The High-Affinity Phosphodiesterase BcPde2 Has Impact on Growth, Differentiation and Virulence of the Phytopathogenic Ascomycete Botrytis cinerea

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Abstract

Components of the cAMP signaling pathway, such as the adenylate cyclase Bac and the protein kinase A (PKA) were shown to affect growth, morphogenesis and differentiation as well as virulence of the phytopathogenic fungus Botrytis cinerea. While loss of Bac caused drastically reduced intracellular cAMP levels, deletion of the PKA resulted in extremely increased cAMP concentrations. To regulate the intracellular level of the second messenger cAMP, a balance between its biosynthesis through adenylate cyclase activity and its hydrolysis by phosphodiesterases (PDEs) is crucial. Here, we report the functional characterization of the two PDEs in the ascomycete B. cinerea, BcPde1 and BcPde2. While deletion of bcpde2 resulted in severely affected vegetative growth, conidiation, germination and virulence, the bcpde1 deletion strain displayed a wild-type-like phenotype. However, the double bcpde1/2 deletion mutant exhibited an even stronger phenotype. Localization studies revealed that BcPde2 accumulates at the plasma membrane, but is also localized in the cytoplasm. BcPde1 was shown to be distributed in the cytoplasm as well, but also accumulates in so far unknown mobile vesicles. Overexpression of bcpde1 in the Δbcpde2 background rescued the deletion phenotype, and in addition an increased transcript level of bcpde1 in the Δbcpde2 strain was observed, indicating redundant functions of both PDEs and an interdependent gene expression.

Introduction

Botrytis cinerea is a phytopathogenic ascomycete causing gray mold disease in more than 200 plant species. Infection occurs through penetration followed by invasive growth, subsequent maceration of the plant tissue and generation of asexual conidia. In inappropriate conditions survival is ensured through formation of sclerotia, which can either germinate vegetatively or serve as female partner in sexual reproduction [1–3].

External signals, such as humidity, pH, osmotic stress or nutrient availability, have to be sensed and transduced via a diversity of signaling cascades, which can be activated via stimulation of a heterotrimeric G protein. Besides the Ca2+-signaling pathway [4–6], for example the Gz-subunit Bcg1 is able to stimulate the 3’-5’-cyclic adenosine monophosphate (cAMP)-mediated pathway. Activation of adenylate cyclase (AC) activity leads to formation of the second messenger cAMP, which is subsequently bound by the regulatory subunits of the protein kinase A (PKA). In its inactive state, the PKA is a heterotetramer formed by two catalytic and two regulatory subunits. Binding of cAMP induces the release of the catalytic subunits of the PKA which in turn may phosphorylate downstream targets such as transcription factors [7].

In B. cinerea, several components of this pathway, including two of the three Gz subunits (Bcg1 and Bcg3), the AC (Bac), and the catalytic and regulatory subunits of the PKA have been characterized in the last years. Bcg1 controls light-dependent conidiation, sclerotia formation and invasive growth in plant hosts [8], whereas Bcg3 was shown to regulate conidiation and carbon-source-induced germination [9]. The cytosolic cAMP concentration in both mutants was lower than that of the wild type [9,10], indicating that both Gz-subunits stimulate Bac activity.

Like other fungi, B. cinerea possesses one gene encoding the regulatory PKA subunit (bcpka1), and two genes encoding catalytic subunits (bcpka2, bcpka3). BcPka1 appears to be the major subunit whose deletion led to significant growth retardation, delayed germination and reduced in vitro sporulation, as it was also observed for the bac deletion mutant [11,12]. However, in contrast to the Δbac mutant which has lost the ability to form sclerotia and to sporulate in planta, Δbcpka1 mutants are able to conidiate during infection in a wild type-like manner and to form sclerotia. These significant differences between the Δbac and Δbcpka1 mutants indicate that additional cAMP-binding proteins beside the PKA must exist which transduce signals to other downstream targets. Unexpectedly, deletion of BcPkaR did not result in a constitutively active PKA, as in other fungi, but instead in reduced PKA activity and hyperaccumulation of cAMP [11].

cAMP is the key second messenger of this pathway and its synthesis and degradation needs tight regulation. Inactivation of cAMP to AMP is carried out by hydrolysis through phosphodiesterase (PDE) activity and counteracts and resets the cAMP/PKA cascade. Saccharomyces cerevisiae possesses two PDEs, Pde1 and Pde2, with unrelated primary sequences [13]. Pde1 is described as a low-
Phosphodiesterases in Botrytis cinerea

affinity PDE that downregulates agonist-induced cAMP accumulation in a PKA-controlled negative feedback loop, whereas Pde2 (high-affinity) controls the basal intracellular cAMP level [14,15]. Both PDEs are at least partially redundant [16–18]. In all studies performed so far, deletion of the high-affinity Pde2 revealed a much stronger phenotype than deletion of pde1 [17,18].

