujikuroi, F. verticillioides genomes), exhibiting approximately 40% identity with erythrose reductase of Yarrowia lipolytica (accession number AAG15954), the enzyme producing erythritol; on the other hand, FFUJ_00595, FVEG_00541, FGSG_00655 and FOXG_00973 exhibit approximately 52% identity with L-arabitol dehydrogenase from Aspergillus niger (XP001397484). The occurrence of these genes suggests that Fusarium spp. are able to produce these polyalcohols in addition to glycerol. Likewise, the synthesis of other classes of osmoprotectants, such as trehalose, cannot be discounted. The Fusarium spp. genomes also contain orthologues for tpsA and orlA of A. nidulans, encoding the trehalose-6-phosphate synthase and phosphatase needed for trehalose production from glucose-6P (F. fujikuroi orthologues: FFUJ_13242 and FFUJ_13709, with approximately 77% and 43% identical positions with TpsA and OrlA, respectively). However, trehalose is not accumulated in response to osmotic stress in A. oryzae (Ruijter et al., 2004), and in A. nidulans it is rather accumulated in sexual and asexual spores (Ni & Yu, 2007), pointing to arabitol and erythritol as more likely osmotic-stress-induced osmolytes in F. fujikuroi.

The biochemical basis of glycerol production has been investigated in detail in S. cerevisiae, where this polyalcohol is synthesized from dihydroxyacetone phosphate through its conversion to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase, and the subsequent dephosphorylation of this product by glycerol-3-phosphate dehydrogenase to yield glycerol (Fig. 7). S. cerevisiae contains two glycerol-3-phosphate (GPP) isoforms, with 92.4% identical positions and encoded by genes gpp1 and gpp2 (Pahlman et al., 2001). The Fusarium spp. genomes contain a single gene putatively orthologous to gpp1 and gpp2, encoding a protein with only 32% and 33% coincident positions, respectively (comparison with FUJ_02752 of F. fujikuroi in Fig. S2). Glycerol also may be produced by a parallel pathway, in which dihydroxyacetone phosphate is first dephosphorylated to dihydroxyacetone by a putative GPP, and then converted to glycerol by glycerol dehydrogenase. This alternative pathway seems to function in A. nidulans, as indicated by the persistence of glycerol accumulation in mutants of the glycerol-3-phosphate dehydrogenase gene gldA (Fillinger et al., 2001b). Moreover, the null gldA mutants exhibit growth defects under standard growth conditions, but not under hyper-osmotic stress, and a role of this enzymic activity in cell wall integrity has been proposed.

In F. fujikuroi, cutA is linked and co-regulated with gldB, encoding the putative NADP⁺-dependent glycerol dehydrogenase, i.e., the enzyme converting dihydroxyacetone into glycerol. The gene gldB was previously investigated in A. nidulans, where the null mutants were totally devoid of glycerol dehydrogenase activity and exhibited impaired growth under hyper-osmotic conditions (De Vries et al., 2003). Moreover, glycerol dehydrogenase activity was markedly enhanced under hyper-osmotic stress in the wild-type, but not that of glycerol-3-phosphate dehydrogenase, explaining the incapacity of the gldB mutant to
grow under hyper-osmotic conditions. Taken together, these results are consistent with the synthesis of glycerol through dihydroxyacetone instead of glycerol-3P, suggesting that \textit{A. nidulans} and \textit{S. cerevisiae} have diverged in their biochemical mechanisms to produce this osmolyte. The similar regulation by osmotic stress of \textit{gldB} in \textit{F. fujikuroi} suggests the same biochemical scenario in this fungus.

If glycerol biosynthesis proceeds via dihydroxyacetone in filamentous fungi, the enzyme producing this precursor from dihydroxyacetone-3P remains to be identified. Recently, a HAD enzyme of \textit{Corynebacterium glutamicum} (cgR_2128 or Hdpa) was found to catalyse the dephosphorylation of dihydroxyacetone phosphate to produce dihydroxyacetone (Jojima \textit{et al.}, 2012). Hdpa is much shorter than CutA (274 and 494 amino acids, respectively), and as typically found in HAD enzymes, exhibits low similarity (60 coincident positions, Fig. S3). We propose that CutA catalyses the same enzymic reaction to produce dihydroxyacetone in \textit{Fusarium} spp. (Fig. 7). Supporting this hypothesis: (i) many enzymes from the HAD family exhibit phosphatase activities on a large variety of substrates, including among them dihydroxyacetone phosphate, and (ii) \textit{cutA} is divergently transcribed and coregulated with \textit{gldB}, encoding the predicted dihydroxyacetone-metabolizing enzyme. Fitting this hypothesis, and as found for the \textit{ΔcutA} mutant of \textit{F. fujikuroi}, intracellular glycerol levels were strongly decreased in the \textit{gldB} mutants of \textit{A. nidulans} compared to the wild-type (De Vries \textit{et al.}, 2003).

In summary, our results are consistent with \textit{cutA}/\textit{gldB} in \textit{F. fujikuroi} as a coordinated transcriptional set in charge of dihydroxyacetone-mediated glycerol biosynthesis, responding to different environmental cues associated to osmotic stress and desiccation, such as sun exposure and elevated temperatures. The HOG regulatory cascade is likely to play a central role in this regulation, including the induction by light. Moreover, the faster WcoA-dependent photoinduction exhibited by the \textit{hogA} gene compared to \textit{cutA} and \textit{gldB} is consistent with a sequential regulatory mechanism. Thus, \textit{hogA} could be activated by light by WcoA, and \textit{hogA} could in turn activate \textit{cutA}/\textit{gldB}. Confirmation of this hypothesis will require a deeper investigation of this regulatory system. The same genetic organization of \textit{cutA} and \textit{gldB} orthologues in other ascomycetes suggests a common origin from an early ancestor of this biochemical and regulatory pattern for glycerol biosynthesis.

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