Role of the transcription factor ChAP1 in cytoplasmic redox homeostasis: imaging with a genetically encoded sensor in the maize pathogen *Cochliobolus heterostrophus*

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SUMMARY

The redox-sensitive transcription factor *ChAP1* [*Cochliobolus heterostrophus* *YAP1* (Yeast Activator Protein 1) orthologue] of *C. heterostrophus* is required for oxidative stress tolerance. It is not known, however, to what extent the intracellular redox state changes on exposure of the fungus to oxidants, and whether *ChAP1* is involved in the return of the cell to redox homeostasis. In order to answer these questions, we expressed a ratiometric redox-sensitive fluorescent protein sensor, pHyper, in *C. heterostrophus*. The fluorescence ratio was sensitive to extracellular hydrogen peroxide (H₂O₂) concentrations that had been shown previously to inhibit the germination of conidia and growth of the pathogen in culture. *chap1* mutants showed a slower return to redox homeostasis than the wild-type on exposure to H₂O₂. Plant extracts that mimic oxidants in their ability to promote nuclear retention of ChAP1 reduced, rather than oxidized, the fungal cells. This result is consistent with other data suggesting that *ChAP1* responds to plant-derived signals other than oxidants. pHyper should be a useful reporter of the intracellular redox state in filamentous fungi.

INTRODUCTION

In fungi, the intracellular redox state determines the nuclear retention of a bZIP transcription factor belonging to the APETALA-1 (AP-1) family (Moye-Rowley *et al.*, 1989). The mechanism of oxidant sensing was elucidated in yeast where, in the absence of stress, *YAP1* (Yeast Activator Protein 1) is rapidly exported from the nucleus. Oxidation results in the formation of an intramolecular cysteine–cysteine disulphide bond, which prevents the interaction of *YAP1* with the nuclear export factor *CRM1* (Chromosomal Region Maintenance 1). The oxidation of *YAP1* is indirect. Hydrogen peroxide (H₂O₂) oxidizes a peroxiredoxin, which then reacts with *YAP1* (Delaunay *et al.*, 2000, 2002; Okazaki *et al.*, 2005). The domain structure of the proteins encoded by orthologues of *YAP1* in filamentous fungi is similar to that of the yeast protein. Experiments with green fluorescent protein (GFP) fusions have shown

that they are likewise retained in the nucleus in response to oxidants. Loss of ascomycete or basidiomycete *YAP1* orthologues results in hypersensitivity of the mutants to oxidants (Guo *et al.*, 2011; Huang *et al.*, 2011; Kim *et al.*, 2009; Lessing *et al.*, 2007; Lev *et al.*, 2005; Lin *et al.*, 2009; Molina and Kahmann, 2007; Temme and Tudzynski, 2009). Furthermore, the mutants fail to show the induction of target genes (belonging to the *YAP1* regulon) encoding enzymes that function in overcoming oxidative stress. These phenotypes suggest that *YAP1* is essential for the return to redox homeostasis during and following exposure to oxidative stress. This hypothesis has been tested in *Botrytis cinerea*, where the recovery of the reduced glutathione pool (*GSH*) was followed with a genetically encoded ratiometric fluorescent sensor: in a mutant lacking the *YAP1* orthologue *BAP1*, recovery of the *GSH* pool was affected following repeated exposure to H₂O₂ (Heller *et al.*, 2012). pHyper, a sensor in which a circularly permuted yellow fluorescent protein (YFP) was inserted into the regulatory domain of the *Escherichia coli* H₂O₂ sensor protein OxyR, was developed to report intracellular H₂O₂ levels (Belousov *et al.*, 2006). In this study, we constructed a fungal expression vector for the expression of pHyper in order to directly report H₂O₂ levels.

The maize pathogen *Cochliobolus heterostrophus*, agent of Southern corn leaf blight, causes necrotic lesions on leaves and is particularly damaging to maize lines with T cytoplasm, and therefore sensitive to the polyketide T-toxin produced by race T of the fungus (see Turgeon and Baker, 2007; Yoder, 1988). *Cochliobolus heterostrophus* is a necrotroph, killing plant host tissue. Necrotrophs apparently thrive in the presence of reactive oxygen species (ROS) produced by both the plant and the pathogen (see Heller and Tudzynski, 2011). Interestingly, loss of the *C. heterostrophus* *YAP1* orthologue, *ChAP1*, does not have a major effect on virulence (Lev *et al.*, 2005). In *Botrytis cinerea*, *BAP1* is also dispensable for virulence, and its target genes are not expressed *in planta* (Temme and Tudzynski, 2009). In *Ustilago maydis* (Molina and Kahmann, 2007) and *Alternaria alternata* (Lin *et al.*, 2009), however, the *YAP1* orthologues are required for full virulence. Thus, the function of this conserved transcriptional regulator appears to have diverged in the co-evolution of plant pathogens with their hosts, or may depend on additional experimental factors. In *C. heterostrophus*, phenolic compounds that are found in the plant host promote the nuclear retention of a functional

