Does *Botrytis cinerea* Ignore H₂O₂-Induced Oxidative Stress During Infection? Characterization of *Botrytis* Activator Protein 1

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Botrytis cinerea is a phytopathogen infecting a broad range of plants including strawberries and grapevine. During infection, the necrotrophic fungus is exposed to reactive oxygen species (ROS) released by the oxidative burst, an early plant defense reaction. *B. cinerea* even produces ROS itself in planta. This raises questions about how the pathogen senses and responds to the host defense reaction and which role the pathogen's oxidative stress response systems play. Functional analysis of the AP-1 transcription factor Bap1 confirmed its role as a pivotal regulator of ROS detoxification in vitro. Macroarray analysis revealed 99 H₂O₂-induced Bap1 target genes, of which several genes encoded ROS-degrading enzymes as well as other central components of the cellular redox status. However, Bap1 is not essential for pathogenesis. In planta analyses revealed that the Bap1 target genes were not expressed 2 days postinoculation although H₂O₂ was detectable, proving that the normal virulence of the Δ bap1 mutant is not due to alternative regulation of the major oxidative stress response system in planta. The fungus obviously does not suffer H₂O₂-induced oxidative stress in planta, questioning classical ideas about the role of the oxidative burst in the infection process.

Reactive oxygen species (ROS) play a major role in pathogen-host interactions. Under pathogen attack, plants as well as neutrophils and mammalian macrophages (Quinn et al. 1992) use the so-called oxidative burst as an early defense reaction, and rapid accumulation of ROS induces several host defense mechanisms, like cross-linking of cell walls and the hypersensitive response (HR). This can result in local cell death of the penetration site tissue. Thus, the oxidative burst is an effective process to combat biotrophic pathogens that depend on living host cells (Rivas and Thomas 2005). However, it does not protect the host against necrotrophs.

The phytopathogenic ascomycete *Botrytis cinerea* not only shows resistance against the oxidative burst but even stimulates it on host plants. Its virulence correlates with the intensity of this plant defense reaction (Tudzynski and Kokkelink 2008; von Tiedemann 1997). *B. cinerea* even produces ROS in planta, presumably via NADPH oxidases and superoxide dismutases; during the infection process, accumulation of O₂^{•−} was identified in fungal hyphal tips and H₂O₂ was generated in and

around the penetrated cell wall as well as in the plant plasma membrane (Schouten et al. 2002; Tenberge et al. 2002). These oxidative processes probably result in a massive perturbation of the plant redox status and could, therefore, contribute to the disease progress in the host (Lyon et al. 2004). Investigations on the infection process of *B. cinerea* on *Arabidopsis thaliana* revealed that the fungus requires an active cell-death program in the host and infection induces an oxidative burst and hypersensitive response resulting in host cell death. In addition, infection of *B. cinerea* was suppressed in a HR-deficient *A. thaliana* strain (Govrin and Levine 2000). As *B. cinerea* depends on this programmed cell death, it seems to require the active participation of the host to achieve full virulence (Govrin and Levine 2000; van Kan 2006).

The increased ROS level during the infection not only affects the host but might influence the redox status of the fungus as well. Besides its ROS generating systems, *B. cinerea* possesses effective ROS-detoxification systems like catalases and peroxidases, but their role in the infection process is open. In contrast to the H₂O₂-producing (and O₂^{•−}-detoxifying) superoxide dismutase BcSod1 that impacts pathogenesis (Rolke et al. 2004), deletion of the catalase BcCatB did not result in reduced virulence (Schouten et al. 2002). However, the presence of multiple catalase genes in the genome of *B. cinerea* (discussed below) makes it rather unlikely that a single deletion would have a drastic effect. Further investigations on the regulation of ROS degradation pathways led to analysis of the system's key transcription factor activator protein 1 (AP-1), which has been characterized as a regulator of ROS resistance in mammalian cells as well as in fungi.

AP-1 like transcription factors belong to the basic region-leucine zipper (bZIP) superfamily of eukaryotic transcription factors binding the AP-1 site (TGACTCA) or related DNA sequences. In mammalian cells, the AP-1 transcription factors include the Jun, Fos, and ATF family proteins forming homo- or heterodimers to regulate diverse processes such as stress response and also proliferation, differentiation, and apoptosis (Shaulian and Karin 2001).

The mode of action of these transcription factors is based on redox-sensitive nuclear export; H₂O₂ mediates the oxidation of a peroxiredoxin, which oxidizes the highly conserved Cys-residues of the transcription factor, causing its nuclear accumulation. A reversion of its oxidative state by thioredoxin systems that are, in turn, transcriptionally upregulated by the transcription factor presents a negative feedback mechanism (Delaunay et al. 2000; Vivancos et al. 2004). Characterization in both budding and fission yeast revealed different regulatory mechanisms in these organisms. Yap1 from *Saccharomyces cerevisiae* is activated under all H₂O₂ concentrations assayed, due to its acti-

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vating peroxiredoxin, Gpx3, which is not susceptible to over-oxidation. So far, no relationship has been reported between the transcription factor and the stress-activated mitogen-activated protein (MAP) kinase (SAPK) Hog1 that is induced upon ROS exposure as well (Delaunay et al. 2000, 2002).

