The cAMP-Dependent Signaling Pathway and Its Role in Conidial Germination, Growth, and Virulence of the Gray Mold *Botrytis cinerea*

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In *Botrytis cinerea*, some components of the cAMP-dependent pathway, such as α subunits of heterotrimeric G proteins and the adenylate cyclase BAC, have been characterized and their impact on growth, conidiation, germination, and virulence has been demonstrated. Here, we describe the functions of more components of the cAMP cascade: the catalytic subunits BcPKA1 and BcPKA2 and the regulatory subunit BcPKAR of the cAMP-dependent protein kinase (PKA). Although $\Delta bcpka2$ mutants showed no obvious phenotypes, growth and virulence were severely affected by deletion of both *bcpka1* and *bcpkaR*. Similar to Δbac , lesion development of $\Delta bcpka1$ and $\Delta bcpkaR$ was slower than in controls and soft rot of leaves never occurred. In contrast to Δbac , $\Delta bcpka1$ and $\Delta bcpkaR$ mutants sporulated in planta, and growth rate, conidiation, and conidial germination were not impaired, indicating PKA-independent functions of cAMP. Unexpectedly, $\Delta bcpka1$ and $\Delta bcpkaR$ showed identical phenotypes, suggesting the total loss of PKA activity in both mutants. The deletion of *bcras2* encoding the fungal-specific Ras GTPase resulted in significantly delayed germination and decreased growth rates. Both effects could be partially restored by exogenous cAMP, suggesting that BcRAS2 activates the adenylate cyclase in addition to the G α subunits BCG1 and BCG3, thus influencing cAMP-dependent signal transduction.

The gray mold *Botrytis cinerea* Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is an important plant pathogen causing serious pre- and postharvest losses in more than 230 crop species worldwide. Soft rots, accompanied by collapse and water-soaking of parenchyma tissues, followed by a rapid appearance of gray masses of conidia, are the most typical symptoms on leaves and soft fruit. Despite the recent introduction of new antifungal compounds, *Botrytis cinerea* is difficult to control because of its broad host range, high genetic variation, and ability to survive as a saprophyte or for extended periods as sclerotia in crop debris (Williamson et al. 2007). Due to the great economic damage it causes and the variety of

attack methods on different host plants and organs, *B. cinerea* has become one of the model systems in molecular phytopathology. The release of the genome sequence of strain B05.10 by the Broad Institute has opened new opportunities and perspectives for basic research to study the mechanisms of plant infection in *B. cinerea*.

Several molecular analyses have been performed to study early penetration events and the establishment of plant infection. Enzymes involved in cell wall degradation, such as endopolygalacturonases, xylanases, and a pectin methylesterase (Brito et al. 2006; ten Have et al. 1998, 2001; Valette-Collet et al. 2003), as well as detoxification of phytoalexins (Schoonbeek et al. 2001) or detoxification or generation of reactive oxygen species (ROS) (Rolke et al. 2004; Segmüller et al. 2008), were shown to affect the speed of plant colonization. However, only the deletion of genes for the two mitogen-activated protein kinases (MAPK) BMP1 (Zheng et al. 2000) and BcSAK1 (Segmüller et al. 2007) and the tetraspanin BcPLS1 (Gourgues et al. 2004) resulted in nonpathogenic mutants. Other signaling components, such as the histidine kinase BOS1 (Viaud et al. 2006) and the adenylate cyclase BAC (Klimpel et al. 2002), are necessary for full virulence.

Deletion of *bcg1*, encoding one of the three α subunits of heterotrimeric G proteins, also had a strong effect on pathogenicity in *B. cinerea*: mutants penetrated the plant surface, causing small necrotic spots, but failed to produce secondary spreading lesions and soft rots (Schulze Gronover et al. 2001, 2004). In contrast, both $\Delta bcg2$ (Schulze Gronover et al. 2001) and $\Delta bcg3$ (Döhlemann et al. 2006a) mutants were only slightly delayed in host infection compared with the wild type. Two of the *B. cinerea* G α subunits, BCG1 and BCG3, stimulate the formation of cAMP by activating the adenylate cyclase BAC (Döhlemann et al. 2006a; Schulze Gronover et al. 2001). Although the phenotype of the $\Delta bcg3$ mutant (characterized by altered growth, less reduced conidiation, and impaired conidial germination) can be almost completely restored by supplementation with cAMP, several phenotypes of the *bcg1* mutant (e.g., protease secretion and botrydial production) cannot be restored by cAMP. Recently we have shown that BCG1 controls at least one additional signaling pathway, the Ca²⁺/calcineurin pathway (Schumacher et al. 2008b). This explains why the *bcg1* mutant is much more affected in its virulence than the adenylate cyclase (*bac*) mutants: it can only penetrate plant tissue producing small primary lesions, whereas the *bac* mutants are able to invade plant tissue. However, in the *bac* mutant, lesion extension stops before the stage of soft rot formation and in planta conidi-

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ation does not occur (Klimpel et al. 2002). Therefore, the cAMP-dependent pathway in *B. cinerea* is not essential for penetration. Similarly, in *Mycosphaerella graminicola*, a pathogen which penetrates its host through stomata, the cAMP-dependent protein kinase (PKA) signaling cascade was shown to be important only in later stages of infection: deletion mutants of *MgTpk2* encoding a catalytic subunit of the PKA were able to germinate, penetrate, and colonize tissue but were unable to produce pycnidia (Mehrabi and Kema 2006). In contrast to these fungi, the deletion of the adenylate cyclase or the PKA resulted in nonpathogenic mutants in appressoria-producing fungi such as *Magnaporthe grisea* (Choi and Dean 1997; Mitchell and Dean 1995; Xu et al. 1997), *Colletotrichum trifolii* (Yang and Dickman 1999), and *C. lagenarium* (Takano et al. 2001; Yamauchi et al. 2004). These examples show that this pathway can be involved in different processes in certain fungi, including growth (Ni et al. 2005), sporulation (Takano et al. 2001), conidial germination (Fillinger et al. 2002; Liebmann et al. 2003; Yamauchi et al. 2004; Zhao et al. 2006), secondary metabolism (Roze et al. 2004; Shimizu et al. 2003), nutrient sensing (Bahn et al. 2007; Thevelein et al. 2005), stress response (Ni et al. 2005; Zhao et al. 2006), sclerotia formation (Jurick and Rollins 2007), and pathogenicity or virulence (Xu et al. 1997; Yang and Dickman 1999; Mehrabi and Kema 2006) despite the high conservation of the signaling components of the cAMP-dependent pathway in fungi.

The best-studied cAMP-dependent signaling cascade in filamentous fungi is that of *Aspergillus nidulans*. Deletion of genes encoding the $G\alpha$ subunit GanB (Lafon et al. 2005), the adenylate cyclase CyaA (Shimizu and Keller 2001), or one of the catalytic subunits of the PKA, PkaA (Fillinger et al. 2002; Ni et al. 2005), causes severe defects in vegetative growth and conidial germination. The reason for the delayed germ tube formation was shown to be a decrease in trehalose degradation (D'Enfert et al. 1999; Fillinger et al. 2002). Single deletion of the second catalytic subunit, PkaB, does not cause any apparent phenotypic changes, whereas the absence of both *pkaB* and *pkaA* is lethal, indicating that PkaB and PkaA are the sole PKA catalytic subunits that are essential for viability of *A. nidulans* (Ni et al. 2005). The mutation of the regulatory subunit in *A. nidulans* has not been reported. However, the deletion of the corresponding gene in *A. fumigatus* resulted in mutants with increased cAMP-insensitive PKA activities (Zhao et al. 2006).

Little is known about the regulation of the cAMP pathway by components other than heterotrimeric G proteins in phytopathogenic fungi. In *Saccharomyces cerevisiae*, the activation of the adenylate cyclase is mediated by the RAS-homologues RAS1 and RAS2 (Broek et al. 1985; Toda et al. 1985). On the other hand, low-affinity and high-affinity cAMP phosphodiesterases (Pde1 and Pde2, respectively) antagonize G protein-Cyr1-cAMP signaling through enzymatic inactivation of cAMP after activation of the cAMP/PKA pathway (Santangelo 2006). Although deletion of RAS-encoding genes in *A. nidulans* and *Fusarium graminearum* resulted in impaired conidial germination (in addition to other effects), no interconnection to the adenylate cyclase/cAMP/PKA cascade has been found. RasA and the cAMP pathway control both germination and aflatoxin production in *A. nidulans* independently (Fillinger et al. 2002; Shimizu et al. 2003) and *F. graminearum* Δ ras2 mutants showed neither altered intracellular cAMP levels nor response to exogenous cAMP (Bluhm et al. 2007).

In this study, we wanted to further investigate the cAMP-dependent pathway in *B. cinerea* and its possible interconnection to other signaling components such as small GTPases. To better understand its involvement in growth, development, conidial germination, and virulence, we cloned and deleted the genes encoding the two different catalytic subunits of the

cAMP-dependent protein kinase A (BcPKA1 and BcPKA2), the regulatory subunit BcPKAR, and BcRAS2, a member of the superfamily of RAS-GTPases. Both PKA catalytic subunits are dispensable for conidiation, germination, penetration, initial colonization of bean leaves, and toxin production, but BcPKA1 is essential for normal growth on agar media, colony morphology, and full virulence. Surprisingly, the deletion of *bcpkaR*, which was expected to result in mutants with a constitutively active PKA, yielded mutants with a phenotype nearly identical to that of the *bcpka1* mutants. *Bcras2* mutants displayed a delay in conidial germination similar to the *bac* mutant. This germination defect can be partially restored by exogenous cAMP, suggesting a functional connection between BcRAS2 and the cAMP/PKA signaling pathway in *B. cinerea*.

RESULTS

Cloning of *bcpka1*, *bcpka2*, *bcpkaR*, and *bcras2*.

