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Redox-sensitive GFP2: use of the genetically encoded biosensor of the redox status in the filamentous fungus *Botrytis cinerea*

JENS HELLER[1], ANDREAS J. MEYER[2] AND PAUL TUDZYNSKI[1,*]

[1]*Institut fuer Biologie und Biotechnologie der Pflanzen, Westf. Wilhelms-Universitaet, Hindenburgplatz 55, D-48143 Muenster, Germany*
[2]*INRES—Chemical Signalling, Rheinische Friedrich-Wilhelms-Universitaet Bonn, Friedrich-Ebert-Allee 144, D-53113 Bonn, Germany*

SUMMARY

The production of reactive oxygen species (ROS) is part of the defence reaction of plants against invading pathogens. The effect of ROS on filamentous fungi is still unclear. In this study, ratiometric redox-sensitive green fluorescent protein (roGFP) was introduced as a tool for *in vivo* measurement of the cellular redox status in filamentous fungi. A fungal expression system for roGFP2 was constructed. Expressed in *Botrytis cinerea*, roGFP2 reversibly responded to redox changes induced by incubation with H_2O_2 or dithiothreitol, which was determined by confocal laser scanning microscopy imaging and fluorometry. As the sensor detects the redox potential of the cellular glutathione pool, it was used to analyse the kinetics of GSH (glutathione, reduced form) recovery after H_2O_2 treatment. The transcription factor Bap1 is the main transcriptional regulator of H_2O_2-scavenging proteins in *B. cinerea*. When compared with the wild-type, GSH recovery in the Δbap1 deletion mutant was affected after repeated H_2O_2 treatment. ROS and intracellular redox changes can be used by fungi for signalling purposes. *In planta* experiments, performed in this study, indicated that redox processes seem to be important for the differentiation of penetration structures. During the penetration of onion epidermal cells, the status of the cellular glutathione pool differed between appressoria-like structures and infecting hyphae, being reduced in the presence of infecting hyphae and more oxidized around appressoria-like structures.

INTRODUCTION

Botrytis cinerea Pers.:Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is a necrotrophic plant pathogen that causes grey mould disease in more than 200 crop species, leading to serious pre- and post-harvest losses worldwide (Williamson *et al*., 2007).

An initial broad and nonspecific defence reaction of plants against invading pathogens is the oxidative burst, a process in which plants produce large amounts of reactive oxygen species (ROS) around the infection site (reviewed in Heller and Tudzynski, 2011). As *B. cinerea* induces such an oxidative burst in all analysed host tissues and, in particular, because *Botrytis* seems to enhance the oxidative burst by producing its own ROS (Tenberge *et al*., 2002; Tiedemann, 1997), the question arises as to whether a plant's defence reaction causes oxidative stress for this fungus during infection. To examine this question, several components of the fungal oxidative stress response system have already been identified and characterized.

However, divergent results in these classical deletion approaches (Rolke *et al*., 2004; Schouten *et al*., 2002; Segmüller *et al*., 2007; Temme and Tudzynski, 2009) indicate that they are limited in their ability to explain the role of ROS in the plant–fungus interaction. To elucidate whether there is oxidative stress for *B. cinerea* during infection, it is probable that progress will depend on the development of high-precision tools for the quantification and experimental manipulation of defined redox processes *in vivo*. A possibility for the imaging of redox processes has emerged from the development of genetically encoded yellow fluorescent protein (YFP)- and green fluorescent protein (GFP)-based transgenic fluorescent reporters (Dooley *et al*., 2004; Østergaard *et al*., 2001; reviewed in Meyer and Dick, 2010).

Additional cysteine residues located on two adjacent β-strands have been engineered into the protein barrel of redox-sensitive GFP2 (roGFP2), which are able to form disulphide bridges (Dooley *et al*., 2004). The formation or release of these disulphide bridges depends on the redox environment of the protein and leads to slight conformational changes in the protein barrel, which alter the protonation state of the fluorophore. Accordingly, the oxidized and reduced forms of roGFP2 are preferentially excited at two different wavelengths (395 and 488 nm), facilitating quantitative ratiometric imaging of redox dynamics (Fig. S1, see Supporting Information).

However, as pointed out by Meyer *et al*. (2007), roGFP2 does not sense the overall redox status of the cell, but specifically senses the redox potential of the cellular glutathione pool using glutaredoxin (GRX) as a mediator of reversible electron flow between glutathione and roGFP2. The tripeptide glutathione (γ-glutamyl-L-cysteinyl-glycine), which can be reversibly converted from its reduced form (GSH) to the oxidized form (GSSG), is one of the key metabolites involved in maintaining a reduced intracellular redox milieu. GSH is the basic low-molecular-weight thiol in most eukaryotic and prokaryotic cells, with concentrations in the low millimolar range in plasmatic compartments (Foyer and

**Correspondence*: Email: tudzyns@uni-muenster.de

Noctor, 2005). The thiol redox potential, which is reflected in the $[GSH]^2/[GSSG]$ ratio, is highly regulated within various cellular compartments. Changes in the redox equilibrium of GSH can reversibly modify redox-sensitive thiol groups of target proteins, activating various cellular processes (Meyer, 2008). Accordingly, GSH has long been suggested to be part of signalling cascades transducing environmental signals to the nucleus and, in this context, plays a role as an anti-oxidant (May *et al*., 1998).

The molecular sensor roGFP has already been used successfully in HeLa cells, *Arabidopsis thaliana*, tobacco and yeast cells to determine cellular redox changes (Dooley *et al*., 2004; Jiang *et al*., 2006; Morgan *et al*., 2011; Schwarzländer *et al*., 2008; Yu *et al*., 2009). In this study, it is shown for the first time that roGFP2 is also functional in filamentous fungi and can be used to determine changes in the glutathione redox potential of *B. cinerea* hyphae *in vivo*. The sensor reversibly responds to the addition of oxidizing agents, such as H_2O_2, and reducing agents, such as dithiothreitol (DTT). Therefore, it can be used to analyse the kinetics of GSH recovery after H_2O_2 treatment in different strains. It has been shown recently that the AP1 transcription factor Bap1, which is the main transcriptional regulator of H_2O_2-scavenging proteins, is not essential for the virulence of *B. cinerea* (Temme and Tudzynski, 2009). The data presented here suggest that, although Bap1 controls the genes for GSH recovery on a transcriptional level, the *bap1* deletion mutant and the wild-type (WT) do not differ in the kinetics of GSH recovery after a single oxidative stress treatment: differences could be detected only when the strains were exposed to oxidative stress repeatedly. *In planta* experiments indicated that the redox status of the cellular glutathione pool was dependent on the structure being analysed, with appressoria-like structures being more oxidized and infecting hyphae more reduced.

RESULTS

Redox-dependent changes in roGFP2 fluorescence in living *Botrytis* hyphae can be visualized by confocal laser scanning microscopy (CLSM)

As it has been shown *in vitro* that roGFPs reversibly respond to changes in the redox potential of different buffers, these molecular sensors can be used to determine redox changes of their specific environment (Hanson *et al*., 2004). Furthermore, roGFPs have been used successfully in different organisms to measure cellular redox changes *in vivo* (Dooley *et al*., 2004; Jiang *et al*., 2006; Yu *et al*., 2009). To analyse the redox status of living *B. cinerea* hyphae, a fungal expression system for roGFP2 was constructed, consisting of the gene encoding roGFP2 under the control of the *bcglnA* (glutamine synthetase) promoter and terminator. This promoter–terminator combination was used as the gene *bcglnA* is constitutively expressed in *B. cinerea* (Schumacher *et al*., 2008).