Characterization of the AP-1 homolog Cap1 in *Candida albicans* revealed its importance for tolerance to H₂O₂ and diamide (Alarco et al. 1999; Zhang et al. 2000) independently of the Hog1 cascade (Enjalbert et al. 2006). In another *Candida* species, *C. glabrata*, resistance to oxidative stress is mediated by the concerted role of the Yap1 homolog, the response regulator Skn7, and the transcription factor Msn4 (Cuéllar-Cruz et al. 2008). In these opportunistic human pathogenic yeasts and in the filamentous airborne fungus *Aspergillus fumigatus*, deletion of the Yap1 homologs did not result in impaired virulence. However, deletion of this AfYap1-encoding gene resulted in drastically increased sensitivity to H₂O₂ and menadione (Lessing et al. 2007). The same is true for the AP-1-like transcription factor Chap1 from the maize pathogen *Cochliobolus heterostrophus* (Lev et al. 2005). Only in the biotrophic phytopathogen *Ustilago maydis* does the Yap1 homolog play a striking role during pathogenesis, with Yap1 preventing accumulation of H₂O₂ in the vicinity of the hyphae of *U. maydis* during early infection stages (Molina and Kahmann 2007).

This information raises challenging, captivating, and intriguing questions about how pathogens sense and respond to the defending host reaction and whether or not ROS released by the host tissue affect the pathogen.

Characterization of this pivotal transcription factor of the ROS detoxification system in the broad-host range phytopathogen *B. cinerea* should provide more information to answer these questions. Therefore, the Yap1 homologous *Botrytis* activator protein 1 (Bap1) was identified and characterized to investigate the role of the fungal ROS detoxification system during infection.

RESULTS

bap1 encodes an AP-1 transcription factor.

In the Broad Institute *B. cinerea* B05.10 database, an AP-1 homolog protein was identified that was annotated as hypothetical protein BC1G_14094.1. Additional analyses revealed an open reading frame with 2,009 bp and three introns of 56, 93, and 54 bp coding for a protein of 601 amino acids. It was called Bap1 (accession number FM957543).

The predicted protein contains a basic region leucine zipper domain (BRLZ) and two C-terminal cysteine-rich domains (c-CRD). The eukaryotic bZIP transcription factors (Ellenberger 1994; Hurst 1995) are proteins that contain a basic region mediating sequence-specific DNA binding followed by a leucine zipper region required for dimerization (Landschulz et al. 1988). Both the BRLZ as well as the c-CRD domains are highly conserved among AP-1 transcription factors. An alignment of the BRLZ region and the two c-CRD domains of Bap1 with AP-1 transcription factors from different ascomycetes presents high sequence identities of these domains (Supplementary Fig. S1). Beside these conserved regions, homologies are of negligible significance. Amino-acid sequence similarities of Bap1 and the homologous protein SS1G_09561 from *Sclerotinia sclerotiorum* are at 84.4%. Comparisons with more distantly related AP-1 homologs of *Gibberella zeae* (51.4% amino acid similarity with Bap1), *Magnaporthe grisea* (51.5%), and *Aspergillus fumigatus* (39.2%) presented many fewer similarities.

As the bZIP transcription factor was chosen for detailed characterization due to its putative role in redox regulation, its own expression under exposure to H₂O₂ and menadione was analyzed via Northern blot experiments. The wild-type B05.10 was cultivated under standard cultivation conditions in Czapek

Dox (CD) medium in the presence or absence of 5 and 10 mM H₂O₂ as well as 250 and 500 μ M menadione. No differences on transcriptional level were detected; *bap1* was slightly expressed independent of the cultivation conditions tested (data not shown).

Generation of Δ bap1 mutants.

To investigate the function of the putative Bap1 transcription factor in *B. cinerea*, a *bap1* deletion strain was created using a replacement approach. The open reading frame of *bap1* in the wild-type strain B05.10 was replaced by a nourseothricin resistance cassette (Fig. 1). Homologous integration was analyzed for several transformants via diagnostic polymerase chain reaction (PCR). Single-spore isolation resulted in homokaryotic strains of deletion mutants that were screened for the absence of the *bap1* wild-type allele by PCR. Southern blot analysis confirmed homologous integration into the *bap1* locus without further ectopic integration events of the replacement cassette into their genomes for the two chosen transformants, termed Δ bap1_1 and Δ bap1_6 (Supplementary Fig. S3). Both independent mutants exhibited the same phenotypes and were chosen for further studies.

Bap1 is essential for ROS resistance.

The role of the transcription factor in ROS resistance was analyzed by plate assays. Complete medium (CM) was supplemented with H₂O₂ and menadione (2-methyl-1,4-naphthoquinone). Although both H₂O₂ and menadione generate ROS, they act differently; H₂O₂ can be reduced by metals via the Fenton reaction to form hydroxyl radicals, whereas menadione can form superoxide, H₂O₂, and semiquinone radicals by entering flavoprotein-catalyzed redox cycles with dioxygen. This results in the formation of O₂⁻ (McCord and Fridovich 1969). The different effects of these breakdown products were analyzed by the sensitivity profile they caused, and we observed the growth rate of the Δ bap1 mutant declined with increasing H₂O₂ concentrations compared with wild type. Growth rates were drastically reduced at 5 mM H₂O₂ when compared with growth on CM (23% relative growth), and no growth was detectable when 10 mM H₂O₂ was added to the medium. Comparable results were obtained with menadione; on agar plates containing 1 mM menadione, there was hardly any growth of the Δ bap1 mutant (15% relative growth) (Fig. 2).

Complementation of the Δ bap1 mutant with the *bap1* gene under control of its own promoter and terminator in the *bcniaD* locus restored wild-type growth on 10 mM H₂O₂ (Supplementary Fig. S2). These data provide evidence for an essential role of the transcription factor Bap1 in ROS resistance, most probably by regulation of ROS detoxification and thus of maintenance of the (intra?) cellular fungal redox status.

Bap1 has no impact on pathogenicity and differentiation.