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GFP:ChAP1 fusion protein, but do not induce the accumulation of ChAP1-dependent transcripts (Shalaby *et al.*, 2012; Shanmugam *et al.*, 2010). In yeast, some phenolics have been found to indirectly activate *YAP1* by causing the accumulation of H_2O_2 (Maeta *et al.*, 2007). This does not seem to be the mechanism in *C. heterostrophus*, because: (i) catalase treatment of plant extracts did not remove their ability to cause nuclear retention of a GFP:ChAP1 fusion protein (Lev *et al.*, 2005); and (ii) if this were the case, one should observe the induction of ROS-detoxifying target genes on exposure to phenolics. In order to understand the connection between phenolics as small-molecule signals, the response to oxidants and their combined role in virulence, it is essential to follow the intracellular redox state in living cells. As a step towards answering these questions, and to develop a generally useful redox reporter for fungal biology, we expressed pHyper in *C. heterostrophus* and followed the ratiometric fluorescence signal on exposure to H_2O_2 or to phenolic compounds in wild-type and *chap1* strains.

RESULTS AND DISCUSSION

To express pHyper (Belousov *et al.*, 2006) in *C. heterostrophus*, the coding sequence was cloned downstream of the *Aspergillus nidulans* promoter region PtpC, and a fungal transcription terminator region, also from *A. nidulans*, was introduced downstream of the pHyper coding sequence. The construct, which also contained a cassette for hygromycin B resistance expressed under the control of the PtpC promoter, was transformed into *C. heterostrophus* by the polyethylene glycol (PEG) method, and transformants were selected for resistance to hygromycin B. Transformants showing strong fluorescence were purified by the isolation of single conidia. The excitation spectrum of the fluorescent protein encoded by pHyper has two maxima: a broad one near 400–420 nm and a well-defined one at 500 nm. The excitation spectrum depends on the H_2O_2 concentration. Fluorescence emitted on excitation with blue or green light is recorded in separate channels of the confocal microscopic image. The fluorescence emitted by excitation at 500 nm (green) increases strongly on exposure to H_2O_2 , whereas the fluorescence emitted by excitation at 400 nm (blue) on treatment with oxidant decreases slightly. The ratio of fluorescence measured on excitation with green light relative to that measured on excitation with blue light is therefore a sensitive measure of intracellular H_2O_2 levels (Belousov *et al.*, 2006).

Confocal images of germinating conidia of a *C. heterostrophus* strain expressing pHyper were obtained by allowing conidia to germinate in water on a glass slide. Fluorescence emission was recorded (collection band >510 nm) with laser excitation at 405 nm (blue channel) or 488 nm (green channel). Confocal images of germinating conidia of a pHyper-expressing strain are shown in Fig. S1 (see Supporting Information), in which each pair of images shows the green and blue channels. It is important to

note that the emitted fluorescence is always measured in the same wavelength band, whereas it is the excitation wavelength that differs between the two channels, which are coded by blue or green false colour in the images.

The fluorescence excited by the longer wavelength is enhanced by incubation with H_2O_2 relative to the H_2O control or a maize leaf extract. The increase in oxidant-dependent, long-wavelength excitation of pHyper fluorescence is clearly evident by inspection of the 405- and 488-nm channels (Fig. S1).