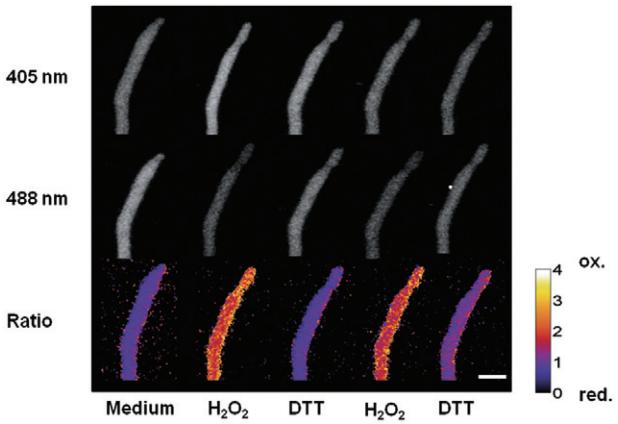

Fig. 1 The redox-dependent fluorescence of the ratiometric redox-sensitive green fluorescent protein (roGFP2) expressed in the cytosol of *Botrytis cinerea* hyphae is fully reversible. Conidial suspensions were germinated overnight and analysed microscopically. *Z*-Stacks of images were taken by confocal laser scanning microscopy (CLSM) with excitation at 405 nm and 488 nm, respectively. Average projections of these *Z*-stacks (top and middle row) were used for the calculation of the ratio images (bottom row). The colour scale for the ratio values indicates reduced roGFP2 in blue and oxidized roGFP2 in yellow. Medium, hypha growing in minimal medium before the addition of H_2O_2; H_2O_2, hypha 10 min after the addition of 10 mM H_2O_2; DTT, hypha 10 min after the addition of 10 mM dithiothreitol. Scale bar, 10 μm.

Seventeen hygromycin-resistant transformants were generated after transformation of the construct into the WT B05.10. Diagnostic polymerase chain reaction (PCR) and Southern blot analysis confirmed the integration of the whole expression cassette in these transformants (Fig. S2, see Supporting Information). All strains were used for two rounds of single-spore isolation to increase the proportion of transformed nuclei. Northern blot analyses confirmed that roGFP2 was expressed in all the strains (Fig. S3, see Supporting Information). The strain showing the strongest signal by fluorescence microscopy (B05.10:roGFP2.1) was analysed phenotypically; as it did not behave differently from the WT B05.10 in terms of its growth rate on different media, differentiation and pathogenicity (not shown), it was considered to be suitable for use in microscopic analyses.

Hyphae of the strain B05.10:roGFP2.1, expressing roGFP2 in the cytosol, showed strong fluorescence in a CLSM analysis when excited at 405 and 488 nm, respectively (Fig. 1). The ratio of the two images was not modified significantly by perfusing the hyphae with DTT solution, indicating that roGFP2 was almost fully reduced in the undisturbed hyphae. With the goal of investigating the feasibility of dynamic redox measurements, hyphae were successively treated with sublethal concentrations of H_2O_2 and DTT, respectively. The addition of 10 mM H_2O_2 to the incubation solution resulted in immediate oxidation of roGFP2 in *B. cinerea* hyphae, reflected by an increase in the fluorescence ratio; the successive addition of 10 mM DTT resulted in immediate reduction of roGFP2 (Fig 1; Movie S1, see Supporting Information). These results

indicate that roGFP2 can be used as a reporter gene system for cellular redox changes in *B. cinerea*.

Characterization of roGFP2 expressed in *B. cinerea* hyphae

The feasibility of using a fluorometer for the measurement of the redox potential in *Arabidopsis* leaves has been demonstrated previously by comparing four *Arabidopsis* transgenic lines expressing roGFPs in the cytosol (cyt-roGFP1), mitochondria (mit-roGFP1, mit-roGFP2) and plastids (pla-roGFP2) (Rosenwasser *et al*., 2010). Therefore, it was tested whether fluorometer-based measurements can also be used to determine redox changes in filamentous fungi. Liquid malt medium was inoculated with a conidial suspension of the strain B05.10:roGFP2.1 and incubated overnight. Samples were analysed in a plate reader. The fluorescence intensities at 510 nm were determined following excitation at 395 and 488 nm, respectively, for the resting state and after the addition of different oxidizing agents and DTT (Fig. 2). As already shown for CLSM, the fluorescence intensity with excitation at 395 nm increased with H_2O_2 application and decreased with DTT application, whereas the intensity with excitation at 488 nm showed the opposite behaviour (Fig. 2A). Although measurements by CLSM and by the fluorometer cannot be compared directly (as CLSM observes single hyphae, whereas the fluorometer detects a mean value of different colonies), the general characteristics of roGFP2 can be determined using the fluorometer:

1. The amplitude of the ratio change correlates with the concentration of H_2O_2 added to the medium (Fig. 2B). After the addition of 1 mM H_2O_2 to the cells, the ratio increased by 0.16 from about 0.45 to about 0.61, after 10 mM H_2O_2 by 0.7 to about 1.15, and after 100 mM H_2O_2 by 0.85 to about 1.3.
2. The reaction of roGFP2 is specific for oxidizing agents, but not for H_2O_2 (Fig. 2C). The amplitude of the ratio change after treatment with the oxidizing agent NaClO (0.3%) was similar to the ratio change after treatment with H_2O_2 (10 mM), whereas the nonoxidizing stressors NaCl (1 M) and DTT (10 mM) had no effect on the ratio. Interestingly, the chemical menadione, which is described as an intracellular O_2^- generator (Hassan and Fridovich, 1979), did not influence the ratio of the sensor.

In a control experiment, *B. cinerea* B05.10 and a strain expressing a GFP that was codon optimized for *B. cinerea* (Leroch *et al*., 2011) in the cytosol were analysed (Fig. S4, see Supporting Information). Although *B. cinerea* B05.10 showed some autofluorescence in the 395-nm channel, there was hardly any autofluorescence in the 488-nm channel (Fig. S4A). Accordingly, the ratio was rather high, but did not change after the addition of oxidizing or reducing agents. In the strain expressing GFP in the cytosol, fluorescence in the 488-nm channel was high and fluorescence in the 395-nm channel was low. No correlation of the ratio change with the addition of oxidizing or reducing agents could be observed (Fig. 4B). These results confirm that the ratio changes in response to changing redox environments are specific for roGFP2.

To specify the characteristics of roGFP2 expressed in *B. cinerea* hyphae, a washout experiment was performed. Coverslips were inoculated with 10 μL of a conidial suspension of the strain B05.10:roGFP2.1 and conidia were allowed to germinate overnight prior to microscopic analyses.

As observed previously, the molecular sensor was reduced in untreated hyphae, indicated by a low 405/488-nm ratio of about 0.45 (Fig. 3). On addition of medium containing H_2O_2 (100 mM), the ratio increased rapidly to about 1.4, indicating that the sensor was being oxidized. As soon as the H_2O_2-containing medium was replaced by fresh medium, the sensor was gradually reduced again. However, full reduction could only be achieved by the addition of medium containing reducing agents (2 mM DTT). The whole reaction was repeatable, as shown by a second oxidation of the sensor after the addition of H_2O_2-containing medium (increase in the ratio to about 1.6), and of reduction after the addition of DTT-containing medium (decrease in the ratio to about 0.45). These analyses show that, in addition to CLSM analyses, fluorometer-based measurements are suitable for the characterization of roGFP2 expressed in filamentous fungi, and that hyphae of *B. cinerea* maintain the cytosol in a reduced state.

Fusion of Grx1 to roGFP2 accelerates the reaction of the sensor

The fact that Meyer *et al*. (2007) were able to show that the reaction of roGFP2 in living plant cells was more rapid than the reaction *in vitro* suggests that, in living cells, roGFP2 is not oxidized by H_2O_2 directly, but rather by GRXs that mediate the redox equilibration of roGFPs with the cellular glutathione redox buffer, acting as a mediator of reversible electron flow between glutathione and roGFP2. Therefore, the intracellular response of nonfused roGFP2 may be limited by the availability of endogenous GRX, which appears to be insufficient for rapid equilibration in many systems and situations. This problem was solved by Gutscher *et al*. (2008), who created a fusion construct in which the redox catalyst Grx1 was covalently attached to roGFP2. To analyse whether there are differences between roGFP2 and Grx1-roGFP2 expressed in the cytosol of *B. cinerea* hyphae, an expression cassette for the improved sensor was constructed and transformed into *B. cinerea*. Transformation of the linearized vector into *B. cinerea* B05.10 yielded 20 transformants; transformant B05.10:Grx1-roGFP2.12 was chosen for further analyses as it gave the strongest fluorescence (not shown).