Bcpka1 was cloned by polymerase chain reaction (PCR) followed by gene library screening (before release of the genome sequence of *B. cinerea* B05.10). The gene has an open reading frame (ORF) of 1,785 bp; is interrupted by three introns of 51, 222, and 48 bp; and encodes a protein of 487 amino acids. The N-terminus (approximately 150 amino acids) of the protein shows low similarity to the catalytic subunits of the PKA of other fungi, whereas the residual protein sequence is highly conserved, with approximately 90% sequence identity to the catalytic subunits CPKA of *M. grisea* (AAA93199), PKAC-1 of *Neurospora crassa* (XP_326095), and PkaA of *A. nidulans* (AAF75762). Therefore, BcPKA1 belongs to class I of fungal PKA (Fig. 1A). Conserved motifs characteristic for catalytic subunits were found in the sequence of BcPKA1 (e.g., the nucleotide binding site GTGSFG, amino acids 183 to 188; the catalytic loop RDLKPEN, amino acids 298 to 304; and the motif DFGFAK, amino acids 317 to 322), which is involved in Mg^{2+} binding (Hanks and Hunter 1995; Knighton et al. 1991). In addition, three amino acids which were shown to be required for the association between catalytic and regulatory subunits based on x-ray crystal structure of mouse PKA-C α (Knighton et al. 1991) were also found in the *B. cinerea* BcPKA1 (Fig. 1B).

For the identification of the second catalytic subunit (BcPKA2), the sequence of *A. fumigatus* PKAC2 (XP_753780) (Nierman et al. 2005) was used for a similarity search (BlastX) (Altschul et al. 1997) in the protein database of *B. cinerea* B05.10. The predicted BcPKA2-encoding gene (annotated protein BC1G_07542.1) has an ORF of 1,560 bp and contains six introns (90, 60, 53, 115, 53, and 67 bp) whose positions were identified by reverse-transcriptase PCR. The corresponding protein is composed of 373 amino acids (Fig. 1B). A putative nucleotide binding site (GTGTFA) has been found at amino acids 58 to 63, which differs from the conserved motif of BcPKA1. Also, the catalytic loop RDMKPEN (amino acids 180 to 186) differs by 1 amino acid from the typical domain, whereas the motif DFGFAK (amino acids 199 to 205) is identical to that in other PKA. Interestingly, the conserved amino acids H100, Y212 (instead of the conserved tryptophan W329 in BcPKA1), and L214 that are probably required for the association between the catalytic and regulatory subunits (Fig. 1B) are also found in BcPKA2 but not in the PKA-like protein kinase BPK2 (Fig. 1B). The highly conserved catalytic domains of the *B. cinerea* PKA extend from amino acids 175 to 430 and 50 to 315 in the BcPKA1 and BcPKA2 proteins, respectively. These domains also show a high degree of sequence similarity to the previously identified *B. cinerea* homologue of the *S. cerevisiae* Sch9 Ser/Thr protein kinase BPK2 (Tudzynski and Schulze Gronover 2004) (Fig. 1B). However, from the three amino acids responsible for interac-

tion with the regulatory subunit, only the histidine can be found at the conserved position. In addition, BcPKA1 and BcPKA2 contain extensions to Ser/Thr-type protein kinase domains at the C-termini, which are conserved in numerous PKA catalytic subunits (Hanks and Hunter 1995).

The gene encoding the *B. cinerea* regulatory subunit BcPKAR was identified by BlastX analysis against the *B. cinerea* B05.10 sequence database using the sequence of *A. fumigatus* PkaR (AF401202) (Oliver et al. 2002). The putative *bcpkaR* gene (annotated protein BC1G_10590.1) has an ORF of 1,458 bp, contains one intron (52 bp), and encodes a 468-amino acid protein. The deduced protein shows the characteristics of a type II regulatory subunit, including the RII α motif (amino acids 9 to 43) and the two cAMP-binding domains (amino acids 212 to 335 and 334 to 453, respectively).

The BcRAS2-encoding gene (annotated protein BC1G_04437.1) was identified in the *B. cinerea* genome sequence due to its similarity to RasB of *A. fumigatus* (AY266674) (Fortwendel et al. 2004). *Bcras2* has an ORF of 757 bp and is interrupted by one intron that is 58 bp in length. The 232-amino-acid protein shows 70% sequence identity to *A. fumigatus* RasB (AY266674) and 74% to RAS-2 of *N. crassa* (BAA03708).

Deletion of *bcpka1*, *bcpka2*, *bcpkaR*, and *bcras2*.

For deletion of the four genes, replacement vectors carrying the hygromycin (p Δ *bcpka1*, p Δ *bcpka2*) or the nourseothricin (p Δ *bcpkaR*, p Δ *bcras2*) resistance cassette were constructed as described below and shown in Supplementary Figure S1A through D. The replacement cassettes were used to transform protoplasts of the wild-type strain B05.10. The transformants were screened for homologous integration by diagnostic PCR and purified to homokaryons by single-spore isolation, hyphal-tip isolation, or preparation of protoplasts. Three homokaryotic knock-out mutants for each gene were subject to Southern blot analyses, and all of them were shown to have a single-copy integration of the replacement cassette. Because all mutants for one knock-out revealed the same phenotype based on colony morphology, growth behavior, and virulence, one isolate for each replacement was chosen for further studies.

The cAMP pathway and BcRAS2 are involved in conidial germination.

In several fungi, conidial germination is regulated via the cAMP pathway. In addition, small GTPases of the RAS family were shown to be involved in germination in *A. nidulans*

(Fillinger et al. 2002), *A. fumigatus* (Fortwendel et al. 2004), and *F. graminearum* (Bluhm et al. 2007). In *B. cinerea*, the deletion of the adenylate cyclase-encoding gene *bac* and the G α subunit-encoding gene *bcg3* resulted in significantly reduced germination rates (Döhlemann et al. 2006a). To determine if this is also a characteristic feature for mutants of the other components of the cAMP pathway, and if BcRAS2 is involved in germination, we compared the germination rates of the Δ *bac*, Δ *bcpka1*, Δ *bcpka2*, Δ *bcpkaR*, and Δ *bcras2* mutants and the wild-type B05.10 in media with different carbon sources and in water on hydrophobic surfaces.

Sugar-induced germination on glass surfaces was monitored after 6 and 24 h of incubation in the presence of 10 mM fruc-

Fig. 1. cAMP-dependent protein kinase (PKA) catalytic subunits BcPKA1 and BcPKA2 of *Botrytis cinerea*. **A**, Phylogenetic tree of BcPKA1, BcPKA2, and BPK2 of *B. cinerea* (AM948966, AM948967, and CAC03748); PKA1 and PKA2 of *Sclerotinia sclerotiorum* (EDN98718 and AAS59253); CPK1 of *Colletotrichum lagenarium* (BAD04044); PKAC of *C. trifolii* (AAC04355); PKAC-1 and PKAC-2 of *Neurospora crassa* (XP_326095 and XP_324862); CPKA and CPK2 of *Magnaporthe grisea* (AAA93199 and AAO21201); TPK2 of *Mycosphaerella graminicola* (ABD92791); PKAC1 of *Aspergillus niger* (CAK37565); PKAC1 and PKAC2 of *A. fumigatus* (AAL02131 and XP_753780); PKAA, PKAB, and SCH4 of *A. nidulans* (AAF57562, EAA60759, and AAK71879); and TPK1, TPK2, TPK3, and SCH9 of *Saccharomyces cerevisiae* (NP_012371, NP_015121, NP_012755, and CAA040853). Sequence alignment of the fungal proteins and tree construction were done with ClustalW by use of MegAlign, a program of the Lasergene package (DNASTAR). **B**, Alignment of BcPKA1, BcPKA2, and BPK2 (only amino acids 300 to 840). The amino acids of the serine/threonine protein kinase (catalytic) domains are gray-shaded; important motifs for catalytic subunits are dark-gray-shaded. Invariant residues in all three proteins are marked by asterisks; identical residues of BcPKA1 and BcPKA2 are marked by lines. Alignment of protein sequences was done with ClustalW of the HUSAR-Sequence Analysis Package.

tose (Table 1; Fig. 2A). Although almost all wild-type conidia were already germinated after 6 h, the numbers of germinated conidia of *bac* and *bcras2* mutants were significantly lower after 6 h (29.3 and 59.0%, respectively) and also after 24 h of incubation (74.0 and 85.7%, respectively). However, the germination rate of the PKA mutants was either not affected (*Δbcpka1* and *Δbcpka2*) or only delayed after 6 h, and wild-type-like germination rates were observed after 24 h of incubation (*ΔbcpkaR*).

Germination of *B. cinerea* conidia on hydrophobic surfaces, such as polypropylene foil, is characterized by the formation of short germ tubes (Fig. 2B). The conidia of the *bac* mutant showed abnormal germ tubes or no germination, resulting in the low germination rate of 49.3% after 24 h of incubation. Shorter germ tubes compared with the wild-type were also observed for *bcpkaR* and *bcras2* mutants, although the total germination rates were not significantly affected (Table 1; Fig. 2B). To determine if the germination defect of *Δbcras2* conidia

is due to a decreased intracellular cAMP level, we studied the effect of adding extracellular cAMP on fructose-induced germination. The mutant responded to the addition of 8-CPT-cAMP to the medium at 100 μg/ml with increased germination rates (Fig. 3), indicating that BcRAS2 affects conidial germination in a cAMP-dependent manner. Thus, germination of *B. cinerea* conidia is dependent on BcRAS2 and BAC (cAMP) but is not directly dependent on PKA.

Intracellular cAMP levels are modulated by BcRAS2 and the PKA subunits.