As ROS are present during the infection process of *B. cinerea* on its host plants (Tenberge 2004; von Tiedemann 1997), regulatory proteins of the cellular ROS balance were assumed to be involved in the infection process. Tests were performed on different hosts, namely on leaves of *Phaseolus vulgaris* and *A. thaliana* as well as on fruits of *Lycopersicon esculentum* (no fungicide treatment) and on wounded pieces of *Malus domestica* 'Holsteiner Cox'. However, deletion of the AP-1 transcription factor did not lead to reduced virulence of *B. cinerea* in any of the host systems tested (Fig. 3).

Furthermore, the *bap1* deletion mutant showed a B05.10-like phenotype during differentiation, i.e., sporulation on potato dextrose agar (PDA) plus bean medium plates, germination in the presence of fructose and sucrose, hyphal morphology on different standard media, as well as sclerotia formation on CM

after 4 weeks in the dark were comparable to the wild type (data not shown).

Identification of Bap1 target genes.

Since the only phenotype of the $\Delta bap1$ mutant observed was its increased sensitivity against oxidative stress, we analyzed in detail the role of Bap1 as a redox-regulator. Several Bap1-dependent genes were identified by testing a selection of oxidative stress response (OSR) genes. Axenic cultures were exposed to oxidative stress mediated by H_2O_2 (10 mM) for a short time period of 30 min. At this concentration, growth reduction of the wild-type strain by nearly 50% indicated severe stress. Growth of the $\Delta bap1$ mutant stopped at this H_2O_2 level (discussed above). The short incubation period guaranteed cells could be harvested that were still living, even in the changed fatal-growth conditions. For a set of pivotal OSR genes, induction was detected in the wild type but not in the $\Delta bap1$ mutant (Fig. 4A). These Bap1 targets included genes encoding the catalaseB BcCatB (Schouten et al. 2002), the glutathione reductase BcGlr1, a thioredoxin-like protein, a peroxiredoxin 2D, a glutathione peroxidase BcGpx, and a Ryp1-like protein BcRLP (C. Michielse and P. Tudzynski, *unpublished data*). Macroarray analyses yielded additional Bap1-dependent target genes. Transcripts of 99 genes were identified that were only detectable in the wild type after exposure to H_2O_2 but not in the $\Delta bap1$ mutant. The majority of these genes encoded proteins involved in normal metabolic processes and protein biosynthesis (44). There were some genes involved in

regulatory mechanisms (10) as well as in differentiation and growth (4), transport (4), cell-wall degradation (3), and in general stress response (1). Six genes encode proteins probably directly involved in the oxidative-stress response in *B. cinerea*. The data obtained for these Bap1-induced OSR genes could be verified by Northern blot experiments (Fig. 4A). These genes were encoding the catalaseC BcCatC (BC1G_01968.1), the thioredoxin reductase BcTrr1 (BC1G_01592), a glutaredoxin BcGR (BC1G_05109), the glutathione-S-transferase1 BcGst1 (BC1G_07391) (Prins et al. 2000), the thioredoxin BcTR (BC1G_14403), and a hypothetical class gamma glutathione-S-transferase (BC1G_05767). Furthermore, exemplary genes of the other categories mentioned were included in these experiments. Transcriptional regulation of an ascorbate peroxidase (BC1G_08301) probably also involved in ROS detoxification, a ferric-chelate reductase (BC1G_00264), a NADPH dehydrogenase (BC1G_02693), a heat-shock protein 12, and an ABC transporter homolog (BC1G_08185) with or without 10 mM H_2O_2 in the wild type and in the $\Delta bap1$ mutant are shown in Supplementary Table S1. Expression of the H_2O_2 -regulated genes encoding the opsin Bop1 (BC1G_02456) (J. Heller, *unpublished data*) and the oxalacetate acetylhydrolase BcOahA (BC1G_03473) (J. van Kan, *personal communication*) appeared to be independent of Bap1. Surprisingly, when considering the H_2O_2 -dependent mode of activation of AP-1 transcription factors, there was a group of genes whose expression was Bap1-dependent in a H_2O_2 -independent manner. This group includes genes like the catalaseA BcCatA and the glutathione-S-trans-

Fig. 1. Replacement strategy for deletion of *bap1*. The flanking regions of *bap1* were amplified via polymerase chain reaction. Modified primer integrated specific restriction sites for endonucleases into the flanking regions, allowing for directed cloning of these fragments into the pNR1 replacement vector. A linear fragment received by digestion of pDbap1 with *SacI* and *XhoI* was used for transformation of *Botrytis cinerea* wild-type strain B05.10. uni/rev = sequencing primer, F/R = primer for *bap1* wild type control (F = bcap1_CF_1, R = bcap1_CR_1), OliC promoter from *Aspergillus nidulans*, *nat1* = nourseothricin acetyltransferase (*Streptomyces noursei*), and T-tub = terminator from *B. cinerea*.

ferase2 BcGst2 (Fig. 4B). Both can be related to oxidative stress and seem to be constitutively negatively regulated by the transcription factor independent of the amount of ROS present in or around the fungal mycelium.

ROS regulation in planta.