To test whether the behaviour of roGFP2 and Grx1-roGFP2 expressed in *B. cinerea* WT hyphae differed after H_2O_2 treatment, analyses using a fluorometer were performed (Fig. 4). The strains

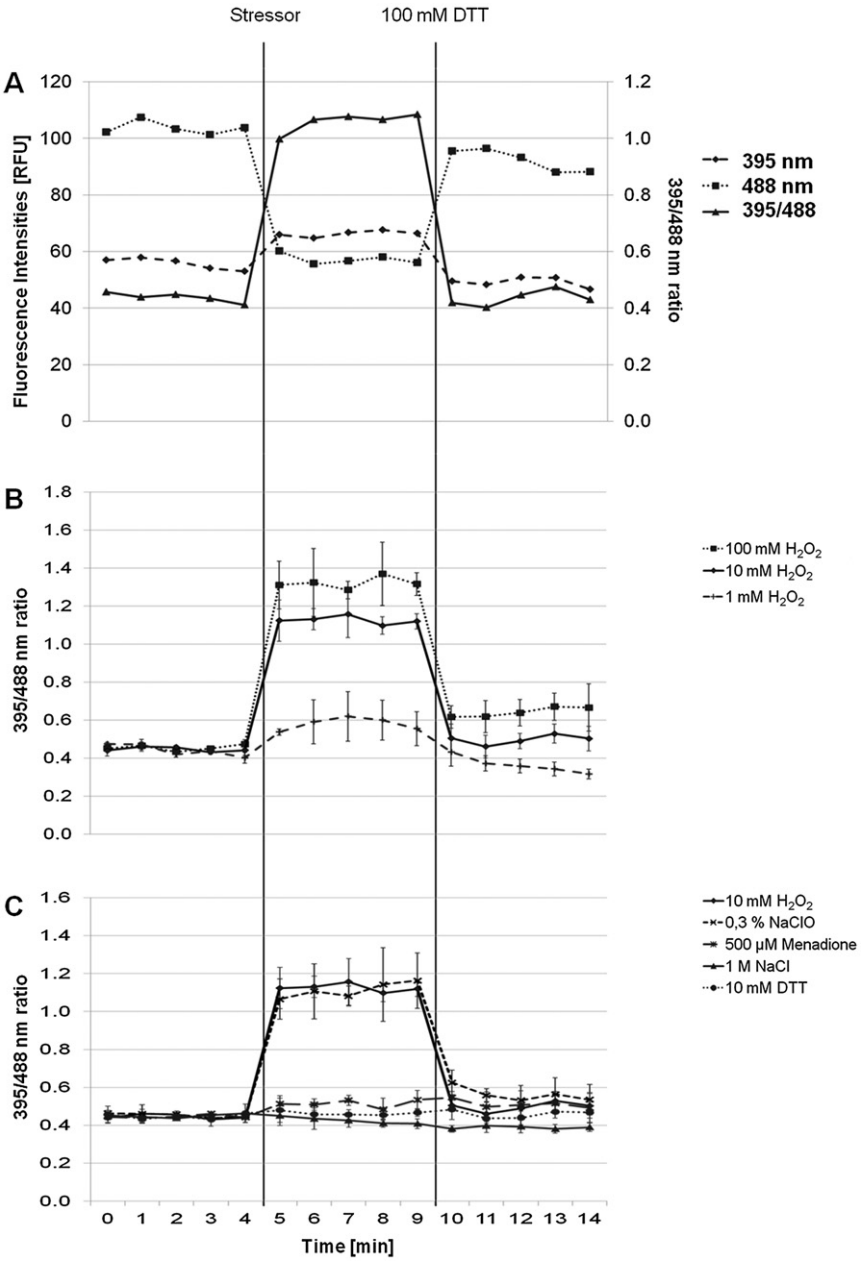

Fig. 2 Characterization of the ratiometric redox-sensitive green fluorescent protein (roGFP2) expressed in *Botrytis cinerea* hyphae using a fluorometer. Germlings of a roGFP2-expressing wild-type (WT) strain were used for fluorescence measurements at a microplate reader. Relative fluorescence units were measured with 3 × 3 reads per well after excitation with a wavelength of 395 ± 5 nm for the oxidized state and 488 ± 5 nm for the reduced state of roGFP2. Colonies were initially grown in GB5 medium to which a stressor (stressor) and 100 mM dithiothreitol (DTT), respectively, were added. (A) Example of the measurement in one well. After the addition of the stressor (10 mM H_2O_2), the fluorescence intensity in the 488-nm channel decreases and the fluorescence intensity in the 395-nm channel increases. As a result, the ratio (395 nm/488 nm) increases. With the addition of DTT, the initial status is obtained. (B) Correlation of the amplitude of the ratio change with the H_2O_2 concentration. (C) Specificity of the roGFP2 reaction to oxidizing agents. (B, C) The indicated values are the means of five different experiments; standard deviations are indicated by error bars.

B05.10:roGFP2.1 and B05.10:Grx1-roGFP2.12 were cultivated as indicated previously and the behaviour of the sensor in both strains was followed after the induction of oxidative stress using 5 mM H_2O_2. These analyses indicated that in *B. cinerea* the fusion of Grx1 does not influence the reaction of roGFP2 in a similar manner to that described for HeLa cells (Gutscher *et al*., 2008). There was only a marginal effect: the reaction of the sensor after H_2O_2 treatment in the strain expressing the Grx1-roGFP2 construct