To determine the impact of BcRAS2 and the PKA on cAMP signaling, we assayed the intracellular cAMP levels in the different knock-out strains. Unexpectedly, germinated conidia of the adenylate cyclase mutant showed cAMP levels similar to those of the wild type. In contrast, in 3-day-old mycelium grown in minimal medium with 10 mM fructose as single carbon source, the *bac* mutant had significantly less cAMP than the wild type (Table 2), which is consistent with previous studies (Klimpel et al. 2002). In accordance with our finding that *Δbcras2* mutants responded to exogenous cAMP with increased germination rates, the cAMP concentration in germinated conidia was reduced by 35% compared with the wild type. Due to increased cAMP concentrations in 3-day-old mycelium, we propose that the regulation of intracellular cAMP levels by BcRAS2 is stage dependent and more pronounced during

Fig. 2. Morphology of germinated conidia of the wild-type B05.10 (WT) and the different *Botrytis cinerea* mutants. **A**, Sugar-induced germination on glass surfaces was monitored in liquid Gamborg-B5 medium amended with 10 mM fructose as the single carbon source. Photographs were taken after 8 h. **B**, Hydrophobicity-induced germination was monitored in double-distilled water on polypropylene foil. Pictures were taken after 24 h of incubation.

Table 1. Sugar- and hydrophobicity-induced germination of *Botrytis cinerea* mutants

Strain ^c	Sugar-induced germination (%) ^a		Hydrophobicity-induced germination (%) ^b
	6 hpi	24 hpi	
WT:B05.10	96.7 ± 2.2	98.8 ± 1.6	97.3 ± 0.6
<i>Δbac</i>	29.3 ± 3.5	74.0 ± 4.8	49.3 ± 3.8
<i>Δbcpka1</i>	94.0 ± 2.3	98.0 ± 2.5	98.3 ± 1.5
<i>Δbcpka2</i>	92.2 ± 2.1	96.0 ± 3.2	97.0 ± 1.0
<i>ΔbcpkaR</i>	67.7 ± 3.4	97.2 ± 2.6	94.0 ± 3.5
<i>Δbcras2</i>	59.0 ± 7.1	84.3 ± 7.0	85.7 ± 2.1

^a Conidia were incubated on glass slides in the presence of 10 mM fructose. Germinated conidia were counted at 6 and 24 h postincubation (hpi).

^b Conidia (in double-distilled water) were incubated for 24 h on polypropylene foil.

^c WT = wild type.

Fig. 3. Germination rates of *bcras2* conidia were increased by exogenous cAMP. Germination was monitored in liquid Gamborg-B5 medium amended with 10 mM fructose, with or without the addition of 8-CPT-cAMP at 100 μg/ml. The germination assay of the *bcras2* mutants was repeated four times, and the diagram represents one experiment with triplicate counting.

conidial germination. Furthermore, the extremely increased cAMP concentrations in $\Delta bcpka1$, $\Delta bcpka2$, and $\Delta bcpkaR$ mutants in germinated spores as well as in conidia-derived mycelia indicate a deregulation of cAMP generation or degradation in these strains. Taken together, the intracellular cAMP level in *B. cinerea* is positively affected by the *Ga* subunits BCG1 (Schulze Gronover et al. 2001) and BCG3 (Döhlemann et al. 2006a) and the GTPase BcrAS2, and is negatively regulated by the PKA.

BcPKA1, BcPKAR, and BcrAS2 are essential for full virulence of *B. cinerea*.

In *B. cinerea*, cAMP signaling is important in different stages of host infection. Thus, the *bac* mutants infect living bean plants and form spreading lesions but wild-type-like continuous invasive growth culminating in soft rot of the leaves is prevented, and in planta conidiation never occurs (Klimpel et al. 2002). These data demonstrate the importance of BAC, especially during the late stages of plant colonization, although an impact on the early stages of infection is also suggested due to the germination defect of the *bac* mutant.

To compare the early events of plant–fungus interactions between the wild-type and mutant strains, we performed scanning electron microscope (SEM) analyses of germinating conidia on the bean leaf surface. Most of the conidia of the Δbac mutant were germinated after 22 h (in contrast to in vitro conditions) and formed abnormally long and branched germ tubes compared with the wild type, leading to slightly delayed penetration of the plant surface by the Δbac mutant (Fig. 4). The $\Delta bcpka1$ (Fig. 4) and $\Delta bcpkaR$ (data not shown) strains display an identical phenotype on bean leaf surfaces. In addition to direct penetration events, many conidia form elongated germ tubes that show an abnormal branching at the end of the out-growing hyphae. The deletion of *bcpka2* had a less pronounced effect on penetration: the germ tubes were bent and slightly elongated but formed typical thickenings at the penetration site. The *bcras2* mutants showed a penetration pattern similar to the wild type, with only a slight elongation of the germ tube prior to penetration of the cuticle. Therefore, although all the studied deletion strains show an altered germination and penetration behavior compared with the wild type, BAC, BcPKA1, and BcPKAR seem to play a more important role in plant penetration than BcPKA2 and BcrAS2.

To test whether the following infection process of the PKA and *bcras2* mutants proceeds in a manner and time course similar to the Δbac mutants, we performed infection assays on bean plants, pepper fruit, and detached tobacco leaves to compare the infection process of the wild type with that of all mutants (Fig. 5). Whereas the $\Delta bcpka2$ mutants showed wild-type

infection patterns leading to full maceration of bean leaves within 6 days, deletion of *bcpka1* and *bcpkaR* resulted in impaired virulence. Both mutants were able to produce secondary lesions but the infection process was delayed compared with the wild type. Colonization was arrested before spreading lesions developed, and yellow chlorotic spots appeared around the primary lesions, similar to the *bac* mutant. However, in contrast to Δbac , the $\Delta bcpka1$ and $\Delta bcpkaR$ mutants were able to sporulate in planta (data not shown). The $\Delta bcras2$ mutant was able to complete the whole in planta life cycle, including spreading lesion formation, soft rot, and conidiation, but with a significant delay of 3 to 4 days. Similar observations were made for the different strains using pepper fruit and detached tobacco leaves as hosts (Fig. 5B and C). Therefore, BcPKA2 is dispensable, whereas BAC, BcPKA1, BcPKAR, and BcrAS2 are required for full pathogenicity of *B. cinerea*.

BAC and PKA mutants display distinct phenotypes.

Because Δbac on the one hand and $\Delta bcpka1$ and $\Delta bcpkaR$ mutants on the other hand differ significantly in their intracellular cAMP content, it is not surprising that these differences have consequences for vegetative growth, conidial germination, and plant infection processes in these mutants. Therefore, we compared the phenotypes of the different mutants with those of the wild type in more detail.

Although no apparent phenotypic changes were caused by deletion of *bcpka2*, the mutants deleted in the other PKA sub-

Table 2. cAMP concentrations in the different strains

Strain ^b	Concentration (fmol of cAMP per milligram of dry weight) ^a	
	Germlings ^c	Mycelium ^d
WT:B05.10	393 ± 1 (100%)	942 ± 70 (100%)
Δbac	465 ± 6 (118%)	299 ± 16 (32%)
$\Delta bcpka1$	462,8 ± 303 (1176%)	26,926 ± 985 (2858%)
$\Delta bcpka2$	534 ± 34 (136%)	3,960 ± 346 (420%)
$\Delta bcpkaR$	2,479 ± 117 (630%)	4,678 ± 351 (497%)
$\Delta bcras2$	254 ± 6 (64%)	847 ± 27 (90%)

^a The cAMP concentrations are average data from three technical replications.
^b WT = wild type.
^c Conidia were spread on cellophane-covered complete medium agar and incubated for 24 h for germination.
^d Liquid minimal medium (Gamborgs B5 + 2% fructose) was inoculated with conidia (5 × 10⁶) and incubated for 3 days.

Fig. 4. In planta penetration assay. Low- and high-resolution (left and right, respectively) scanning electron microscopy images of germinating spores of the wild type (WT) and various mutants on the surface of bean leaves (22 h postinoculation).

units revealed a pronounced growth inhibition on all tested synthetic and undefined complete media (Fig. 6A). *Bcpka1* and *bcpkaR* mutants always grew as small compact colonies and, even after extended incubations of 2 weeks, the colony diameters were restricted to approximately 2 cm. Addition of different nitrogen or carbon sources or plant compounds (bean extract) did not restore the wild-type growth rate and colony

morphology. The Δbac mutants were less impaired in their growth rate but showed a different morphology on agar plates compared with the wild type: the colonies grew with an irregular indistinct margin.

Like the $\Delta bcpka1$ and $\Delta bcpkaR$ mutants, the $\Delta bcras2$ mutant also grew on most media as small, dense, and intensively sporulating colonies. However, on complete medium and po-

Fig. 5. Virulence of the different mutants in comparison with the wild-type B05.10 (WT). **A**, Time course of infection of French bean (*Phaseolus vulgaris*). Primary leaves were inoculated with 7- μ l droplets of conidial suspensions (2×10^5 /ml in Gamborgs B5 + 2% glucose). Progression of infections was documented daily until 9 days postinoculation (dpi). After this, no further development of disease was observed. **B**, Infection of yellow pepper (*Capsicum annuum*) and tobacco (*Nicotiana tabacum*). Pepper fruit were wounded with a needle prior to the inoculation with conidial suspensions of the different strains. Infected areas (9 dpi) are highlighted by dashed lines (left panel). Detached tobacco leaves were inoculated with 10- μ l droplets of conidial suspensions. Photograph was taken 15 dpi (right panel).

tato dextrose agar (PDA) with chopped bean leaves (PDAB), the colonies expanded after some days, accompanied by the formation of sclerotia (Fig. 6A). The addition of cAMP (8-CPT-cAMP at 100 µg/ml) partially restored the vegetative growth of the *bcras2* mutant, suggesting an interconnection between BcrAS2 and the cAMP-dependent signaling pathway (data not shown). In contrast, the wild-type strain showed slightly diminished vegetative growth on media supplemented with cAMP. The $\Delta bcpka1$ strain did not show any noticeable alteration in vegetative growth with cAMP supplementation (data not shown).