The data obtained by growth assays and macroarray analyses underlined the crucial effect of Bap1 on H₂O₂ detoxification and ROS regulation. As the presence of H₂O₂ during the infection process was shown in previous microscopical studies (Lyon et al. 2004; Segmüller et al. 2007; Tenberge 2004) and has been visualized by 3,3-diaminobenzidine (DAB) staining as brown precipitate in primary lesions in this work (Fig. 5A),

we addressed questions related to the fungal redox-status regulation in planta. Therefore, RNA was isolated from primary lesions of bean leaves infected with conidiospores of the wild type and the $\Delta bap1$ mutant and expression of Bap1 target genes was investigated at 2 days postinoculation (dpi). Expression of the genes encoding the metalloprotease BcMP1 (SSH8) (Schulze-Gronover et al. 2004) and the superoxide dismutase1 BcSod1 (Rolke et al. 2004) that had shown to be induced in planta were used as positive controls. Constitutively expressed BcActA served as a loading control. The data obtained revealed no Bap1-dependent induction of ROS-degrading enzymes like the catalaseB BcCatB, the thioredoxin reductase BcTrr1, or the glutathione reductase BcGlr1 in primary le-

Fig. 2. Growth of the $\Delta bap1$ mutant and the wild-type B05.10 on oxidative stress-inducing media after 3 days. Complete medium (CM) was supplemented with H₂O₂ (5 mM, 10 mM) and menadione (1 mM). **A**, Growth of the wild-type B05.10 and the $\Delta bap1$ strain. **B**, Growth of the wild-type B05.10 (black) and the $\Delta bap1$ strain (stripes) (growth radius on stress-inducing media [cm], three plates per experiment).

sions. Bap1 target genes were only slightly constitutively or even not expressed at all at this stage of infection, neither in the $\Delta bap1$ mutant nor in the wild type (Fig. 5B).

Induction of ROS target genes.

To exclude the possibility that the H_2O_2 concentrations tested in axenic cultivations have been too high and therefore did not represent the physiological in planta situation, mycelial growth and expression of Bap1 target genes was analyzed at different H_2O_2 concentrations (0.5 to 10 mM). Plate assays for growth tests as well as axenic cultivation with or without exposure to H_2O_2 for half an hour were performed as described above. The experiments revealed an induction of ROS detoxification genes that started with the increasing sensitivity of the $\Delta bap1$ mutant towards H_2O_2 at 0.5 mM H_2O_2 . The transcription factor is needed for induction of OSR genes at all H_2O_2 concentrations analyzed (Fig. 6). The relatively low H_2O_2 con-

centrations used may also be present on the host plant leaf and thus reflect H_2O_2 exposure in growth conditions on the host.

DISCUSSION

Characterization of the AP-1 transcription factor Bap1 in *B. cinerea* revealed new insights in the fungal-host interaction. Bap1 controls pivotal ROS detoxification networks of major importance for ROS degradation in *B. cinerea*. Due to that fact, one could assume a crucial role of Bap1 regulating the fungal defense of the host's oxidative burst during infection. This hypothesis was refuted, as we showed that the activator protein 1 is, in fact, of no importance for *B. cinerea*'s pathogenicity, although H_2O_2 is present in and around lesions caused by the fungus. Bap1 target genes, including those that encode ROS detoxification enzymes, were not induced during expansion on and maceration of the host plant tissue.

Fig. 3. Pathogenicity assay of the $\Delta bap1$ mutant on different host plants and tissues in comparison with the wild-type B05.10. Conidiospore suspensions were dropped on leaves of **A**, *Phaseolus vulgaris* and **B**, *Arabidopsis thaliana* as well as on wounded pieces of **C**, *Malus domestica* 'Holsteiner Cox' and on fruits of **D**, *Lycopersicon esculentum*. dpi = days postinfection.

Fig. 4. H₂O₂-dependent gene regulation in the $\Delta bap1$ strain and in wild-type B05.10. **A**, Bap1-dependent H₂O₂-induced genes as well as **B**, H₂O₂-induced Bap1-independent genes and Bap1-dependent H₂O₂-independent genes. Cultivation was performed in Czapek Dox medium with 10 mM H₂O₂ (+) or without H₂O₂ (–) for half an hour. rDNA was used as loading control. Asteriks (*) indicate common target genes of Bap1 and the stress- and mitogen-activated protein kinase BcSak1.

Bap1 is essential for fungal survival on high concentrations of both H_2O_2 and menadione in vitro. It might, therefore, act as a generally important response element to various species of reactive oxygen. Growth and expression assays with different amounts of H_2O_2 demonstrated a decreasing growth rate of the $\Delta bap1$ mutant inversely proportional to the expression level of ROS detoxification genes in the wild type. The expression level of Bap1 target genes was higher with increasing levels, indicating a role of Bap1 of greater importance at exposure to very high levels of ROS when the cellular redox balance was completely disturbed. From 5 mM H_2O_2 , the growth rate of the deletion mutant was drastically reduced, and 10 mM H_2O_2 was absolutely lethal. Until that concentration, other SOS systems might be active that compensate for the absence of Bap1. The same is true for exposure to high levels of menadione. With up to 500 μM menadione, the $\Delta bap1$ mutant is still able to grow on the quinone, although with drastically reduced growth rates compared with the wild type (data not shown); from 1 mM menadione, the deletion strain is unable to grow. Yet, this growth effect was not accompanied by induction of Bap1-de-

pendent ROS detoxification genes (data not shown). So far no menadione-specific Bap1 target genes were identified.

Deletion of *bap1* did not result in an aberrant phenotype concerning growth rate on standard media, sporulation, germination, or sclerotia formation. None of these differentiation processes were affected by the *bap1* deletion, although ROS regulatory systems are known to control various differentiation processes. The data obtained from the macroarrays performed in this work revealed that Bap1—apart from its essential role in ROS detoxification—also is involved in the regulation of a notable number of genes encoding proteins involved in metabolic processes and differentiation. It is unclear why this does not result in a more drastic phenotype of the mutant.