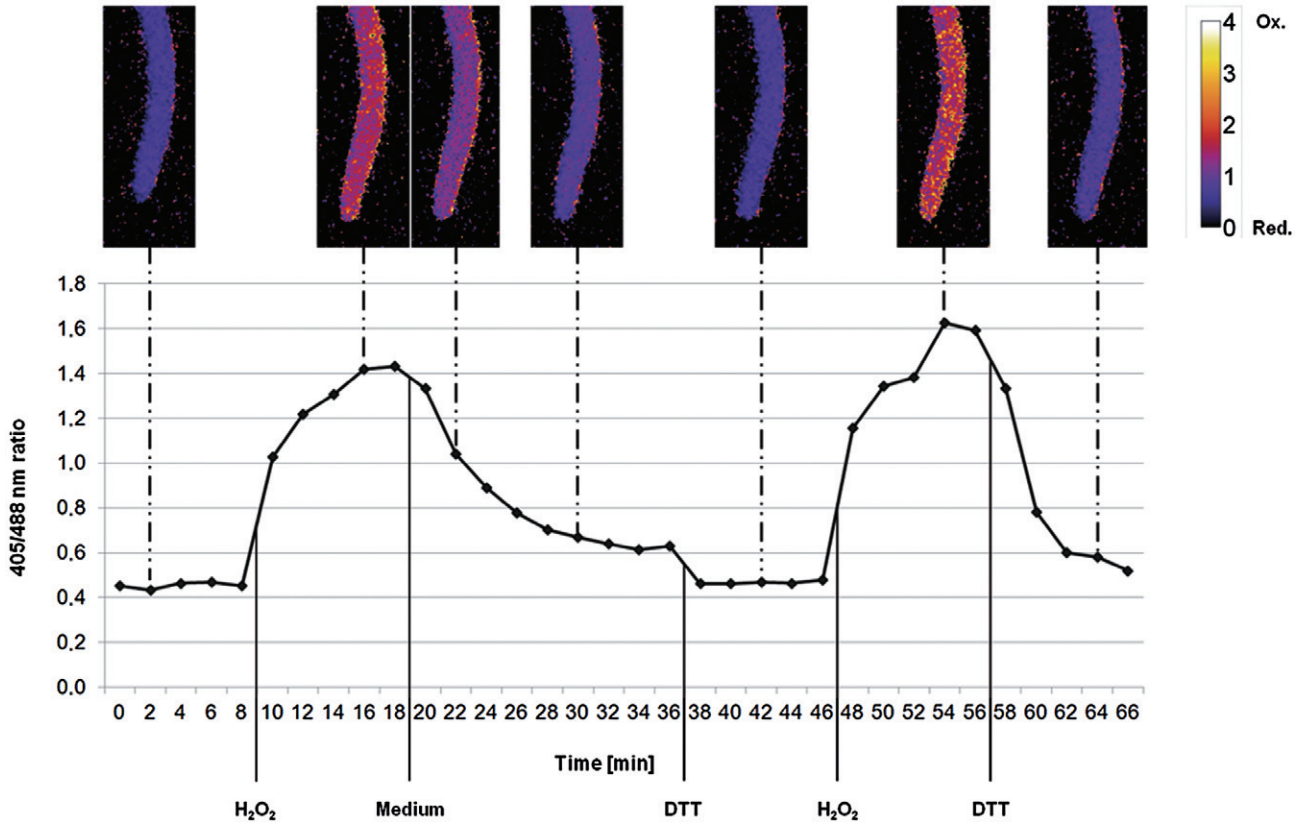

Fig. 3 Washout experiment showing the independent reduction of the glutathione pool in *Botrytis cinerea* hyphae after treatment with H_2O_2. *Z*-Stacks of images were taken by confocal laser scanning microscopy (CLSM) with excitation at 405 nm and 488 nm, respectively. Average projections of these *Z*-stacks were used for the calculation of the ratio images. The colour scale for the ratio values indicates reduced roGFP2 (ratiometric redox-sensitive green fluorescent protein) in blue and oxidized roGFP2 in yellow. Numerical ratio values were calculated using the software ImageJ and plotted against the time. Hyphae were initially grown in GB5 medium. Oxidative stress was induced by replacing the medium with GB5 containing 100 mM H_2O_2 (H_2O_2). To observe self-sufficient reduction of the glutathione pool, this medium was replaced by fresh GB5 medium again (medium). A reducing environment was created using 2 mM dithiothreitol (DTT). The graph shows an example of five different experiments with similar results.

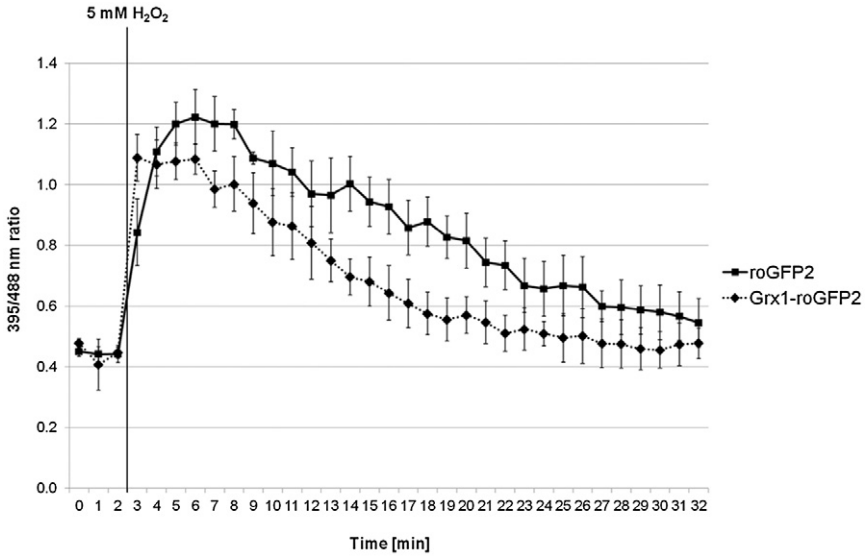

Fig. 4 Comparison of the reaction of the ratiometric redox-sensitive green fluorescent protein (roGFP2) and Grx1-roGFP2 expressed in *Botrytis cinerea* hyphae using a fluorometer. The response of Grx1-roGFP2 is faster than the response of roGFP2, as shown by the faster induction of the ratio to the maximum level after 3 min. The indicated values are the means of five different experiments; standard deviations are indicated by the error bars.

was slightly accelerated, i.e. there was a faster oxidation of the sensor after H_2O_2 treatment (Fig. 4, 3 min).

The sensor roGFP2 can be used to compare different mutants

There are several signalling components in *B. cinerea* involved in ROS signalling (reviewed in Tudzynski and Kokkelink, 2009). With the help of roGFP2, a new method was introduced for the further characterization of the corresponding mutants. Because roGFP2 senses the redox potential of the cellular glutathione pool, it can be used to analyse the kinetics of GSH recovery after H_2O_2 treatment in these strains. The transcription factor Bap1, a homologue of Yap1 from *Saccharomyces cerevisiae*, is the major regulator of genes involved in the oxidative stress response at the transcriptional level in *B. cinerea* (Temme and Tudzynski, 2009). Accordingly, the deletion mutant shows a strong phenotype on media containing oxidative stressors and is incapable of growth on media containing H_2O_2 (Fig. 5A). However, apart from the sensitivity to oxidative stress, Δbap1 does not show any further phenotype with regard to growth rates on different media, differentiation and pathogenicity (Temme and Tudzynski, 2009). Nevertheless, among the genes induced by Bap1 after H_2O_2 treatment are genes involved in GSH homeostasis (i.e. *bcgr1* encoding for a glutathione reductase, *bcgpx* encoding for a glutathione peroxidase, *bcgst1* encoding for a glutathione-*S*-transferase, *bcgrx1* encoding for a GRX; Temme and Tudzynski, 2009). To test whether GSH recovery is disturbed in Δbap1, the roGFP2 expression cassette was transformed into the deletion mutant. Transformation of the construct into the Δbap1 deletion mutant yielded over 30 transformants. Transformant Δbap:roGFP2.22 showed the brightest fluorescence and was therefore used for further characterization (data not shown).

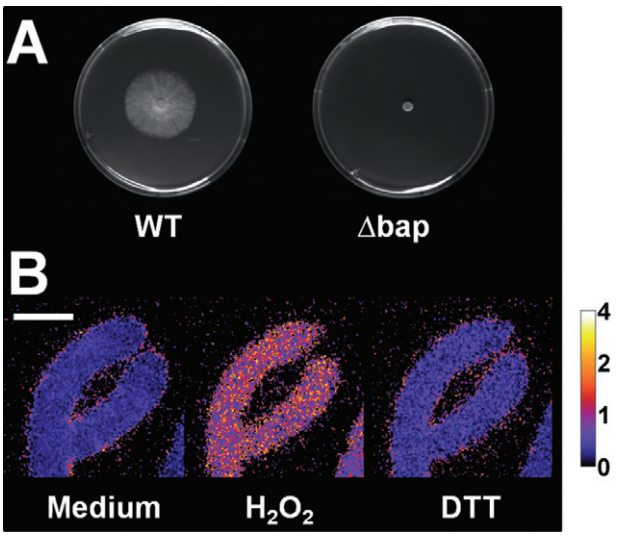

Fig. 5 Characterization of the strain Δbap1:roGFP2.22. (A) H_2O_2 sensitivity of the strain Δbap1:roGFP2.22 (Δbap) in comparison with the strain B05.10:roGFP2.1 (wild-type, WT). Strains were cultivated for 3 days on complete medium (CM) supplemented with 10 mM H_2O_2. (B) Redox-dependent fluorescence ratio of the ratiometric redox-sensitive green fluorescent protein (roGFP2) expressed in the cytosol of *Botrytis cinerea* strain Δbap1:roGFP2.22. *Z*-Stacks of images were taken by confocal laser scanning microscopy (CLSM) with excitation at 405 nm and 488 nm, respectively. Average projections of these *Z*-stacks were used for the calculation of the ratio images. Colour scale: reduced roGFP2, blue; oxidized roGFP2, yellow. Scale bar. 10 μm.

CLSM analyses indicated that roGFP2 was functional in strain Δbap:roGFP2.22 as the sensor was reduced in untreated hyphae, could be oxidized by the addition of H_2O_2 and was reduced back to the point of origin by the addition of DTT (Fig. 5B). To test whether this strain and WT differed in the kinetics of GSH recovery after oxidative stress treatment, analyses using a fluorometer were performed (Fig. 6). Both strains were cultivated as indicated previously and the behaviour of roGFP2 in both strains was followed after induction with 10 mM H_2O_2. Surprisingly, no differences between the strains were detectable. Although Bap1 is responsible for the induction of *bcgr1*, GSH recovery was not affected in the deletion mutant. However, as there is no induction of *bcgr1* in the mutant, the reduction of the glutathione pool should be slower after a second H_2O_2 treatment. To test this hypothesis, another washout experiment was performed by CLSM (Fig. 7). Indeed, this analysis showed that there are differences between the strains: whereas the independent reduction of the 405/488-nm ratio of roGFP2 after H_2O_2 treatment was similar in WT and Δbap1 (although, in this experiment, the reduction of the sensor was already slightly retarded in the mutant after the first H_2O_2 treatment), only a minor reduction of the sensor could be observed in the mutant when creating reduced conditions using DTT after the second H_2O_2 treatment. Accordingly, at the end of the experiment, the sensor was in a reduced state in WT, but was still in an oxidized state in Δbap1.