Interestingly, the described growth defect of *bcpka1*, *bcpkaR*, and *bcras2* mutants was only visible on solid media. In liquid cultures inoculated with equal numbers of conidia, these mutants showed growth rates comparable with the wild type whereas the *bac* mutant displayed only poor growth under these conditions (data not shown), probably due to the reduced cAMP level. Furthermore, the *bac* mutant produced significantly less conidia than the wild type and the other mutants (approximately 30%) when grown on complete medium (CM) agar plates (Table 3). In addition to the limited capacity to form conidia efficiently in planta and in vitro, *bac* mutants displayed an abnormal behavior when they were incubated for several weeks in darkness. Instead of formation of sclerotia, the mutants produced aerial hyphae and conidia in the dark, demonstrating the importance of cAMP signaling for normal

differentiation (Fig. 6B). In contrast, the *bcpka1*, *bcpka2*, *bcpkaR*, and *bcras2* mutants were still able to form sclerotia, and conidiation was repressed in darkness. However, *bcpkaR* mutants produced less sclerotia, and the formation of aerial mycelium was increased compared with $\Delta bcpka1$ and $\Delta bcras2$.

In summary, the *bac* mutants differ significantly from the *bcpka1* or *bcpkaR* deletion strains and also from *bcras2* mutants, suggesting additional targets of cAMP besides the regulatory subunit of the PKA.

Table 3. Amounts of conidia produced by the different *Botrytis cinerea* strains

Strain	Conidia ($\times 10^8$)/petri dish ^a	Conidia ($\times 10^6$)/cm ²	WT (%) ^b
WT:B05.10	3.70 $\pm$ 0.66	5.78	100.0
Δbac	1.07 $\pm$ 0.12	1.67	28.8
$\Delta bcpka1$	2.93 $\pm$ 0.25	4.58	79.3
$\Delta bcpka2$	3.00 $\pm$ 0.30	4.69	81.1
$\Delta bcpkaR$	2.73 $\pm$ 0.71	4.27	73.9
$\Delta bcras2$	2.67 $\pm$ 0.25	4.17	72.1

^a Complete medium agar plates were inoculated with 5×10^4 conidia. After 2 weeks, conidia were harvested and counted. Values are means of three petri dishes (diameter of 9 cm).

^b WT = wild type.

Fig. 6. Comparison of growth, conidiation, and sclerotia formation; WT = wild type. **A**, Strains were incubated for 1 week under light (near UV) for conidiation on minimal medium (CD; Czapek Dox), complete medium (CM), potato dextrose agar supplemented with homogenized bean leaves (PDAB), and grape juice agar. **B**, Strains were cultivated on CM for 4 weeks in darkness for sclerotia formation.

Differential expression of BCG1/BAC target genes in the PKA and *bcras2* mutants.

To further investigate the distinct phenotypes of *bac* and PKA mutants, we studied the in planta expression of several known BCG1 target genes in the different strains. Bean leaves were inoculated with conidial suspensions and the infected plant material was harvested in equal infection stages (primary lesions). As probes, we used genes which were previously shown to be up- or downregulated in both the *bcg1* and *bac* mutants (e.g., the xylanase-encoding genes SSH C09 and SSH B03), and those which are downregulated (e.g., SSH 22 and SSH 8) or upregulated (SSH[–] B01) only in the *bcg1* mutant in a BAC-independent manner (Schulze Gronover et al. 2004). We also included genes identified as BAC-dependent (contig

1310, contig 63, and *bcgst3*) or BcPKA1-dependent (*bcpgl*) in a macroarray approach (discussed below).

The expression studies showed that several analyzed genes were differentially expressed in the PKA and *bcras2* mutants compared with Δbac . Thus, the xylanase-encoding genes SSH C09 and SSH B03 as well as SSH H08 and SSH A06, all BCG1 and BAC target genes, showed a higher expression level in the different PKA and *bcras2* mutants than in the *bcg1* and *bac* mutants. However, the transcript levels were slightly lower in the $\Delta bcpka1$ and $\Delta bcpkaR$ mutants than in the wild type and the *bcpka2* mutant (Fig. 7). The extreme upregulation of two genes (*contig63* and *bcgst3*) in Δbac and the specific downregulation of *bcpgl* only in the *bcpka1*, *bcpkaR*, and *bcras2* mutants further underline the fact that significant differences in gene expression patterns exist between *bac* and *bcpka1/bcpkaR* mutants and that interconnections between BcRAS2 and components of the cAMP/PKA pathway must exist.

BcPKA1 is the predominant catalytic PKA subunit.

Because only the deletion of the *bcpka1* gene caused significant differences in vegetative growth and virulence compared with the wild-type B05.10, we concluded that BcPKA1 is the predominant catalytic subunit of the PKA in *B. cinerea*. Unexpectedly, the phenotype of *bcpkaR* mutants was almost identical to the *bcpka1* mutants' phenotype. This observation was contradictory to the described constitutive activation of the PKA in mutants of the regulatory subunit in other fungi (Mehrabi and Kema 2006; Zhao et al. 2006). To study whether the deletion of *bcpkaR* affected the PKA activity, we monitored basal and total PKA activity in crude cell extracts of *B. cinerea* wild-type and mutant strains (Fig. 8). In fact, no PKA activity was detectable in *bcpka1* and *bcpkaR* mutants whereas $\Delta bcpka2$ and $\Delta bcras2$ showed PKA activities comparable with the wild type. Therefore, the deletion of the regulatory subunit of the PKA does not result in a constitutively active PKA but causes a strong downregulation of total PKA activity in *B. cinerea*. The observation that no PKA activity was detectable in the *bcpka1* mutant underlines that BcPKA1 is indeed the predominantly active catalytic PKA subunit.

Sclerotia formation as well as botrydial and oxalic acid production are not regulated by PKA.

The α subunit BCG1 was shown to be an upstream activator of the adenylate cyclase BAC. Some of the features (e.g., colony morphology) were shown to be identical in both mutants and some of the BCG1 target genes are also affected in the *bac* mutant (e.g., xylanase-encoding genes) (Schulze Gronover et al. 2004). Here, we show that the formation of sclerotia is also regulated by BCG1 via BAC. Both the *bcg1* and *bac* mutants do not form sclerotia but produce macroco-

Fig. 7. In planta expression studies in the wild-type B05.10 (WT) and the *bcp1*, *bac*, *bcpka1*, *bcpka2*, *bcpkaR*, and *bcras2* mutants. Total RNA from infected plant material (bean leaves; harvested either 65 h postinoculation [hpi] [WT, $\Delta bcp1$, and $\Delta bcpka2$] or 90 hpi [Δbac , $\Delta bcpka1$, $\Delta bcpkaR$, and $\Delta bcras2$] to obtain the same stage of infection) was used for Northern blot analyses. The following genes were used as probes: SSH C09 (BC1G_03590.1, xylanase XYN11A) (Brito et al. 2006), SSH B03 (BC1G_00576.1, xylanase BcXYL2), SSH H08 (BC1G_08363.1, predicted protein), SSH A06 (BC1G_05864.1, maltose permease), SSH 22 (BC1G_03070.1, aspartyl protease), SSH 8 (BC1G_09180.1, metalloprotease BcMP1), and SSH(-) B01 (BC1G_09891.1, monosaccharide transporter) and were identified by a suppression subtractive hybridization approach (SSH) as differentially expressed genes in planta (WT B05.10 versus $\Delta bcp1$) (Schulze Gronover et al. 2004). *Bcpgl* (endopolygalacturonase 1) (Ten Have et al. 1998) was found to be downregulated in $\Delta bcpka1$ compared with WT in planta by a macroarray approach. Genes named *contig63* (expressed sequence tag clone; not found in any public database), *bcgst3* (BC1G_15497.1, glutathione S-transferase 3) (Schulze Gronover et al. 2005), and *contig1310* (BC1G_05503.1, CipC-like antibiotic response protein) were found to be differentially expressed in Δbac and WT in planta by a macroarray approach. *bcoahA* (BC1G_03473.1, oxaloacetate acetylhydrolase) (Han et al. 2007) and *bchxt* (BC1G_10092.1, monosaccharide transporter) were identified as BCG1-dependent genes under axenic conditions by a macroarray approach (Schumacher et al. 2008b). *BcactA* encoding actin and rRNA were used as loading controls.

Fig. 8. cAMP-dependent protein kinase (PKA) activities of the wild-type B05.10 (WT) and the different mutants. PKA activities in germinated conidia were monitored by gel electrophoresis. The phosphorylated PKA substrate moves toward the anode, and the activator contained cAMP. Conidia were incubated on cellophane-covered complete medium agar for 24 h.

nidia in darkness in contrast to the wild type, suggesting an impact of cAMP signaling on light-dependent developmental processes (Fig. 6B) (data not shown).

In addition to these similarities, most of the BCG1-controlled processes seem to be cAMP-independent. Previously, we have shown that the expression of several protease genes strictly depends on BCG1 in a BAC-independent manner (Schulze Gronover et al. 2004). As expected, the secretion of extracellular proteases on milk agar was also not affected in the PKA mutants characterized by the formation of visible halos around the colonies (data not shown).

A second characteristic feature of the $\Delta bcg1$ mutant is the loss of the ability to produce botrydial (Schulze Gronover et al. 2004). Botrydial is a *B. cinerea*-specific sesquiterpene causing chlorotic lesions on plant organs. Recently, we have shown that the expression of the botrydial biosynthesis gene *bcbot1*, encoding a P450 monooxygenase, and three more putative biosynthesis genes is controlled by BCG1 (Siewers et al. 2005) (data not shown). The analyses of botrydial production in Δbac , $\Delta bcpka1$, $\Delta bcpka2$, $\Delta bcpkaR$, and $\Delta bcras2$ mutants revealed that all these mutant strains were still able to produce botrydial and its derivatives, in contrast to $\Delta bcg1$ (Table 4). Therefore, biosynthesis of botrydial is dependent on BCG1 in a cAMP- and PKA-independent manner.