To obtain quantitative measurements, the ratio of the fluorescence excited at 488 nm to that excited at 405 nm was calculated from sets of images of fluorescence detected in the 405-nm (blue) and 488-nm (green) channels. We expressed pHyper in the wild-type (strain C4) and *chap1* mutant backgrounds. The exposure of germinating conidia of the wild-type or *chap1* mutant to 4 mM H_2O_2 resulted in a large shift in the fluorescence ratio, at the earliest recordable time point, within seconds of replacing the solution (Fig. 1A). In the wild-type, the intracellular redox state, as reported by the pHyper fluorescence excitation ratio, almost returned to the initial value by 30 min after the addition of H_2O_2 (Fig. 1A). In the *chap1* mutant, in contrast, intracellular ROS levels remained elevated for a longer period. We therefore chose 30 min as a convenient time point at which to see the resolution between the wild-type and *chap1* mutant. A concentration curve measured for external H_2O_2 at 30 min showed that there was a concentration range of about 1–10 mM external H_2O_2 within which the mutant cytoplasm remained oxidized (as measured by a higher 488-nm/405-nm ratio, Fig. 1B). A much higher range of extracellular peroxide concentrations was needed to shift the pHyper excitation spectrum *in vivo* relative to the purified fluorophore protein *in vitro* (Belousov *et al.*, 2006), probably as a result of the activity of catalases, the uptake of peroxide and its reaction with intracellular redox buffers, such as *GSH*. Furthermore, in *in vivo* experiments with *E. coli* (Belousov *et al.*, 2006), exposure to 2.5–50 μ M H_2O_2 was sufficient to cover the dynamic range of pHyper. Three orders of magnitude higher H_2O_2 levels were required in *C. heterostrophus* (Fig. 1B). H_2O_2 in the millimolar range is, likewise, needed to inhibit growth and to activate nuclear retention of ChAP1 (Lev *et al.*, 2005), suggesting that the fungus possesses efficient mechanisms to prevent extracellular oxidants from changing the intracellular redox state. Secreted catalase activity could be one such mechanism and, once H_2O_2 has entered the cell, it is likely to be degraded intracellularly as well. The pHyper construct described here for the ratiometric detection of H_2O_2 in filamentous fungi should be a useful tool, and can be compared with the previously described reporter of the state of the glutathione pool (Heller *et al.*, 2012).

In both the wild-type and *chap1* mutant, exposure to plant extract decreased the 488-nm/405-nm ratio (Fig. 2). Quantitative measurement indicated that the cytoplasm was, surprisingly, less oxidized than in water (Fig. 2). After 30 min of incubation with

Fig. 1 Intracellular redox state as reported by pHyper, following exposure of wild-type (WT) and *chap1* *Cochliobolus heterostrophus* strains to hydrogen peroxide (H_2O_2). (A) Time course: the x-axis indicates minutes after replacement of the water in which the conidia germinated with 4 mM H_2O_2 ; the y-axis shows the fluorescence ratio (488-nm/405-nm excitation); higher values indicate greater oxidation. ddw, H_2O control; data points are means and standard error (SE) for 10 microscopic fields. (B) Concentration dependence: the y-axis shows the 488-nm/405-nm ratio. Increasing concentrations of H_2O_2 (x-axis, mM) were applied for 30 min before imaging. Symbols indicate the averages of two to four independent experiments, each including 10 microscopic fields at each H_2O_2 concentration; error bars indicate the standard error of the mean (SEM) of the experiments. The WT and *chap1* datasets are significantly different ($P = 0.0012$, two-tailed paired Student's *t*-test applied to the complete datasets before calculation of the averages). The curves are least-squares fits of the data points to an exponential saturation curve. The value observed 30 min after replacement of the water covering the conidia with fresh deionized water without H_2O_2 (equivalent to ddw, A) was chosen as the baseline (dotted line).

plant extract or with several phenolic compounds, the intracellular redox state was more reduced than that observed for the H_2O (double-distilled water) controls. This result is perhaps surprising in view of the fact that these same treatments with phenolic

compounds (Shanmugam *et al.*, 2010) or maize leaf extract (Lev *et al.*, 2005 and Fig. S2, see Supporting Information) promoted the nuclear retention of *ChAP1*. It is, however, in agreement with the lack of induction of antioxidant genes by phenolic compounds. Plant extract does induce ROS-detoxifying genes (Lev *et al.*, 2005; Shalaby *et al.*, 2012; Shanmugam *et al.*, 2010). Nevertheless, in the presence of plant extract, the intracellular redox state was more reduced than that observed when conidia germinated in water, and similar to the redox state in the presence of phenolics (Fig. 2). This suggests that plant extract contains other compounds, distinct from phenolics, which can act as signals to induce antioxidant gene expression. The leaf extract was originally fractionated on the basis of GFP:ChAP1 nuclearization (Lev *et al.*, 2005). New fractionation experiments based on the expression of, for example, the thioredoxin gene *TRX2* will probably identify new classes of active compounds. Possible mechanisms for the nuclear retention of ChAP1 in the absence of any obvious oxidant are also under study. Attempts to visualize pHyper *in planta* in *C. heterostrophus* were not successful. Inspection of the images in Fig. S1 provides an explanation for this: in the absence of oxidants, fluorescence from the green channel is predicted, from the *in vitro* results, to be low, particularly in the presence of plant phenolics. Furthermore, excitation by blue light may be hindered by the absorption of plant tissue. In the interaction of wild-type *C. heterostrophus* with maize, there is no indication that the plant produces a strong oxidative burst. The introduction of pHyper to other pathosystems in which there is a clear oxidative burst should be promising.