The cellular redox status of *B. cinerea* hyphae varies between different structures during penetration

As ROS and redox signalling play important roles in the differentiation processes of filamentous fungi, especially during pathogenesis (reviewed in Heller and Tudzynski, 2011), it was tested whether differences in the redox status of *B. cinerea* hyphae could be observed during the penetration of onion epidermal cells. During the penetration of plant tissue, *B. cinerea* differentiates thickened structures at the apices of the growing hyphae—so-called 'appressoria-like structures'—which are the origin of invading hyphae. Therefore, penetrating *B. cinerea* hyphae can be divided into three different phases during differentiation: (i) hyphae, growing on top of the plant tissue; (ii) appressoria-like structures; and (iii) invading hyphae.

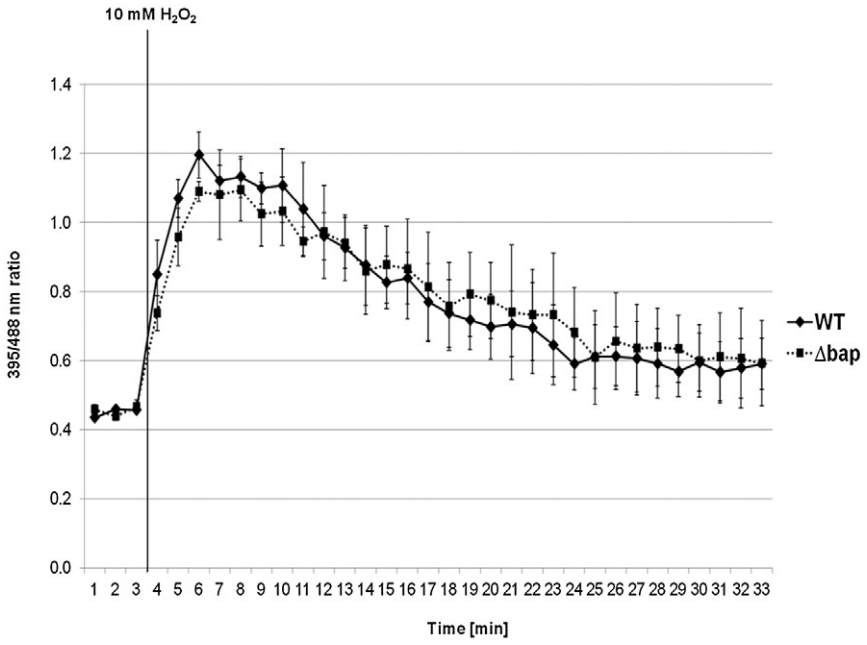

Fig. 6 Comparison of the reaction of the ratiometric redox-sensitive green fluorescent protein (roGFP2) expressed in *Botrytis cinerea* strain B05.10:roGFP2.1 (WT) and strain Δbap1:roGFP2.22 (Δbap) using a fluorometer. Reduction of roGFP2 is displayed after a single oxidation event using 10 mM H_2O_2. There is no difference in glutathione pool recovery between the two strains in this experiment. The indicated values are the means of six different experiments; standard deviations are indicated by the error bars.

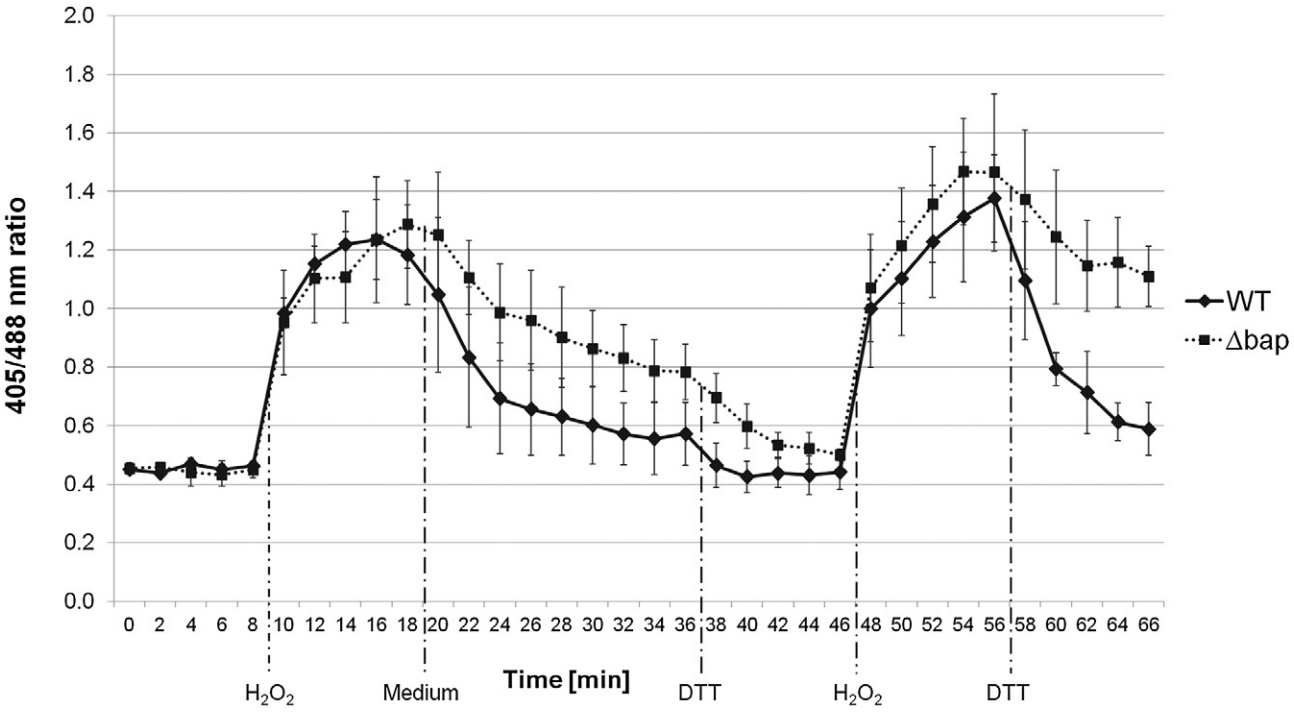

Fig. 7 Comparison of the reaction of the ratiometric redox-sensitive green fluorescent protein (roGFP2) expressed in *Botrytis cinerea* strain B05.10:roGFP2.1 (wild-type, WT) and strain Δbap1:roGFP2.22 (Δbap) using confocal laser scanning microscopy (CLSM). *Z*-Stacks of images were taken by CLSM with excitation at 405 and 488 nm, respectively. Ratio images of average projections of these *Z*-stacks were used for the calculation of numerical ratio values employing the software ImageJ. Oxidation of roGFP2 after H_2O_2 treatment (100 mM) progresses in a similar manner in both strains. However, after a second H_2O_2 treatment, a reduced state of roGFP2 in the strain Δbap1:roGFP2.22 is not achieved, even when dithiothreitol (DTT) is added to create reducing conditions. The indicated values are the means of four different experiments; standard deviations are indicated by the error bars.