The production of oxalic acid is thought to contribute to virulence in *B. cinerea* by affecting the production and activity of secreted enzymes (Han et al. 2007; van Kan 2006). BCG1 controls the expression of *bcoahA* encoding the oxaloacetate

acetylhydrolase in vitro (Schumacher et al. 2008b) and in planta (Fig. 7). This enzyme was shown to be involved in oxalic acid production (Han et al. 2007). By performing plate assays using the pH indicator bromothymol blue, we compared the ability of the wild-type and mutant strains to acidify the agar medium by secreting oxalic acid. Except for $\Delta bcg1$, the other mutants defective in the components of the cAMP pathway were not affected in oxalic acid formation (data not shown). These data indicate that oxalic acid production is controlled by BCG1 in a cAMP-independent manner, similarly to the botrydial production.

DISCUSSION

Our studies aimed to investigate the role of the cAMP/PKA signaling pathway and its components in regulation of vegetative growth, development, and virulence in the phytopathogenic fungus *B. cinerea*. Although no G protein-coupled receptors have been identified in *B. cinerea* so far, the activation of the adenylate cyclase (BAC) by two of the three $G\alpha$ subunits was already demonstrated: BCG3 stimulates cAMP formation, mainly during conidial germination and penetration of the plant surface (Döhlemann et al. 2006a), and BCG1 activates BAC activity and cAMP signaling during growth on solid media in a carbon-source-dependent manner (Schulze Gronover et al. 2001). Consistent with these data, the Δbac mutant shares characteristic features with both *bcg1* and *bcg3* mutants, such as the germination defect with $\Delta bcg3$ and the altered colony

Table 4. Compounds isolated from the studied *Botrytis cinerea* strains

Mutant strains	Isolated metabolites (mg/liter)		
WT: B05.10	1 (7), 2 (5), 3 (4), 8 (3), 9 (5), 10 (1), 11 (0.9)		
$\Delta bcg1$	...		
Δbac	1 (10), 2 (7)		
$\Delta bcpka1$	1 (2), 2 (20.8), 3 (5), 4 (3), 10 (5), 11 (7)		
$\Delta bcpka2$	1 (15), 2 (17), 5 (20), 6 (6), 11 (45)		
$\Delta bcpkaR$	2 (15), 6 (12), 11 (37)		
$\Delta bcras2$	1 (4.5), 6 (5), 7 (4), 10 (4.5), 11 (4)

1	2	3	4
Botrydial	Dihydrobotrydial	4β-Acetoxy-9β,10β,15α-trihydroxyprobotryane	4β-Acetoxy-9β,10β-dihydroxyprobotryane

5	6	7	8
10-oxodihydro-botrydial	15α-methoxy-O-methyldihydrobotrydial	O-Methyldihydro-botrydialone	Dehydrobotrydienal

9	10	11	R ₁
Secobotrytrienediol	Botcinic acid	Botcinin A	

morphology with *Δbcg1*. However, based on the fact that several functions of BCG1 are not related to BAC and cAMP, additional BCG1-dependent pathways have been suggested (Schulze Gronover et al. 2004). Recently, an interconnection of BCG1 and the calcium- and calcineurin-dependent pathway has been demonstrated (Schumacher et al. 2008a,b).

Here, we report on the role of other components of the cAMP pathway, the two PKA catalytic subunits (BcPKA1 and BcPKA2) and the regulatory subunit (BcPKAR), in the life cycle of *B. cinerea*. Although the *Δbcpka2* mutants did not differ from the wild type in any of the parameters tested, both the *Δbcpka1* and *Δbcpkar* mutants were impaired in growth rate, colony morphology, virulence, PKA activity, and intracellular cAMP concentration. Furthermore, modulation of cAMP signaling by the monomeric G protein BcRAS2 is described.

The small GTPase BcRAS2 and the cAMP cascade are interconnected.

In *B. cinerea*, two proteins of the RAS-GTPase superfamily were found, BcRAS1 and BcRAS2. Although the deletion of *bcras1* dramatically affects growth and development (L. Kokkelink, unpublished data), *Δbcras2* mutants display a less severe phenotype characterized by altered colony morphology, delayed conidial germination, and slower infection of bean leaves. Due to the fact that the germination rate in the presence of fructose and the growth rate on solid media were improved by addition of cAMP, an interconnection between BcRAS2 and the cAMP/PKA cascade has been suggested. The decreased cAMP concentrations in germinated *Δbcras2* conidia and, to a lesser extent, in 3-day-old mycelium of *bcras2* mutants, further indicate the impact of BcRAS2 on cAMP signaling.

In *S. cerevisiae*, the GTP-bound monomeric and heterotrimeric G proteins (Ras and Gpa2) bind independently to adenylate cyclase (Cyr1) and stimulate production of cAMP. Ras-GEF (guanine nucleotide exchange factors Cdc25 and Sdc25) and RasGAP (GTPase-activating proteins Ira1 and Ira2) are also present in the Ras/Cyr1 complex and regulate adenylate cyclase activity by controlling the switch from the active to the inactive form of Ras (Santangelo 2006). A similar interaction of a Ras GTPase with the adenylate cyclase has been demonstrated in the human pathogen *Candida albicans*: Ras1 binds to the conserved Ras association (RA) domain of the adenylate cyclase Cdc35, thereby raising the levels of cellular cAMP (Fang and Wang 2006). Because the *B. cinerea* adenylate cyclase (BAC) also contains a conserved RA domain (Klimpel et al. 2002), the binding of BcRAS2 to the RA motif of BAC could be the mechanism by which the monomeric G protein affects cellular cAMP levels in *B. cinerea*. In addition, the fact that both the *bac* and *bcras2* mutants revealed a delayed conidial germination on glass slides in the presence of fructose as the single carbon source supports our suggestion that there might be a functional interconnection between BcRAS2 and BAC.

Therefore, despite the high degree of sequence conservation, the function of RAS proteins is probably not conserved in evolution: in *A. nidulans*, both RasA and the cAMP pathway control conidial germination but in an independent manner (Fillinger et al. 2002), and an interconnection between RAS2 (a BcRAS2 homologue) and cAMP signaling could not be shown in *F. graminearum* (Bluhm et al. 2007).

cAMP/PKA signaling and pathogenicity.

Unlike in appressoria-forming fungi such as *M. grisea* and *Colletotrichum lagenarium*, where the adenylate cyclase and the PKA holoenzyme are essential for rapid degradation of lipid and glycogen reserves and formation of functional appressoria as prerequisites for penetration (Choi and Dean 1997; Thines et al.

2000; Yamauchi et al. 2004), the penetration process per se is not impaired in *B. cinerea* BAC and PKA mutants. However, *bac*, *bcpka1*, and *bcpkar* mutants displayed elongated germ tubes after 22 h of incubation, resulting in delayed penetration. In contrast, wild-type conidia only form short germ tubes that directly penetrate the plant surface. *B. cinerea* mutants defective in either trehalose synthesis (deletion of trehalose-6-phosphate synthase, TPS1) or trehalose degradation (deletion of neutral trehalase, TRE1) showed impaired conidial germination in the presence of sugars but were unaffected in their ability to penetrate plant cells or in subsequent invasive growth. In addition, exogenous cAMP has been shown to induce trehalose mobilization that was accompanied by accelerated germination on hydrophobic surfaces (Döhlemann et al. 2006b). These results suggest a penetration mechanism differing from appressoria-forming fungi and corresponds with the observation that *B. cinerea* can either directly penetrate host tissue or form swollen hyphal tips. These "appressoria-like" structures do not form septa to seal off the germ tube and, therefore, differ from appressoria formed by *M. grisea* (Tenberge 2004). Similarly to *B. cinerea*, *Mycosphaerella graminicola* mutants of the PKA catalytic and regulatory subunits were not affected in penetration and this fungus does not require appressoria for successful infection (Mehrabi and Kema 2006).

However, the *bac*, *bcpka1*, and *bcpkar* deletion strains were unable to complete the in planta life cycle: lesion expansion of *Δbcpka1* and *Δbcpkar* stopped after 5 days of incubation, while the lesions caused by *Δbac* proceeded slowly until 8 days postinoculation. All three mutants produced chlorotic spots around the lesions, probably due to the secretion of botrydial (Siewers et al. 2005). From these data, we conclude that the late stages of colonization are regulated via cAMP and PKA. The *bcras2* mutant is able to complete the infection cycle although the infection process is delayed, again demonstrating that PKA-dependent cAMP signaling is not clearly associated with BcRAS2 activity. This assumption is also supported by the less pronounced delay in host penetration with the BcRAS2 mutant compared with the BAC, BcPKA1, and BcPKAR deletion strains. In contrast, BCG1 controls the early stages of invasive growth in a cAMP-independent manner, because *Δbcg1* mutants are completely unable to spread after primary lesions have formed (Schulze Gronover et al. 2004).

As in other fungi (e.g., *A. nidulans* and *Ustilago maydis*) (Dürrenberger et al. 1998; Ni et al. 2005), the deletion of the gene encoding the second catalytic subunit (class II PKA), *bcpka2*, did not cause any obvious phenotype.

PKA is not the sole effector of cAMP in *B. cinerea*.

In addition to some similarities between *bac*, *bcpka1*, and *bcpkar* mutants, we found several significant differences; unlike *Δbac*, the *Δbcpka1* and *Δbcpkar* mutants were not affected in sugar-induced conidial germination. In *M. grisea*, only the adenylate cyclase (MAC) mutants exhibited delayed germination, whereas mutants lacking the PKA catalytic subunit germinated as the wild type (Choi and Dean 1997; Xu et al. 1997). In contrast, in *C. lagenarium*, the deletion of both the PKA catalytic subunit and the adenylate cyclase resulted in mutants affected in germination on both plant and glass surfaces (Yamauchi et al. 2004).