The macroarray results provided a basis for understanding gene regulation mediated by Bap1 and gave some information about the transcriptome of the *B. cinerea* controlled by the transcription factor. Results shown here confirmed data previously obtained for genes classified as OSR genes. All of these genes are directly involved in the OSR and in ROS degradation, as they encode catalases, a glutathione reductase, a peroxire-

Fig. 5. Regulation of oxidative stress response (OSR) genes in planta. **A**, H_2O_2 detection via 3,3-diaminobenzidine-staining in primary lesions (2 days post-inoculation [dpi]) of leaves infected with the $\Delta bap1$ strain and with the wild-type B05.10. **B**, Analyses of OSR gene regulation in the $\Delta bap1$ strain and in the wild-type B05.10 in primary lesions (2 dpi). Actin was used as loading control.

doxin, a glutathione peroxidase, glutathione-S-transferases, and proteins of the thioredoxin as well as of the glutaredoxin redox systems.

Genes whose expression was H₂O₂-induced but Bap1-independent coded for proteins that were not primarily involved in ROS detoxification but in metabolic and differentiation processes. The most obvious and general regulatory effect of Bap1 is mediated by H₂O₂. But this does not explain the regulatory differences of the wild-type B05.10 and the $\Delta bap1$ mutant on the expression of the catalaseA BcCatA and the glutathione

peroxidase 2 BcGst2 that was independent of H₂O₂ incubation. Expression of these genes seems to be regulated by Bap1 in a H₂O₂-independent manner.

Bap1 has been shown to be of major importance for OSR in vitro but nonessential for pathogenicity, indicating that the ROS-degrading enzymes controlled by Bap1 are of minor importance for the infection process. However, infection of *B. cinerea* does not remain undiscovered on the host, as indicated by ROS production during the plant's oxidative burst, used to defend against invading pathogens (von Tiedemann 1997; Tenberge 2004;

Fig. 6. Sensitivity of the $\Delta bap1$ strain and the wild-type B05.10 towards increasing H₂O₂ concentrations (0 to 10 mM H₂O₂) related to oxidative stress response gene expression at comparable growth conditions. **A**, Growth of the wild-type B05.10 (black) and the $\Delta bap1$ strain (stripes) (growth radius on stress-inducing media [cm], five plates per experiment). **B**, Expression analyses of Bap1-dependent H₂O₂-induced genes. Czapek Dox medium was supplemented with 0 to 10 mM H₂O₂. rRNA (representative for all filters compared in these figures) was used as loading control.

Williamson 2007). Especially during expansion of primary lesions, significant H₂O₂-formation is observed; yet no Bap1 target genes could be detected. It was shown here and elsewhere that the fungus is sensitive to ROS (Tudzynski and Kokkelink 2008), and it is not known how the fungus protects itself on the host when the most prominent in vitro protection system is not activated and dispensable. Thus, there must be other infection-specific ROS protection systems that are not switched on in axenic culture to shield the fungus or the fungus does not sense oxidative stress. Maybe there are differences in the composition of the fungal extracellular matrix on the plant surfaces when compared with that under axenic cultivation conditions. However, the differentiation of a special protective coat cannot be mimicked by a plant extract only, as plate assays on media with PDA plus bean leaves supplemented with H₂O₂ did not compensate for the loss of Bap1 (data not shown). Development of guarding structures—if existing—must be triggered by the living plant cells, indicating a direct interaction of host and pathogen as proposed by van Kan (2006).

The data obtained for Bap1 are in line with the results shown for the homologous protein AfYap1 from the human pathogenic ascomycete *Aspergillus fumigatus*. No influence of the transcription factor on pathogenicity was detected during in vitro killing assays with human polymorphonuclear leukocytes (PMN) and in an infection model applying immunosuppressed neutropenic mice, although ROS were involved in the killing of fungal hyphae by PMN (Lessing et al. 2007). In the maize pathogen *Cochliobolus heterostrophus*, the AP-1 homolog Chap1 was shown to be nuclear localized during infection of host plants, indicating a role of the transcription factor in the fungal pathogenicity. Yet, deletion of the encoding gene did not result in reduced virulence (Lev et al. 2005). Only in the biotroph *U. maydis*, which also infects maize but without killing the host, the ROS detoxification system regulated by its Bap1-homolog Yap1 might help to overcome the oxidative burst induced by pathogen-associated molecular patterns. Molina and Kahmann (2007) concluded that Yap1 is responsible for preventing H₂O₂ accumulation in the vicinity of hyphae during early infection stages. Thus, the function of AP-1 as a pathogenicity factor is, so far, limited to biotrophs and its role in pathogenic development of necrotrophs is not evident, in spite of the oxidative burst they normally initiate. Here, we investigated for the first time the transcriptional status and the regulatory function of Bap1 in planta and showed that the role of Bap1 as major player in response to H₂O₂-caused oxidative stress in vitro is not taken over by an alternative control system but that the whole response system is not activated in planta, a new and unexpected observation.

Another aspect needs to be kept in mind, probably explaining the negligible role of the transcription factor during invasion of the host. It seems that ROS generation regulated by enzymes like the superoxide dismutase BcSod1 or the Nox complex with BcNoxA/B and BcNoxR rather than ROS degradation is essential for host plant infection, as BcSod1 and the Nox complex proteins have shown to be involved in pathogenicity (Rolke et al. 2004; Segmüller et al. 2008). Alternatively, the role of BcSod1 during infection could be due to its O₂-degrading function, if superoxide anions rather than H₂O₂ were the toxic ROS affecting the fungus in planta. The role of superoxide in pathogen defense has recently been substantiated in *Candida* spp. (Frohner et al 2009). The data obtained in our work indicated that H₂O₂ is not a major factor limiting fungal growth in planta.