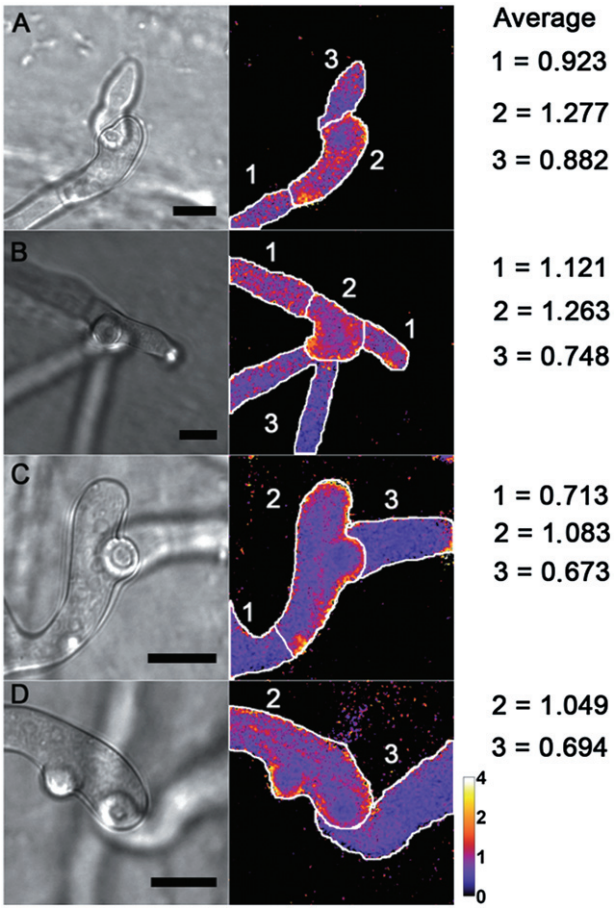

Fig. 8 Analysis of the redox status of the ratiometric redox-sensitive green fluorescent protein (roGFP2) expressed in *Botrytis cinerea* during the penetration of onion epidermal cells. *Z*-Stacks of images were taken by confocal laser scanning microscopy (CLSM) with excitation at 405 nm and 488 nm, respectively. Average projections of these *Z*-stacks were used for the calculation of the ratio images. The colour scale for the ratio values indicates reduced roGFP2 in blue and oxidized roGFP2 in yellow. Numerical ratio values were calculated using the software ImageJ. Infecting hyphae were divided into three parts: 1, hyphae growing on the surface of the onion epidermis; 2, appressoria-like structures; 3, penetrating hyphae. A selection from four experiments is shown (A–D). Further experiments were performed that gave similar results. Scale bars, 10 μm.

To analyse whether the different phases differ in their redox status, onion epidermal strips were inoculated with 10-μL droplets of a conidial suspension of the strain B05.10:roGFP2.1. To ensure that no defence reaction of the plant cells occurred that might influence the sensor, the epidermal strips were killed prior to infection. After 24 h of incubation, the germlings were analysed using CLSM (Fig. 8).

These analyses showed that there are differences in the redox status of roGFP2 in the different parts of the penetrating hyphae. Although the sensor was reduced in hyphae growing on top of the plant tissue and, especially, in invading hyphae, it was oxidized in appressoria-like structures (Fig. 8). Although the absolute numerical values varied between different experiments, the tendency was always the same. Thus, the redox sensor seems to be suitable for the visualization of redox-dependent processes, which might be important for the development and maintenance of developmental structures in *B. cinerea*.

DISCUSSION

Plants attacked by pathogens react by the production of ROS in a defence reaction called the oxidative burst. The impact of ROS on necrotrophic fungi is still elusive and sometimes even contradictory. Although several reports have shown that some fungi require an effective ROS-scavenging machinery to overcome the plant's oxidative burst, others have demonstrated that fungi are completely unaffected by plant-derived ROS (Chi *et al*., 2009; Guo *et al*., 2011; Lev *et al*., 2005; Lin *et al*., 2009; Schouten *et al*., 2002; Temme and Tudzynski, 2009; Yang *et al*., 2009). These contradictory results show that the development of new tools for the *in vivo* quantification of defined redox processes is necessary to determine the role of ROS in the plant–fungus interaction. In addition, ROS signalling has been proposed to be important for several differentiation processes in filamentous fungi, such as conidiation and the formation of appressoria, sclerotia or fruiting bodies (Lara-Ortíz *et al*., 2003; Malagnac *et al*., 2004; Semighini and Harris, 2008; for a review, see Aguirre *et al*., 2005). The investigation of the exact role of ROS in signalling during these differentiation processes requires high-precision tools for the visualization of intracellular redox changes.

The expression of roGFP redox sensors has become a multifunctional tool for dynamic *in vivo* measurements of the cellular redox environment in different organisms. The probes can be expressed in cells and targeted to all subcellular compartments (Dooley *et al*., 2004; Jiang *et al*., 2006; Schwarzländer *et al*., 2008; Yu *et al*., 2009).

In this study, we showed that roGFP2 can be functionally expressed in *B. cinerea* hyphae and is a promising tool for other filamentous fungi. The genetically encoded sensor is suitable for redox state measurements of single hyphae performed by CLSM and for measurements of whole colonies performed in a fluorometer. The results from the two methods of measurement were similar, although slight differences in the absolute values were detectable. This was not surprising because, in the case of fluorometer-based measurements, mean values of different colonies were recorded, which obviously differ from the specific values detected in single hyphae via CLSM. However, in both cases, the sensor was almost completely reduced under resting conditions, as it could not be modified by treatment with the reducing agent DTT. This result indicates that *B. cinerea* maintains the cytosol in a reduced state under normal growth conditions. However, it is notable that the estimated absolute ratio values of the sensor

under resting conditions were dependent on the age of the hyphae or colonies, with higher ratio values in older cultures. As the absolute numerical increase in the ratio, which was observed within seconds after the addition of oxidizing agents, was almost the same in all experiments, the values indicated here were corrected so that, under resting conditions, the mean value of 0.45 was maintained. This ensured that the ratio differences after H_2O_2 treatment and the course of ratio change could be compared between different time course experiments, but the absolute ratio values could not be compared directly. However, the reaction of the sensor was found to be specific to oxidizing agents in *B. cinerea* hyphae and was dependent on the concentration of the oxidizing agents, which could be confirmed in several experiments.

The effective concentration for H_2O_2-induced oxidation of roGFPs was much lower when expressed in HeLa cells than the concentration required *in vitro* (Dooley *et al*., 2004). According to this observation, the authors suggested that the oxidation of roGFPs within cells is a catalysed process. Experiments performed in neuronal cells, as well as in *A. thaliana*, confirmed that roGFP2 is a quantitative biosensor for the redox potential of the cellular glutathione pool rather than of the overall redox environment (Meyer *et al*., 2007; Vesce *et al*., 2005). Accordingly, when using roGFP2, it is the impact of different stressors on the redox status of the GSH pool in *B. cinerea* that is analysed. In addition, Meyer *et al*. (2007) demonstrated that GRXs mediate the redox equilibration of roGFPs with the cellular glutathione redox buffer, acting as a mediator of reversible electron flow between glutathione and roGFP2. To unify the reaction in different compartments or regions of the cell with different GRX concentrations, an improved molecule was introduced, in which Grx1 was fused to roGFP2 (Gutscher *et al*., 2008). In our study, both versions of the biosensor have been used. Although the ratio increase in both sensors was similar when compared directly with each other, a slightly faster reaction of Grx1-roGFP2 was detected when inducing oxidative stress using H_2O_2. However, this accelerated reaction was not as strong as described for HeLa cells (Gutscher *et al*., 2008). A reason for this finding might be that there are high endogenous GRX levels in *B. cinerea* and that the enzyme is distributed in the cytosol equally. This might expedite the reaction of free roGFP2 in the fungus. Indeed, the gene *bcgrx* is strongly induced after H_2O_2 treatment in *B. cinerea* (Temme and Tudzynski, 2009), and hence the influence of additional Grx1 coming from the fusion construct might be low. Nevertheless, we suggest using the fusion construct Grx1-roGFP2 for all further investigations, especially when performing time course experiments, because, in this case, a unified, fast and precise reaction is necessary.

When transformed into deletion mutants involved in ROS homeostasis or ROS signalling, the sensor can be used to determine differences in the reaction of these mutants to different oxidizing agents. However, the interpretation of such experiments must be carried out with caution, because the concentration of oxidative stress-causing agents within the cytosol may differ from the extracellular concentration in specific strains as a result of differences in cell wall composition or enzyme activity.