Even more striking in this study are the significant differences in growth rate and colony morphology between the *Δbac* and *Δbcpka1* mutants. The model of the cAMP-dependent pathway suggests that a decrease in cAMP level (e.g., by deletion of the adenylate cyclase gene) would result in an inactive PKA; therefore, similar phenotypes of mutants affected in either cAMP formation or PKA activity were expected. However, although the *Δbac* mutants showed a significant germination delay in vitro

(Döhlemann et al. 2006a), reduced conidiation, decreased growth rate on agar plates, and loss of ability to sporulate in planta (Klimpel et al. 2002), the *Δbcpka1* mutants were unaffected in all these processes. Furthermore, whereas the *bcg1* and *bac* mutants lost the ability to produce sclerotia and developed conidia in darkness, the PKA-defective mutants produced sclerotia in a wild-type-like manner. A cAMP-dependent and PKA-independent regulation model also has been postulated for sclerotial development in *S. sclerotiorum* (Chen and Dickman 2005). In this fungus, the deletion of the adenylate cyclase gene *sac1* resulted in aberrant sclerotia demonstrating an inhibitory role of cAMP in regulation of sclerotial initiation (Jurick and Rollins 2007). cAMP-mediated sclerotial inhibition was shown to be modulated through MAPK (Chen and Dickman 2005). However, the mechanisms by which cAMP inhibits MAPK remain unclear. The authors suggest that a PKA-independent signaling pathway mediates MAPK (Smk1) inhibition by cAMP via activation of the monomeric G protein Rap1.

In addition to the differences in vegetative growth, germination of conidia, and sclerotia formation, significant differences in the expression of a set of genes in the *Δbac* mutant on one hand and *Δbcpka1* and *ΔbcpkaR* mutants on the other have been obtained. Some of the genes were specifically upregulated only in the *Δbac* but not in the wild type or other mutant strains (Fig. 7).

One explanation for these differences between the *bac* and *bcpka1/bcpkaR* mutants could be the presence of the second catalytic subunit, BcPKA2, and an additional PKA-like protein kinase, BPK2 (Tudzynski and Schulze Gronover 2004), which could take over some essential functions when the major catalytic subunit is deleted. In *A. nidulans*, it has been shown that the absence of both *pkaB* and *pkaA* is lethal, although the deletion of *pkaB* alone does not cause any apparent phenotypic changes. Overexpression of *pkaB* rescues the growth defects caused by deletion of *pkaA*, indicating that PkaB plays a role in vegetative growth processes (Ni et al. 2005). In *B. cinerea*, however, the PKA activity assay clearly showed that the deletion of *bcpka1* resulted in total loss of detectable PKA activity, demonstrating that BcPKA1 encodes the major PKA in *B. cinerea*. BcPKA2 activity is probably very low and cannot suppress the dramatic growth defect in *bcpka1* deletion mutants. It is likely that the differences in activity of both catalytic subunits are due to differences in conserved domains of both proteins (e.g., in the catalytic loop [RDMKPEN instead of RLKPEN] or in the three amino acids responsible for the association with the regulatory subunit [Y212 instead of the conserved W329]). These amino acid differences in BcPKA2 may result in lower activity and preferential binding of the regulatory subunit to BcPKA1. If BcPKA2 could suppress some effects of the *bcpka1* deletion, we would rather expect a less pronounced phenotype for the *Δbcpka1* mutant. Therefore, it is likely that cAMP binds to additional target proteins and controls an as-yet-unidentified regulatory pathway in addition to the PKA pathway.

In human cells, exchange proteins activated directly by cAMP (Epac proteins), a family of cAMP-regulated guanine nucleotide exchange factors (cAMP-GEF), mediate PKA-independent features of cAMP signal transduction. By serving as a cAMP-binding protein with GEF activity, Epac couples cAMP production to the activation of Rap and Ras GTPases, thereby affecting, for instance, MAPK signaling. Two Epac proteins are known, containing either a single (Epac1) or two (Epac2) cAMP-binding domains within their N-terminal regulatory regions (Holz et al. 2006). However, such signaling components have not been cloned and characterized in filamentous fungi thus far. Although no Epac-homologous proteins were found in the *B. cinerea* databases, at least three different proteins in addition to the PKA regulatory subunit contain the highly conserved

cAMP-binding domain: BC1G_01623.1 and BC1G_14322.1 each contain two tandem copies, whereas BC1G_12094.1 contains a single copy of the cAMP receptor domain.

Summarizing our results, it is most likely that cAMP is a second messenger regulating cellular processes in both a PKA-dependent and a PKA-independent manner, thereby activating additional signaling pathways in *B. cinerea*.

Deletion of the PKA regulatory subunit BcPKAR results in loss of PKA activity.

In most fungi studied so far, opposite phenotypes have been described for mutants of the PKA catalytic and regulatory subunits. For example, *U. maydis* mutants lacking the regulatory subunit (Ubc1) display decreased filamentous growth and a multiple-budded phenotype, whereas mutants of the catalytic subunit (Adr1) show a constitutive filamentous phenotype similar to the adenylate cyclase (Uac1) mutants (Dürrenberger et al. 1998; Gold et al. 1997). In *A. niger*, disruption of *pkaR* revealed the most dramatic phenotype: the mutant formed small colonies and failed to sporulate. In contrast, mutants lacking the catalytic subunit PkaC were slightly reduced in germination but showed wild-type-like sporulation (Saudohar et al. 2002). Deletion of the PKA regulatory subunit (MgBcy1) in *Mycosphaerella graminicola* resulted in mutants showing altered osmosensitivity and reduced melanization, whereas mutants of the catalytic subunit (MgTpk2) displayed accelerated melanization (Mehrabi and Kema 2006). Surprisingly, and in contrast to these filamentous fungi, the respective *B. cinerea* mutants behave very similarly. Thus, in contrast to the *Δbac* mutant, *Δbcpka1* and *ΔbcpkaR* mutants formed small compact colonies on all tested solid media, penetrated plant tissue, performed invasive growth up to a certain stage, sporulated in planta, and showed a similar gene expression pattern. The nondetectable PKA activity in *bcpkaR* mutants gives an explanation why *Δbcpka1* and *ΔbcpkaR* display almost identical phenotypes. However, the molecular mechanism by which the deletion of *bcpkaR* causes the loss of detectable PKA activity remains unknown. One possibility is a decreased transcription of the respective gene or the post-translational degradation of the BcPKA1. The latter mechanism has been described for subunits of heterotrimeric G proteins in *Cryptococcus parasitica* and *N. crassa*. Disruption of genes encoding either a Gα or Gβ subunit consistently reduced the level of its presumptive partner protein (Krystofova and Borkovich 2005; Parsley et al. 2003).

Regulation of cAMP levels by the PKA subunits.

B. cinerea mutants deleted in one of the three different PKA subunits had extremely increased cellular cAMP concentrations compared with controls, indicating that the PKA regulates its activity by negatively affecting cAMP levels. The fact that *bcpka1* mutants showed the highest cAMP concentrations is consistent with the predominant role of the catalytic subunit BcPKA1. However, the impact of the *bcpka2* deletion on cellular cAMP indicates that BcPKA2 also may act as a cAMP effector, although the corresponding gene is not essential in the wild-type background.

In *S. cerevisiae*, intracellular cAMP concentrations are influenced by the activity of two cAMP-degrading enzymes: Pde2, the high-affinity phosphodiesterase (PDE), regulates basal cAMP levels while Pde1, the low-affinity PDE, regulates cAMP levels induced by the presence of glucose and is activated by PKA catalytic subunits (Ma et al. 1999). A similar PKA-mediated negative feedback loop for modulation of cAMP concentrations exists in *Cryptococcus neoformans*, because mutants of Pka1 exhibited significantly increased cAMP levels and the site-directed mutation of a consensus PKA phosphorylation site re-

duced Pde1 function. In addition, a PKA-independent regulation of Pde1 activity is proposed (Hicks et al. 2005). On this basis, deletion of PDE-encoding genes in *B. cinerea* might result in increased cellular cAMP levels and subsequent constitutive activation of the cAMP/PKA pathway, as described for *S. cerevisiae* and *C. neoformans*. In fact, two genes were identified in the *B. cinerea* database (proteins are annotated as BC1G_07334.1 and BC1G_08417.1; Broad Institute) exhibiting similarities to *S. cerevisiae* Pde1 and Pde2, respectively.

On the other hand, the loss of adenylate cyclase activity after *bac* deletion and subsequent reduction of cAMP level probably results in downregulation of PDE activity, thus maintaining a basic cAMP level. This might be the reason that we did not find a total loss of intracellular cAMP in the *bac* mutant in our previous (Klimpel et al. 2002) and recent (this article) analyses. We suggest that only those conidia of the *bac* mutant are able to germinate which have a relatively high level of cAMP, but that the level of cAMP rapidly decreases during vegetative growth (Table 2).

Conclusion and perspectives.

Summarizing our knowledge about the cAMP-dependent signaling in *B. cinerea*, it is obvious that there are significant differences in comparison with other fungal systems, even though the components of cAMP/PKA signal cascade are highly conserved among fungi and higher eukaryotes. In contrast to the appressoria-forming fungi such as *M. grisea*, in which cAMP signaling regulates penetration and, therefore, determines pathogenicity, the cAMP pathway plays only a minor role in the plant–fungus interaction in *B. cinerea*. Several signaling components were

shown to modulate cellular cAMP levels: the G α subunits BCG1 and BCG3 and the GTPase BcRAS2 stimulate cAMP formation by adenylate cyclase (BAC), whereas PKA negatively affects cAMP levels, possibly through activation of cAMP degradation (Fig. 9). As shown in other fungi, the class I PKA catalytic subunit BcPKA1 is the predominant subunit also in *B. cinerea*, regulating growth on solid media and, mainly, the late stages of the infection. Surprisingly, Δbac and PKA mutants share only some features (e.g., virulence), leading to the assumption that other downstream effectors of cAMP besides PKA must exist that regulate BAC-dependent, PKA-independent processes such as conidial germination and formation of sclerotia. In contrast to other fungi, the deletion of the PKA regulatory subunit did not result in a constitutively active PKA but in the total loss of PKA activity in *B. cinerea*, indicating differing modes of regulation of PKA protein complexes in these fungi. In addition, we demonstrate a possible interconnection of one of the two *B. cinerea* Ras proteins, BcRAS2, with the cAMP-dependent pathway, probably via modulation of BAC activity.