In *B. cinerea* the OSR is not exclusively regulated by Bap1. The transcription factor shares some target genes with the stress-activated MAP kinase BcSak1, which is not only activated by oxidative but also by osmotic stress. Northern blot experiments as well as comparison with macroarray data performed with the $\Delta bcsak1$ mutant (N. Segmüller and J. Heller, unpublished data) revealed a group of common target gene products including the catalases BcCatA, BcCatB, and BcCatC, the glutaredoxin BcGR, the glutathione peroxidase BcGpx, and the Ryp-like protein BcRLP1 (Fig. 4A, asterisks). There might be further genes that need both Bap1 and a transcription factor downstream of the BcSak1 cascade for their activation. This group of genes includes *catB* and genes regulated by both regulatory systems but in different ways, like *catA*, which is constitutively expressed in the $\Delta bap1$ mutant but induced under exposure to H₂O₂ in the $\Delta bcsak1$ strain.

The factors that mediate this crosstalk as well as those proteins that act as sensors for oxidative stress in *B. cinerea* still need to be identified.

MATERIALS AND METHODS

Fungal strains.

Botrytis cinerea Pers.: Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetz] B05.10 (Quidde et al. 1999) was used as recipient strain for the transformation experiments and as a wild-type control. For comparative expression studies, a mutant strain of B05.10 with a deletion of the single MAP kinase-encoding gene $\Delta bcsak1$ (Segmüller et al. 2007) was used.

Bacterial strains.

Subcloning was performed in strain TOP10F⁻ (Invitrogen, Carlsbad, CA, U.S.A.).

Table 1. Primer used in this work.

Number	Primer	Sequence (5'→3')	Melting temperature (°C)
1	Bap1_for-egfp	GCAAGGCTTCCATGGCTACC	64.5
2	Bap1_rev-egfp	GATCAACCATGGATGCCTTATTGGCCGGTG	71.5
3	bcap1_F11_F	GTCCTGCATGAGCTCACGGTTGAG	65.9
4	bcap1_F11_R	CACCATCTGATGAAGTCCGCGGTAAGT	66.1
5	bcap1_F12_F	GATTATCGCGAACCTCAAAATAAGCTTC	62.0
6	bcap1_F12_R	GCCTCGAGGGGCATAATTCTTCATG	64.6
7	cBap1_For	GTCTCGAGGATTGATTGTCCTGCATAAG	66.1
8	cBap1_Rev	GTGATATCGAGATGTATCATGACCATATG	63.2
9	BcBap1_CF_F	CTGGCCCCTCTCTTTATCCGGC	65.6
10	BcBap1_CF_R	CGCCAAAAATATTGCCTTGTCCCTAGC	63.2
11	Bcap1_CF_1	GGCTCAAAATCGTGCCGCCCAACG	67.6
12	Bcap1_CR_1	CTTACCTGGAACACTCATTTGTC	62.9
13	pOliR2	GGTACTGCCCCACTAGTGGCAGCTCGCG	72.0
14	Tub-T	AGACCAGCTTGTTATTGCCAGTGC	62.7
15	Bcniaad-5'-seq-F	CATCAGTTCTTGTATAGACAAGC	57.6
16	Hygro_idF	AGCTGCGCCGATGGTTTCTAC	56.3
17	Hygro_idR	GGGCGTCGGTTTCCACTATCG	60.4

Media and culture conditions.

B. cinerea strains were grown on complete medium (CM) (Pontecorvo et al. 1953) or PDA containing 10% bean leaves at 20°C under near-UV for conidiation. Testing of stress sensitivity was performed by inoculating strains on CM plates supplemented with H₂O₂ (Merck, Poole, U.K.) and menadione (Sigma, St. Louis). Single conidial isolates were obtained by spreading 100 µl of conidial suspension on Gamborg's B5 medium (Duchefa Biochemie BV, Haarlem, The Netherlands) plates containing 70 µg of nourseothricin or hygromycin per milliliter (Sigma). Conidia were germinated for 48 h, and single colonies were transferred individually to new plates containing nourseothricin or hygromycin. For DNA isolation, mycelium was grown for 2 to 3 days at 20°C on CM agar with a cellophane overlay. For axenic culture-shift experiments and subsequent RNA isolation, 100 ml of malt medium (1.5%) was incubated with spore suspension for 14 h at 18°C and 150 rpm. The mycelium was then harvested over a Nytex membrane and was incubated in defined liquid medium (CD) containing 1 instead of 3 g of NaNO₃ per liter and 20 g of glucose per liter instead of sucrose and pH 5.2 for an additional 24 h under the same conditions. After harvest, flasks with CD medium including stress-inducing supplements were each inoculated with 2 to 3 g of mycelium at 18°C and 150 rpm for 30 min.

Conidial germination tests.

For analyses of conidiospore germination on glass surfaces in nutrient dependency (Döhlemann et al. 2005), spores were harvested from CM agar plates, were filtrated over Nytex, and were suspended in 10 ml of sterile water and, then, were washed three times. Spore suspension (25 µl) with a concentration of 5×10^5 spores ml⁻¹ were placed into the center of a coverslip, and 475 µl of B5 nutrient solution was added to the spores, supplemented with fructose and sucrose at a final concentration of 10.5 mM. The coverslips were incubated in the dark at 20°C for several hours. Germination progress was monitored by light microscopy.

Standard molecular methods.

Fungal genomic DNA was isolated as described by Ceniz (1992). Plasmid DNA was isolated using a plasmid DNA preparation kit (Genomed, Bad Oeynhausen, Germany).

Southern blot analysis was performed according to Sambrook and associates (1989). Hybridization was carried out in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 2 mM EDTA [pH 7.7]), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 50 mM phosphate buffer, pH 6.6, at 65°C for 16 to 20 h in the presence of a random-primed [α -³²P]dCTP labeled probe. Membranes were washed in 2× SSPE, 0.1% SDS at 65°C, before being exposed to an autoradiographic film.