Because Bap1 is the main regulator of oxidative stress response genes and, in particular, as it controls the induction of several genes regulating glutathione homeostasis at a transcriptional level in *B. cinerea* (Temme and Tudzynski, 2009), roGFP2 was transformed into the corresponding deletion mutant to analyse whether the lack of an oxidative stress response influences the reaction of the biosensor. Indeed, a difference between the WT strain B05.10:roGFP2.1 and the *bap1* deletion mutant Δbap1:roGFP2.22 was observed. The mutant was unable to reduce the glutathione pool back to the base level when treated with H_2O_2 twice. Instead, the GSH pool remained in an oxidized state. The reason for this may be that, in the Δbap1 deletion mutant, a basic expression level of genes encoding proteins involved in GSH homeostasis can be detected (Temme and Tudzynski, 2009), although the strong induction of these genes that occurs in the WT after H_2O_2 treatment is missing. However, glutathione reductase is a highly active enzyme, which would mean that, under nonstress conditions, and even during a short-term exposure to H_2O_2, there may be sufficient activity available. This basic activity may be strong enough to reduce the glutathione pool after a single oxidation by H_2O_2. In contrast, when the hyphae are exposed to H_2O_2 repeatedly, the mutant is no longer able to recover the GSH pool. This obviously results in a more oxidized GSH pool in the mutant at the end of the experiment. Other mutants involved in GSH homeostasis or intracellular ROS balance, that are currently under construction, will be tested in a similar way.

Hansberg and Aguirre (1990) have suggested that cell differentiation is a response to oxidative stress. According to their idea, under normal growth conditions, ROS levels are low, and their generation and scavenging is balanced. A switch to differentiation occurs when a transient increase in ROS levels, beyond the cellular capability to neutralize them, is produced. The finding that in *Neurospora crassa* increased formation of ROS can be detected at the start of cell differentiation steps leading to the development of conidia supported their concept (Hansberg *et al*., 1993). For other fungi it was shown that, in particular, the ROS-producing NADPH-oxidase complex (Nox) is important for differentiation processes. In *B. cinerea*, the enzymes NoxA and NoxB are necessary for the formation of sclerotia, which are the basis for the development of sexual fruiting bodies (Segmüller *et al*., 2008); in *Aspergillus nidulans*, the single NoxA is necessary for fruiting body development (Lara-Ortíz *et al*., 2003) and in *Podospora anserina*, Nox activity is required to differentiate specific structures involved in cellulose degradation, similar to appressoria in plant pathogens (Brun *et al*., 2009).

To investigate whether roGFP2 can be used for the direct observation of the localized formation of ROS or ROS-dependent redox changes within differentiation structures of the cell, penetration structures of *B. cinerea* on strips of onion epidermis were analysed. These analyses revealed that differences in the redox status of divergent structures in *B. cinerea* can be visualized. The redox status of the cytosol was more oxidized in appressoria-like structures when compared with hyphae growing on the surface of the plant or invading hyphae within the tissue. The fact that the onion epidermis was killed prior to infection, and therefore no defence reactions occurred, indicates that the redox processes driven by fungus-derived ROS are involved in the differentiation of appressoria-like structures. Therefore, with the help of roGFP2, we were able to obtain a first indication that differences in the redox status may be involved in determining the differentiation of *B. cinerea*. Interestingly, BcNoxB of *B. cinerea*, which may be required to create an oxidized status in appressoria-like structures, is necessary for the successful penetration of onion epidermal cells (Segmüller *et al*., 2008). So far, we have been unable to show whether the oxidized redox status in the appressoria-like structure is a transient effect, and when precisely the oxidation starts. To solve these questions, time course experiments will be performed to follow the redox status of *B. cinerea* during penetration.

In conclusion, this study introduced roGFP2 as a genetically encoded biosensor of the redox status of the cellular glutathione pool in *B. cinerea*. In addition to its possible function as an instrument for the characterization of mutants affected in intracellular redox homeostasis, roGFP2 was shown to be a promising tool to answer so far unresolved questions, especially with regard to the role of ROS in the plant–fungus interaction and during differentiation processes in *B. cinerea*, and also for other filamentous fungi.

EXPERIMENTAL PROCEDURES

Fungal strains

Strain B05.10 of *Botrytis cinerea* Pers.:Fr. [*Botryotinia fuckeliana* (de Bary) Whetzel] is a putative haploid strain obtained after benomyl treatment of an isolate from *Vitis* (Büttner *et al*., 1994), and was used as a host strain for transformation and as a WT control in all experiments. All other strains used in this study are listed in Table 1.

Media and culture conditions

WT and mutant strains were grown on several complex media. Potato dextrose agar (PDA) (Sigma-Aldrich Chemie, Steinheim, Germany) was supplemented with 10% homogenized leaves of French bean (*Phaseolus vulgaris*). Synthetic complete medium (CM) was prepared according to Pontecorvo *et al*. (1953). As minimal medium, Czapek-Dox (20 g/L sucrose, 1 g/L KH_2PO_4, 3 g/L $NaNO_3$, 0.5 g/L KCl, 0.5 g/L $MgSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$, pH 5.2) or GB5 [3.3 g/L Gamborg's-B5 (Duchefa Biochemie BV, Haarlem, the Netherlands), 20 g/L glucose, 1 g/L] was used. For conidiation, the strains were incubated for 1 week at 18 °C under light conditions. For DNA preparations, mycelium was grown for 3–4 days at 18 °C on CM agar with a cellulose acetate (Cellophane) overlay. Testing for stress sensitivity was performed by inoculating strains on CM plates supplemented with H_2O_2.

Expression of roGFP2 and Grx1-roGFP2 in *B. cinerea*

The roGFP2 sequence (Hanson *et al*., 2004) was amplified by PCR with the primers 5′-*GATATC*ATGGTGAGCAAGGGCGAGGAGCTG-3′, adding an *Eco*RV restriction site (italic), and 5′-*CCCGGG*TTACTTGTACAGCTC-3′, adding an *Xma*I restriction site (italic), and cloned into the vector pCR2.1-TOPO. After sequence confirmation, the roGFP2 sequence was excised with *Eco*RV and *Xma*I, and cloned into the vector pΔbcniaD_hygR_1108 downstream of the *bcglnA* promoter. In addition to a resistance cassette for hygromycin, this vector contains the *bcniaD*-flanks to replace the nitrate reductase-encoding gene of *B. cinerea*, ensuring a defined integration site for the expression cassette. As the only impact of *bcniaD* deletion is the inability to use nitrate as a single nitrogen source, and no effects on virulence and growth in rich media have been observed, this gene locus is suitable for the targeted integration of reporter gene constructs in *B. cinerea* (Schumacher *et al*., 2008). The *bcglnA* terminator was amplified using the primers 5′-*CCCGGG*ATTGCTTTTCGCGTGTAAC-3′ and 5′-*CCCGG G*CATCTCCGAAATCGCTGGG-3′, which add an *Xma*I restriction site at each end (italic), and cloned into the vector pCR2.1-TOPO for sequence confirmation. The *bcglnA* terminator sequence was excised using the restriction enzyme *Xma*I and cloned behind the roGFP2 sequence of the vector pΔbcniaD_hygR_1108_roGFP2 (Schumacher *et al*., 2008; Fig. S5). The whole expression cassette was excised using the enzymes *Kpn*I/*Sac*I and transformed into the WT strain B05.10 or Δbap1, respectively.

For the construction of pNAH-Grx1-roGFP2, the homologous recombination system in yeast was used, as described previously (Colot *et al*., 2006). The Grx1-roGFP2 sequence was amplified from the vector pBinAR-Grx1-roGFP2 using the primers 5′-*CTCCATCACATCACAATCGATCCAA CC*ATGGCTCAAGAGTTTGTG-3′ and 5′-*CATACATCTTATCTACATACG*TTAC TTGTACAGCTCGTCCATGC-3′. These primers contain sequences homologous to the oliC promoter and the glucanase terminator, respectively (italics), of the vector pNAH-OGG (Schumacher, 2012), so that, in this vector, the fusion construct is controlled by the constitutive *oliC* promoter of *As. nidulans* and the glucanase terminator of *B. cinerea*. The vector contains the *bcniiA*-flanks to replace the nitrite reductase-encoding gene of *B. cinerea*, ensuring a defined integration site. Deletion of this gene has no effect on *B. cinerea* as long as nitrogen sources other than nitrate are

Table 1 Fungal mutant strains used in this study.