Taken together, these data demonstrate that the cAMP/PKA-dependent signaling pathway in fungi can play different roles in growth and development and may interact with other pathways depending on specific life styles, host plants, and penetration mechanisms.

MATERIALS AND METHODS

Fungal strains and growth conditions.

Strain B05.10 of *B. cinerea* Pers. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is a putative haploid strain ob-

Fig. 9. Model of cAMP-dependent signal transduction in *Botrytis cinerea*. Activation of the heterotrimeric G proteins containing the G α subunits BCG1 and BCG3 by as-yet-unknown G protein-coupled receptors (GPCR) leads to increased cAMP concentrations by stimulation of the adenylate cyclase BAC. While BCG3 appears to be active during germination (in the presence of sugars) and early stages of plant infection (Döhlemann et al. 2006a), BCG1 is required during the later stage of lesion expansion (transition from primary to secondary infection) and is essential for the expression of different putative virulence factors, such as phytotoxic secondary metabolites, secretion of proteases, and oxalic acid production (Schulze Gronover et al. 2001, 2004). Recently, an interconnection between BCG1 and the Ca²⁺/calciurein(CN)/BcCRZ1 signaling pathway has been shown (Schumacher et al. 2008a,b). In addition, cAMP formation by BAC is positively affected by the monomeric G protein BcRAS2, because exogenous cAMP partially restores growth and germination defects of *bcras2* mutants. The main downstream effector of cAMP is the cAMP-dependent protein kinase A, consisting of the cAMP-binding regulatory subunit BcPKAR and the catalytic subunits BcPKA1 and BcPKA2, respectively. BcPKA1 is the predominant catalytic subunit in regulation of vegetative growth and virulence, whereas BcPKA2 might be essential in a $\Delta bcpka1$ background. The deletion of the regulatory subunit-encoding gene *bcpkaR* resulted in the total loss of detectable PKA activity. Because cAMP-dependent protein kinase (PKA) mutants, especially $\Delta bcpka1$, display drastically increased cellular cAMP concentrations, a modulation of cAMP levels through a feedback loop is proposed where the active BcPKA1 positively affects cAMP phosphodiesterase (PDE) activities, thereby regulating the degradation of cAMP. Other cAMP-binding proteins besides BcPKAR are assumed which enable cross-talk to other signaling cascades and are responsible for regulation of the BAC-(cAMP)-dependent but PKA-independent features such as growth rate, proper conidiation, sclerotial development, and conidial germination.

tained after benomyl treatment of an isolate of *Vitis* (Quidde et al. 1999). The B05.10-derivative strain Δbac contains a deletion in the adenylate cyclase-encoding gene *bac* and was described in Klimpel and associates (2002). Strain $\Delta bcl1$ is deleted in the respective $G\alpha$ subunit-encoding gene and was characterized by Schulze Gronover and associates (2001). The *Botrytis cinerea* strains were grown in liquid medium or on plates containing PDA amended with 10% (wt/vol) hackled bean leaves (PDAB), synthetic CM agar (Pontecorvo et al. 1953), modified Czapek-Dox (CD) agar as minimal medium (2% sucrose, 0.1% KH_2PO_4 , 0.3% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4 \times 7 H_2O$, 0.002% $FeSO_4 \times 7 H_2O$, pH 5.0), or grape juice agar (100% grape juice, 0.1% yeast extract, pH 5.0). The strains were incubated for 1 week at 20°C under near-UV for conidiation and for 3 to 4 weeks at 21°C in darkness for sclerotia formation. For DNA mini-preparations, mycelium was grown for 3 days at 20°C on CM agar with a cellophane overlay. Single conidial isolates were obtained by spreading 100 μ l of conidial suspensions on CM plates containing nourseothricin at 70 μ g/ml (Werner BioAgents, Jena, Germany) or hygromycin B at 70 μ g/ml (Invivogen, San Diego, CA, U.S.A.). Conidia were germinated and single colonies transferred individually to new plates containing nourseothricin or hygromycin B.

For chemical analyses, *B. cinerea* strains were grown in solid culture in petri dishes at 25°C for 7 days; conidia derived from these cultures were used to inoculate Erlenmeyer flasks. Mycelia were grown at $24 \pm 2^\circ C$ and 140 rpm in 500-ml Erlenmeyer flasks containing 200 ml of modified CD medium. In a typical experiment, 10 to 15 flasks, each containing 200 ml of culture medium, were inoculated with 2×10^7 fresh conidia/flask and incubated for 7 days for mutant strains and 10 days for the $\Delta bcras2$ mutant.

Plate assays.

To test for the secretion of proteases, *B. cinerea* strains were incubated on agar containing casein as the single nutrient source (milk at 12.5 ml/liter and K_2HPO_4 at 2 g/liter, pH 6.5). Protease activity resulted in the formation of transparent halos around the colonies. The production of oxalic acid was determined by a pH indicator assay using CM agar (pH 8.0) with 0.01% bromothymol blue (Sigma-Aldrich, Taufkirchen, Germany). Acidification of the medium caused by the fungus resulted in a color change (from blue to yellow).

Infection assays.

Conidia from the wild type and the mutants were collected from 10- to 14-day-old PDAB plates and resuspended in Gamborg's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 10 mM glucose and 10 mM KH_2PO_4/K_2HPO_4 , pH 6.4, to a final concentration of 2×10^5 conidia/ml. Droplets (7 to 10 μ l) of the conidial suspensions were used to inoculate leaves of French bean (*Phaseolus vulgaris*), tobacco (*Nicotiana tabacum* L. cv. SNN), and pepper (*Capsicum annuum*) fruit. For reproducible infection, the fruit were wounded with a needle and the droplets were placed onto the wound. Living plants, detached leaves, and fruit were incubated in a plastic propagator box at 20°C under natural illumination in the laboratory.

SEM analyses.

For SEM analyses of conidial germination on bean leaves, primary leaves of *P. vulgaris* were sprayed with a 2×10^6 /ml spore suspension in Gamborg's B5 medium (Duchefa) supplemented with 10 mM glucose and 10 mM KH_2PO_4/K_2HPO_4 , pH 6.4. After 22 h of incubation, 5-mm² pieces of the leaves were excised. The samples were aldehyde fixed and dehydrated in an ethanol series as described by Giesbert and associates (1998). For SEM, samples were critical point dried (Emitech K850

Critical Point dryer; Emitech, Ashford, England), gold sputtered (Emitech vacuum sputter device K550x), and examined with a Hitachi S-3000N scanning electron microscope at 15 kV.

Germination assay.

The germination assays were performed as described by Döhlemann and associates (2006a). Strains were grown for 7 to 10 days on PDAB medium. The spores were harvested and subsequently washed at least three times with double-distilled H_2O . Germination assays were incubated at 18°C in darkness and counted at defined intervals. When the germ tube length exceeded the conidial diameter, spores were counted as germinated.

DNA isolation and Southern blots.

Fungal genomic DNA was isolated as described by Cenis (1992) and plasmid DNA was isolated using a plasmid DNA preparation kit (Qiagen GmbH, Hilden, Germany). Fungal genomic DNA and lambda DNA was transferred to Amersham Hybond-N⁺ filters (GE Healthcare Limited, Freiburg, Germany) after digestion with restriction enzymes and size separation on a 1% agarose gel according to Sambrook and associates (1989). Hybridization was performed as described by Schulze Gronover and associates (2001).

RNA isolation, cDNA synthesis, and Northern blot analyses.

Total RNA was isolated from mycelium or *Botrytis* spp.-infected bean leaves using the RNA easy extraction kit (Promega, Mannheim, Germany). Total RNA (1 μ g) was taken for cDNA synthesis using the oligo (dT)12-18 primer and the SuperScript II reverse-transcriptase (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. The amplification was performed by PCR using the BioTherm DNA polymerase (GeneCraft GmbH, Germany) and the following PCR program: 94°C for 4 min; 35 cycles of: 94°C for 1 min, 55°C for 1 min, and 70°C for 2 min; and a final extension at 70°C. Fragments were cloned into the pCR2.1 TOPO vector for sequencing. For Northern blot analysis, samples (25 μ g of total RNA) were transferred to Hybond-N⁺ membranes 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 Na_2HPO_4 , 0.06 M EDTA, 1% N-lauroylsarcosine (Sigma-Aldrich), 10% dextran sulfate (Eppendorf AG, Hamburg, Germany), and 0.01% salmon sperm DNA, pH 6.2, at 65°C in the presence of a random-primed α -³²P-dCTP-labeled probe.

Transformation of *B. cinerea*.

Protocols for protoplast formation and transformation were adapted from an established procedure (ten Have et al. 1998) and are described by Schulze Gronover and associates (2001).

PKA activity assay.

B. cinerea conidia from indicated strains (1×10^6 conidia/plate) were used to inoculate CM agar plates with a cellophane overlay. Young mycelia were harvested after 24 h and ground under liquid nitrogen. Protein extraction was performed as described by Liebmann and associates (2004). Fluorescent dye-coupled kemptide peptide was used to determine PKA activity following the manufacturer's protocol (Pep-Tag Assay for Non-Radioactive Detection of cAMP-dependent Protein Kinase; Promega).

cAMP assay.