For Northern analysis, RNA was isolated from mycelial samples using the RNeasy total RNA isolation system (Promega, Mannheim, Germany) or the Trizol method (Invitrogen). Samples of 15 µg of RNA were transferred to Hybond-N⁺ filters after electrophoresis on a 1% agarose gel containing formaldehyde, according to Sambrook and associates (1989). Blot hybridizations were carried out in 0.6 M NaCl, 0.16 M Na₂HPO₄, 0.06 M EDTA, 1% N-lauroylsarcosine (Sigma), 10% dextran sulphate (Eppendorf AG, Hamburg, Germany), pH 6.5, as described for Southern blots.

Macroarray analyses.

The cDNA macroarrays include *B. cinerea* cDNAs from three different expressed sequence tag collections. One cDNA library was created from the strain *B. cinerea* ATCC 58025, a non-sporulating overproducer of abscisic acid (ABA), under ABA biosynthesis conditions (Siewers et al. 2004). A second library

was derived from a suppression subtractive hybridization approach, used to identify B05.10 genes specifically affected in expression on the host plant *Phaseolus vulgaris* (Schulze Gronover et al. 2004). A third library was derived from germinating conidia of B05.10 and early stages of plant infection (L. Kokkelink, unpublished). Assembling of the 16,525 cDNA sequences by means of the assembly program CAP3 (Huang and Madan 1999) resulted in 1,901 contigs and 3,047 singlets. Thus, the macroarrays used in this work contain 4,948 genes, including genes from plant origin. Sequence analysis for prediction of protein function was done using blastX at the National Center for Biotechnology Information (Altschul et al. 1990). Radiolabeled cDNA probes were prepared in the presence of 30 µCi [α -³²P]dATP using the SuperScript II reverse transcriptase, as described below. Fungal cultivations with the two genotypes (B05.10 and $\Delta bap1$) with or without 10 mM H₂O₂ were repeated three times. The $\Delta bap1$ mutant as well as the wild-type B05.10 was cultivated under standard cultivation conditions in CD medium with or without 10 mM H₂O₂. RNA was extracted and used for the production of radioactively labeled cDNA probes for macroarray filter hybridization. Each RNA sample was hybridized once to the macroarrays, making a total of three biological repeats and, due to the two replicates, six values per cDNA clone. The hybridization images from the Typhoon 8600 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were analyzed for initial data quantification using ARRAYVISION 8.0 (Amersham Pharmacia, Uppsala, Sweden). The Excel macro FiRe (University of Fribourg Faculty of Science website) was used to select candidate genes for differential expression based on their fold-change ratios (Beckers and Conrath 2006; Garcion et al. 2006), using standard parameters (lower threshold 0.5 and upper threshold 2.0). The ratios of the Bap1-dependent H₂O₂-induced genes were, at least in four of six cases, below the threshold of 0.5 (downregulation in $\Delta bap1$) or above the threshold of 2.0 (upregulation in $\Delta bap1$).

DNA sequencing.

DNA sequencing was performed with an automatic sequencer LI-COR 4200 (MWG Biotech, Munich, Germany), using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia) or with the Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.). After the PCR reaction, the samples were purified by column chromatography and were sequenced in an ABI Prism capillary sequencer (model 3730; Applied Biosystems). For sequence analysis and construction of phylogenetic trees, the program DNA Star (Madison, WI, U.S.A.) was used.

Reverse transcription (RT)-PCR.

For confirmation of introns in *bap1*, RT-PCR analyses were performed using primers 1 and 2 (Table 1). Before reverse transcription, 1 µg of total RNA was treated with 1 U Dnase, following the user's instructions to eliminate traces of genomic DNA in the sample. The RNA sample was then denatured with 200 ng of oligo(dT)₁₂₋₁₈ primers (Invitrogen) for 5 min at 70°C and was incubated with 500-nM concentrations of deoxynucleoside triphosphates, 10 mM dithiothreitol, and 200 U of reverse transcriptase (SuperScript II; Invitrogen) at 42°C for 1 h. Subsequent PCR assays contained 1 µl of RT reaction, 50 ng of each primer, 200 nM deoxynucleoside triphosphates, and 1 U *Taq* polymerase (BioTherm; GeneCraft, Lüdinghausen, Germany) and were carried out at 94°C for 4 min, followed by 36 cycles of 94°C for 1 min, 59°C for 1 min, and 70°C for 2 min.

Vector construction.

For deletion of *bap1*, a replacement vector was designed. By using primer pairs 3 and 4 and 5 and 6 (Table 1), the 5' and 3'

flanking regions, respectively, of the gene were amplified. The flanking regions were cloned into vector pCR 2.1. From this product the 5' and 3' fragments were excised with *SacI* and *SacII* and *HindIII* and *XhoI*, respectively, and were cloned into the nourseothricin-resistance vector pNR1 (Malonek et al. 2004) (Fig. 1). The complete replacement fragment was excised with *SacI* and *XhoI* and was used to transform strain B05.10.

For complementation of *bap1*, a $\Delta bap1$ deletion strain was transformed using a 2.8-kb fragment, which was amplified by PCR using primers 7 and 8 (Table 1). The obtained fragment included the *bap1* gene together with 1 kb of the *bap1* promoter and terminator, respectively. The sequence of the PCR product was determined to ensure correctness of the sequence. For construction of the complementation vector, the *Escherichia coli* hygromycin phosphotransferase gene *hph* under control of the *oliC* promoter was cloned into plasmid pDbcniaD (Schumacher et al. 2008), resulting in the basic vector pDbcniaD_hygR_1008. The 2.8-kb *bap1* full-length fragment was cloned in pCR-BluntII-TOPO (Invitrogen), was cut with *EcoRV* and *XhoI*, and was cloned into the corresponding site of pDbcniaD_hygR_1008. By restriction with *SacI* and *KpnI*, the complementation fragment was excised and was used for complementation of $\Delta bap1_6$ in the *bcniaD* locus.