EXPERIMENTAL PROCEDURES

Expression of pHyper

pHyper (purchased from Evrogen, Moscow, Russia) was expressed under the control of a fungal promoter, P_{TrpC}, from pUCATPH (Lu *et al.*, 1994). The plasmid also carries the hygromycin resistance cassette from pUCATPH. To construct the fungal pHyper vector, the original vector HyPer cyto was cut with *HindIII* and *NheI*, and the 1449-bp product [containing the HyPer open reading frame (ORF)] was treated with Klenow fragment (New England Biolabs, Ipswich, MA, USA). The pYFPN vector underwent *SpeI/NotI* restriction and the 7057-bp product was Klenowed and CIP(ed) [the two fragments were ligated (blunt-end ligation)], and the vector containing the correct orientation was transformed into *C. heterostrophus* wild-type strain C4, or the *chap1* mutant, as described previously (see Catlett *et al.*, 2003; Turgeon *et al.*, 1985, 2008, 2010). The *chap1* mutant used here was constructed by replacement of the *ChAP1* gene with a neomycin resistance cassette (M. Ronen, PhD Thesis, Technion, Haifa, Israel, 2011), so that hygromycin B could be used for this second transformation.

Confocal imaging

Samples were imaged with a Zeiss LSM 700 confocal microscope (Carl Zeiss, Jena, Germany), with 405- and 488-nm laser excitation, and

Fig. 2 Plant extract and phenolics shift the intracellular redox state to a more reduced state. Conidia were germinated on a glass slide; after 8 h of incubation, the water was removed and replaced with the indicated solutions; 30 min later, fluorescence was imaged and the 488-nm/405-nm ratio was calculated (y-axis). Bars indicate average and standard deviation of two experiments on independently isolated pHyper-expressing strains; the value from each experiment is the mean of 10 microscopic fields. PE, plant (maize leaf) extract (see Experimental procedures). The left panel shows data for conidia of the wild-type (WT) and the right panel for the *chap1* mutant. ddw, H₂O control; DMC, 3,4 dimethoxycinnamic acid; DMSO, dimethylsulphoxide.

fluorescence was recorded with the standard filter for GFP, transmitting a band above 510 nm. Image ratio analysis was performed using LSM Image Browser Release 4.2 (Carl Zeiss).

Response of the intracellular redox state to phenolic compounds and plant extract

Phenolic compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA), dissolved in dimethylsulphoxide (DMSO) and diluted to the indicated concentrations. Plant extract was prepared by a procedure modified from Lev *et al.* (2005). The leaves from 2-week-old glasshouse-grown maize plants were ground to a fine powder in liquid nitrogen. Leaf powder was stored at -75°C , defrosted immediately before use and suspended in water at 1:1 weight/volume. The suspension was centrifuged at 20 800 *g* for 5 min, and the supernatant was used immediately.

ACKNOWLEDGEMENTS

This study was supported in part by the Israel Science Foundation (ISF 370/08) and the US–Israel Binational Agricultural Research and Development Fund (BARD IS 4167-08C). We are grateful to Dr Maayan Duvshani-Eshet and Dr Nitsan Dahan for expert assistance with confocal microscopy, and the Life Sciences & Engineering (LS&E) infrastructure facility for establishing the imaging and microscopy unit that made this study possible. Professor Yehuda Assaraf's group introduced us to the use of pHyper. We thank Dr Olga Larkov for helpful discussions and suggestions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Confocal images were exported from the LSM software as triple panels showing the green (488-nm excitation), blue (405-nm excitation) and merged images. The green and blue channels are shown here. Each column shows the corresponding green and blue images for 10 microscopic fields of samples for the indicated treatments. Green and blue are false colour codes for excitation at 488 nm and 405 nm, respectively; fluorescence emission is detected for wavelengths >510 nm (see Experimental procedures).

Fig. S2 Localization of GFP:ChAP1 in response to plant extract (PE). Conidia were germinated on glass slides and exposed to PE or water (control) for 30 min, and imaged by confocal microscopy. Merged images of green fluorescent protein (GFP) fluorescence (green channel) and bright field are shown.