Cellular cAMP was measured using the highly sensitive Amersham cAMP Biotrak Enzymeimmunoassay (GE Health-

care Limited) following the manufacturer's instructions. Lyophilized mycelium was treated with lysis reagent 1B for 15 min and centrifuged for 10 min at maximum speed, and the supernatant was used in several dilutions (1:5, 1:10, 1:50, and 1:100) for the enzyme-linked immunosorbent assay. For each strain and each condition, three technical repeats were performed.

Chemical analyses.

^1H and ^{13}C nuclear magnetic resonance (NMR) measurements were obtained on Varian Gemini 300 and Varian Unity 400 NMR spectrometers with SiMe_4 as the internal reference. Mass spectra were recorded on a GC-MS Thermoquest spectrometer (model: Voyager) and a VG Autospec-Q spectrometer. High-pressure liquid chromatography (HPLC) was performed with a Hitachi/Merck L-6270 apparatus equipped with a UV-VIS detector (L 6200) and a differential refractometer detector (RI-71). TLC was performed on Merck Kieselgel 60 F_{254} , 0.2 mm thick. Silica gel (Merck, Poole, U.K.) was used for column chromatography. Purification by means of HPLC was accomplished with a silica gel column (Hibar 60, 7 m, 1 cm wide, 25 cm long). Chemicals were products of Fluka (Milwaukee, WI, U.S.A.) or Sigma-Aldrich. All solvents used were freshly distilled.

The broth was filtered through a nylon 200- μm mesh filter, the culture liquid (2 to 3 liters) was saturated with NaCl, and the aqueous phase was extracted with ethyl acetate. The ethyl acetate extract was washed with H_2O and then dried over anhydrous Na_2SO_4 . Evaporation of the solvent at reduced pressure gave a yellow oil that was separated by means of column chromatography on silica gel, with a mixture of ethyl acetate and petroleum ether (10, 20, 30, 40, 50, 60, 80, and 100% ethyl acetate) as solvent.

Extensive spectroscopic methods, specifically ^1H -NMR and ^{13}C -NMR, were then employed to analyze the toxins present in each fraction obtained. The toxin structures were analyzed by spectroscopic methods and their structures compared with authentic samples previously isolated from strains of *B. cinerea* (Collado et al. 2000). Furthermore, compounds eluted in the abovementioned fractions were isolated by further purifications using semipreparative HPLC to afford compounds 1 to 11 (Table 4) from both the wild-type isolate and mutants of *B. cinerea*.

Cloning and replacement of *bcpka1*.

A 0.6-kb PCR fragment of *bcpka1*, generated by using the primers 1 and 2 (Supplementary Table S1), was used as a probe for screening a genomic λ -EMBL3 library of *B. cinerea* SAS 56 (Quidde et al. 1999). Two overlapping fragments from the hybridizing clone λ -3 that covered the whole ORF and the 5'- and 3'-noncoding regions were cloned into pUC19. A 0.9-kb *Hind*III fragment (pbcpkal-Hind) contained the 5' part and the first 500 bp of the gene, and the 2.0-kb *Sal*I fragment (pbcpkal-Sal) contained almost the whole ORF, including 0.3 kb of the 3' untranslated regions. The cDNA fragments of *bcpka1* were generated by using the primer combinations 1 and 2 and 3 and 4, respectively, and total cDNA as template. The sequencing revealed the presence of three introns in *bcpka1*. The corresponding computer-based annotation (BC1G_10410.1) in the *B. cinerea* B05.10 Database (Broad Institute) is incorrect; therefore, the revised *bcpka1* sequence is stored in the GenBank database (accession number AM948966). For the construction of the gene replacement vector, the plasmid pOliHP (Rolke et al. 2004), carrying the *Escherichia coli* hygromycin B phosphotransferase gene *hph* under control of the *A. nidulans* *oliC* promoter and *trpC* terminator was used as a basal vector. Gene flanks (700 and 500 bp,

respectively) were amplified by using the primers 5, 6, 7, and 8 containing artificial restriction sites for further cloning. The fragments were isolated from pCR2.1-TOPO with *Kpn*I/*Xho*I and *Hind*III/*Eco*RI, respectively, and cloned into the corresponding restriction sites of pOliHP, creating p Δ *bcpka1*. For transformation, the replacement cassette was isolated by restriction with *Kpn*I and *Eco*RI. Transformants were analyzed by diagnostic PCR for homologous integration using the primer combinations 9 and 36 and 37 and 10. Putative knock-out mutants were purified by single-spore isolation and were screened by PCR for the absence of the wild-type allele using primers 11 and 12. For Southern blot analysis, the genomic DNA of strain B05.10 and *bcpka1* transformants was digested with *Hind*III and hybridized to the 3' flank of *bcpka1*. The wild-type fragment with a size of 2 kb was replaced by a 1-kb fragment in the *bcpka1* transformants due to an additional *Hind*III restriction site within the replacement fragment.

Cloning and replacement of *bcpka2*.

The gene *bcpka2* was identified by BlastX search (Altschul et al. 1997) in the genome sequence of *B. cinerea* B05.10 (Broad Institute Database) on the basis of the corresponding *A. fumigatus* *pkaC2* (XP_753780) (Nierman et al. 2005) sequence. The respective *B. cinerea* protein is annotated as BC1G_07542.1. By sequencing a full-length cDNA clone of *bcpka2*, generated by using the primers 19 and 20, the computer-based annotation was revised. The GenBank accession number is AM948967.

For replacement of *bcpka2*, the 5' (0.8 kb) and 3' (0.7 kb) flanks were amplified by using the primers 13, 14, 15, and 16, and the fragments were cloned into the *Kpn*I/*Sal*I and *Hind*III/*Eco*RI restriction sites of pOliHP, creating the vector p Δ *bcpka2*. The replacement fragment was isolated using *Kpn*I and *Eco*RI and transformed into *B. cinerea* B05.10. Primer combinations 17 and 36 and 37 and 18 were used for PCR to detect the homologous integration of the replacement fragment in hygromycin-resistant transformants. The *bcpka2* wild-type allele was detected using primers 19 and 20. For Southern blot analysis, genomic DNA of the strains was digested with *Xba*I and hybridized to the 5' flank. Due to the loss of an *Xba*I restriction site in the knock-out mutants, the wild-type fragment (2 kb) was replaced by a bigger fragment in the mutant strains. For Southern blot analysis, genomic DNA of the strains was digested with *Hind*III and was blotted and hybridized to the 3' flank of p Δ *bcpkaR*. The wild-type 3-kb fragment was replaced by a 1-kb fragment in the deletion mutants, due to an additional *Hind*III restriction site in the gene replacement fragment.

Cloning and replacement of *bcpkaR*.

Gene *bcpkaR* (corresponding protein annotated as BC1G_10590.1; accession number AM948968) was identified due to sequence similarity to *A. fumigatus* *pkaR* (AF401202) (Oliver et al. 2002). The predicted intron was confirmed by sequencing a cDNA fragment that was amplified using primers 25 and 26. To construct p Δ *bcpkaR*, the vector pNR1 (Malonek et al. 2004), containing the *nat1* gene of *Streptomyces noursei* under control of the *oliC* promoter from *A. nidulans*, was used. Gene flanks comprising the 5' and 3' noncoding regions of *bcpkaR* were generated using primers 21, 22, 23, and 24, and cloned into pCR2.1-TOPO. A 1-kb *Sac*I/*Sac*II fragment from the 5' noncoding region (natural *Sac*I restriction site) and a 0.9-kb *Hind*III/*Xho*I fragment from the 3' noncoding region (natural *Xho*I restriction site) were isolated and inserted into the respective cloning sites of pNR1. The replacement fragment was isolated from p Δ *bcpkaR* using *Sac*I and *Xho*I. Transformants were analyzed by PCR for homologous inte-

gration of the replacement fragment (primer combinations 21 and 36 and 38 and 24, respectively) and for *ΔbcpkaR* and wild-type alleles after single-spore isolations (primers 25 and 26).

Cloning and replacement of *bcras2*.

The gene *bcras2* was identified by BlastX search using the sequence of *A. fumigatus rasB* (AY266674) (Fortwendel et al. 2004). The corresponding protein is annotated as BC1G_04437.1 (Broad Institute database).

The replacement vector p Δ *bcras2* was constructed by cloning a 1,065-bp *SacI/XbaI*-fragment from the 5' noncoding region, and amplified by using primers 29 and 30 and a 488-bp *HindIII/SalI* fragment from the 3' noncoding region, generated using primers 31 and 32, into the vector pNR1. For diagnostic PCR to prove the homologous integration of the replacement fragment, the primer combinations 33 and 36 and 38 and 34 were used. The detection of wild-type alleles was done using primers 33 and 35. For Southern analysis, genomic DNA of the transformants and the wild-type was digested with *EcoRI* and hybridized to the 3' flank of the replacement fragment. Due to an additional *EcoRI* site in the replacement fragment, the corresponding hybridizing fragment was 8 kb in the wild type and 3 kb in the transformants with homologous integration.

Macroarray analyses.

To study BAC- and BcPKA1-dependent gene expression in planta, bean leaves were inoculated with conidial suspensions of wild-type strain B05.10 and *bac* and *bcpka1* deletion mutants, as described above. Primary lesions were harvested after 48 to 60 h of incubation and were subsequently used for RNA isolation. Preparation of radio-labeled cDNA probes, hybridization of macroarray membranes, and analyses of expression data were performed as described by Schumacher and associates (2008a). Some of the clones significantly up- or down-regulated in the *bac* mutant were used as probes for Northern blot analyses.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Broad Institute *B. cinerea* B05.10 protein database:
www.broad.mit.edu/annotation/genome/botrytis_cinerea/
- The German Cancer Research Center HUSAR sequence analysis package:
genius.embnat.dkfz-heidelberg.de