Transformation of *B. cinerea*.

Protocols for protoplast formation and transformation were adapted from ten Have and associates (1998) as described by Schulze Gronover and associates (2001).

Homologous integration of the *bap1* replacement fragment was verified via PCR. The primer pair BcBap1_CF_F and pOliR2 was used to amplify DNA fragment F1, consisting of flanking region 1 upstream of *bap1* and the first part of the resistance-cassette promoter region. A second fragment, F2, with the end of the resistance-cassette terminator and flanking region 2 downstream of *bap1* was received by PCR with the primer pair BcBap1_CF_R and T-tub. Amplification of these DNA fragments should only be possible in cases of homologous integration of the replacement fragment into the *bap1* gene locus. As *B. cinerea* protoplasts are multinucleate, homologous integration might occur in one nucleus, whereas other nuclei do still contain the functional Bap1-encoding gene. To investigate whether there were nuclei still containing the original *bap1* gene, a third PCR was done by using a primer pair, bcap1_CF_1 and bcap1_CR_1, binding in the intact *bap1* gene. Diagnostic PCR preparations were separated via agarose gel electrophoresis. Homologous integration occurred in four of six transformants analyzed. After five rounds of single-spore isolation, three homokaryotic $\Delta bap1$ transformants $\Delta bap1_1$, $\Delta bap1_4$, and $\Delta bap1_6$, respectively, were obtained in which no *bap1* wild-type signal could be obtained after several diagnostic PCR assays using the primer pair bcap1_CF_1 and bcap1_CR_1. Further investigations were performed with mutants $\Delta bap1_1$ and $\Delta bap1_6$. To ensure no ectopic integration event of the replacement fragment, Southern blot analysis was performed. Genomic DNA of $\Delta bap1$ transformants as well as of the B05.10 wild type was digested with the endonuclease *HindIII*, resulting in specific restriction fragments that were separated via agarose gel electrophoresis, were blotted on a nylon membrane, and were hybridized with a flanking region 1 fragment. Due to a *HindIII* restriction site in the *bap1* gene that was replaced by the resistance cassette in $\Delta bap1$ transformants, the probe hybridized with a 0.9-kb fragment in wild-type strain B05.10 and with a 2.9-kb fragment in $\Delta bap1$ deletion mutants.

Homologous integration of the *bap1* complementation fragment in the *bcniaD* locus was verified via PCR. The primer

pair *bcniaD*-5'-seq-F and pOliR2 was used to amplify the DNA fragment consisting of the *bcniaD* flanking region 1 upstream of *bcniaD* and the first part of the resistance-cassette promoter region. Amplification of the DNA fragment should only be possible in cases of homologous integration of the complementation fragment into the *bcniaD* gene locus. A second fragment of the hygromycin-resistance gene *hph* was received by PCR with the primer pair *hygro_idF* and *hygro_idR*. To ensure no further ectopic integration event of the complementation fragment, Southern blot analysis was performed. Genomic DNA of $\Delta bap1$ transformants as well as of B05.10 wild type and $\Delta bap1_6$ mutant was digested with the endonuclease *HindIII*, resulting in specific restriction fragments. Due to a *HindIII* restriction site in the *bap1* gene that was replaced by the resistance cassette in $\Delta bap1$ transformants and complemented again, the probe hybridized with a 0.9-kb fragment in wild-type strain B05.10, with a 2.9-kb fragment in $\Delta bap1$ mutants, and with both fragments in $\Delta bap1$ transformants.

Pathogenicity assays.

Primary leaves of *Phaseolus vulgaris* L. genotype N90598 (originating from J. D. Kelly, Michigan State University, East Lansing, MI, U.S.A.) were inoculated with 7.5 μ l of conidial suspensions (2×10^5 conidia ml^{-1}) of *B. cinerea* strains for the standard pathogenicity test, as described by Klimpel and associates (2002). The infected plants were incubated in a plastic propagator box at 20°C under natural illumination. Disease symptoms were scored 24 and 48 h postinoculation (hpi) and 3 dpi. Additionally, leaves of *Arabidopsis thaliana* (Linnaeus) Heynhold, ecotype Columbia (Col-0) (provided by J. Kudla, WWU Münster, Germany) grown with long-day conditions as well as organic *Lycopersicon esculentum* and wounded pieces of *Malus domestica* 'Holsteiner Cox' were used for pathogenicity studies and were incubated under the same conditions while the infection process was monitored and photographically documented.

In planta gene expression assay.

After infection with 7.5 μ l of conidial suspensions (2×10^5 conidia ml^{-1}) of *B. cinerea* strains (discussed above), primary lesions (48 hpi) were isolated, freeze-dried, and used for RNA isolation. RNA was isolated from mycelial samples using the RNeasy total RNA isolation system (Promega).

DAB staining of infected plants.

Bean leaves with primary lesion were stained with DAB and were subsequently bleached in ethanol, according to the protocol of Thordal-Christensen and associates (1997).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute *Botrytis cinerea* B05.10 database:
www.broad.mit.edu/annotation/genome/botrytis_cinerea.2/Home.html
 University of Fribourg Faculty of Science FiRe and associated macros
 homepage: www.unifr.ch/plantbio/FiRe/main.html