Strain	Genotype	Reference
Δbap1	B05.10 Δbap1::nat1	Temme and Tudzynski (2009)
B05.10:roGFP2.1	B05.10::roGFP2 hph	This study
B05.10:Grx1-roGFP2.12	B05.10::Grx1-roGFP2 hph	This study
Δbap1:roGFP2.22	Δbap1:: roGFP2 hph nat1	This study

available (Schumacher, 2012). The PCR product was co-transformed with pNAH-OGG (linearized with *Nco*I/*Not*I) into the uracil-auxotrophic *S. cerevisiae* strain FY834 (Winston *et al*., 1995), in which homologous recombination took place.

Plasmid DNA from uracil-prototrophic yeast colonies was isolated using the SpeedPrep Yeast plasmid isolation kit (DualsystemsBiotech, Schlieren, Switzerland) and transformed into *Escherichia coli*. Single plasmids were isolated and, after sequencing, the expression cassette was excised using the enzymes *Hin*dIII/*Sac*II and transformed into the WT strain B05.10.

For the transformation of *B. cinerea*, protoplasts were generated using a mixture of glucanex (Novozymes, Bagsværd, Denmark), lysing enzyme (Sigma-Aldrich, St Louis, MO, USA) and yatalase (Takara Bio Inc, Shiga, Japan). Protoplasts were transformed according to Schulze Gronover *et al*. (2001) using 20 μg of the linearized vector. Resistant colonies were transferred to agar plates containing GB5 agar, supplemented with 70 μg/mL of hygromycin B (Invivogen, San Diego, CA, USA) or 70 μg/mL of nourseothricin (Werner-Bioagents, Jena, Germany). Single conidial isolates were obtained by spreading conidial suspensions on GB5 plates containing 70 μg/mL of hygromycin B or 70 μg/mL of nourseothricin. After germination of conidia, single colonies were transferred individually to new plates containing the selection marker. Homologous integration of the different transformation constructs was proven by diagnostic PCR and Southern blots.

Fluorometer-based measurements

For fluorometer-based measurements, strains were grown for 1 week in PDA + bean medium at 18 °C in the light to induce sporulation; 100 mL of malt medium (1.5%) was inoculated with conidia and incubated for 12 h at 18 °C and 150 rounds per minute (rpm); 2-mL samples were taken and washed twice in GB5 medium (without yeast extract, but supplemented with 1 mM $(NH_4)_2HPO_4$). A 96-well plate (Microplate pureGrade™ 96-well PS, transparent bottom, black; BRAND GMBH + CO KG, Wertheim am Main, Germany) was inoculated with 200 μL of the washed germlings and used for fluorescence measurements at a Tecan Safire (Tecan Group Ltd, Männedorf, Switzerland). Fluorescence was measured at the bottom with 3×3 reads per well and an excitation wavelength of 395 ± 5 nm for the oxidized state and 488 ± 5 nm for the reduced state of roGFP2. The emission was detected at 510 ± 5 nm. The gain was set to 100. Relative fluorescence units (RFU) were recorded to calculate the Em_{395}/Em_{488} ratio.

CLSM imaging and ratiometric analysis

A glass slide was inoculated with 10 μL of a conidial suspension (1×10^5 conidia/mL) in GB5 medium (without yeast extract but supplemented with 1 mM $(NH_4)_2HPO_4$). Twenty-four hours after inoculation, germinated conidia were analysed microscopically with an inverted microscope (Leica DMIRE2) equipped with a Leica TCS SP2 scan head (Leica Microsystems, Wetzlar, Germany). Images were collected with a 63× water-immersion lens in multitrack mode with line switching and excitation of roGFP2 at 405 nm in the first track and at 488 nm in the second track. For both excitation wavelengths, roGFP2 fluorescence was collected with a bandpass filter of 505–530 nm and averaged from two readings for noise reduction. *Z*-Stacks of optical sections were collected and projected as average projections using CLSM software. Ratiometric analyses of fluorescence images were calculated in ImageJ (v. 1.44f; http://rsb.info.nih.gov/ij/). A Gaussian Blur with a σ value of 2.0 was performed for each image. In order to rule out the use of background pixels in the evaluation, the fluorescence intensity images were corrected for their background, and the corrected 405-nm image was divided by the corrected 488-nm fluorescence intensity image to produce a ratio image on a pixel-by-pixel basis using the plug-in RatioPlus. The clipping value was set to five. The edited image was the final 405/488-nm ratio image for which the mean ratio values were calculated again using ImageJ software. To illustrate the evaluated 405/488-nm ratio, the threshold of intensity ratio images was set to four and the greyscale was converted to colour using the ImageJ look-up table 'Fire'.

Penetration assays

For penetration assays on onion epidermal layers, epidermal strips were peeled, washed with distilled H_2O and incubated at 70 °C for 1 h in a humid chamber (killing). Fungal conidia were harvested, washed three times with double-distilled H_2O and diluted to a concentration of 5×10^4 spores/mL; 10-μL droplets of this suspension were used for inoculation. The samples were kept in a humid chamber at 18 °C for 24 h.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Characteristics of the ratiometric redox-sensitive green fluorescent protein (roGFP2). Depending on the status of two cysteine residues that have been engineered on two adjacent β-strands of roGFP2 the molecular conformation changes, influencing the spectral properties of the molecule, as the reduced form has an excitation maximum at 488 nm and the oxidized form at 395 nm (a hypothetical graph is shown). By ratiometric measurements, the actual status of the molecule can be determined. roGFP2 senses the redox status of the cellular glutathione pool. Under standard conditions, the redox equilibrium between roGFP2 and glutathione is shifted towards more reduced roGFP2 because glutathione reductase continuously reduces GSSG (oxidized form of glutathione). A stable equilibrium is reached when glutathione reductase can no longer reduce the residual GSSG (modified after Meyer *et al*., 2007).

Fig. S2 Integration of the reporter gene construct roGFP2 (ratiometric redox-sensitive green fluorescent protein) at the *bcniaD* locus of *Botrytis cinerea*. (A) Schematic overview of the integration strategy. A linearized vector containing an expression cassette for

roGFP2 and a hygromycin resistance cassette flanked by the 5′ and 3′ regions of *bcniaD* was transformed into *B. cinerea* B05.10. (B) Southern blot showing the successful integration of the reporter gene construct. Genomic DNA was digested with the restriction enzyme *Xba*I. Using the 5′ flank of the *bcniaD* locus as a probe, a 15.5-kb fragment was expected in the wild-type (WT) and a 6.5-kb fragment in the mutant. As remaining BcNiaD in the transformants ensured that they did not show any phenotype, no further purification was carried out.

Fig. S3 Northern blot showing the expression of the ratiometric redox-sensitive green fluorescent protein (roGFP2). Strains were cultivated on complete medium (CM) plates for 3 days prior to RNA isolation. A roGFP2 transcript was detected in all the transformants. Transformant T1 showed the strongest signal and was chosen for further analyses.

Fig. S4 Control experiment showing that the change in fluorescence is specific for the ratiometric redox-sensitive green fluorescent protein (roGFP2). Fluorescence was detected in untreated colonies and after the addition of 100 mM H_2O_2 and 10 mM dithiothreitol (DTT), respectively. (A) Relative fluorescence intensity in the wild-type (WT) *Botrytis cinerea* B05.10 is shown after excitation at 395 and 488 nm, respectively. There is hardly any fluorescence detectable after excitation at 488 nm. The 395/488-nm ratio is high because of stronger autofluorescence in the 395-nm channel, but is not influenced by the addition of oxidizing or reducing agents. One of five individual experiments with similar results is shown. (B) Comparison of the ratio change in a strain expressing Grx1-roGFP2 and stable GFP in the cytosol. The ratio increased after the addition of H_2O_2 in the strain expressing Grx1-roGFP2, whereas hardly any ratio change could be detected in the strain expressing stable GFP.

Fig. S5 Expression vector for the reporter gene construct roGFP2 (ratiometric redox-sensitive green fluorescent protein).

Movie S1 Time course experiment showing the reaction of *Botrytis cinerea* hyphae expressing the ratiometric redox-sensitive green fluorescent protein (roGFP2) in the cytoplasm. Stacks of images were taken by confocal laser scanning microscopy (CLSM) with excitation at 405 nm and 488 nm, respectively. Average projections of these stacks were used for the calculation of the ratio images. Reduced roGFP2 is indicated in blue and oxidized roGFP2 in yellow.

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