Not much is known so far about the role of PDEs in filamentous fungi. In Neurospora crassa, a pde2 deletion mutant failed to produce any conidia indicating that cAMP turnover is required for the transition from aerial growth to proconidial chain formation [19]. Recently, two studies have been published on characterization of PDEs in the rice-blast fungus Magnaporthe oryzae [20,21]. Deletion of both genes showed that PdeH (homolog of yeast Pde2) is a key regulator of sexual and pathogenic development, whereas PdeL (homolog of Pde1) had no obvious function. Loss of PdeH or of both PDEs resulted in elevated intracellular cAMP levels and reduced virulence [20]. Furthermore, both single deletion mutants exhibited defects in their conidial morphology and hyphal branching pattern [21].

In this work we characterized single and double knockout mutants of both phosphodiesterase-encoding genes, bcpde1 and bcpde2, in B. cinerea. Our results show that BcPde2 has a strong impact on virulence, but also on vegetative growth and development. BcPde1 plays only a minor role, but is able to functionally complement the Δbcpde2 phenotype. In contrast to other fungi, deletion of BcPde2 resulted in reduced intracellular cAMP levels.

Materials and Methods

Strains and culture conditions

B. cinerea Pers.:Fr. B05.10 is an isolate from Vitis [22,23], and was used as host strain for transformation. All B. cinerea strains used in this study are listed in Table 1. Wild-type and mutant strains were grown on several complex media: potato dextrose agar (Sigma-Aldrich Chemie, Steinheim, Germany) was supplemented with 10% homogenized leaves of French bean (Phaseolus vulgaris) (PDAB). Synthetic complete medium (CM) was prepared according to Pontecorvo et al. [24]. As minimal medium, GB5 (0.33% Gamborg B5 [Duchefa Biochemie BV, Haarlem, The Netherlands], 2% glucose) was used. For conidiation, strains were incubated for seven days at 20°C under light/dark (12 h/12 h) conditions; for sclerotia formation, three weeks in continuous darkness. Conidiogenesis was quantified by inoculation of CM with a 5 mm disk of an 11-day-old PDAB agar culture, harvested, filtered and the conidia production was determined by microscopic analysis of the filter sample.

Virulence assays

For penetration assays on onion epidermal layers, epidermal strips were washed with double-distilled water and incubated for one hour in a humid chamber at 70°C. Conidia were harvested, washed three times with double-distilled water and diluted to a concentration of 5×10^4 spores/ml. 10 µl-droplets of these suspensions were used for inoculation. After 24 h penetration was monitored microscopically with an AxioScope.A1 microscope (camera AxioCam MRc for color imaging, both Carl Zeiss MicroImaging GmbH, Jena, Germany). Extracellular fungal hyphae were stained using Lactophenol Aniline Blue solution (Sigma-Aldrich, Germany) prior to light microscopy.

Infection assays on primary leaves of Phaseolus vulgaris were performed with conidia from 7-day-old PDAB agar cultures as described previously [10]. Infected plants were incubated in a plastic propagator box at 20°C under natural illumination.

Standard molecular methods

Fungal genomic DNA (gDNA) was isolated as described previously [25]. Southern blot analyses with fungal DNA were performed according to the method of Sambrook et al. [26]. Total RNA was isolated from mycelial samples using the Trizol procedure (Invitrogen, Groningen, Netherlands). 1 µg of total RNA was taken for cDNA synthesis using the oligo(dT)12–18 primer and SuperScript II reverse transcriptase (Invitrogen, Groningen, Netherlands) according to the manufacturer’s instructions. To avoid gDNA amplification, RNA samples were treated with DNaseI (Promega). For sequence analyses, Lasergene v10 software (DNASTar, Madison, WI) was used. BLASTP analysis was performed using the website http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.

All primers used in this study are listed in Table S1. Quantitative real-time PCR was carried out using a one-tenth dilution of the cDNA template in a MyiQ2 Two-Color Real-Time PCR Detection system with the Bio-Rad iQ SYBR Green supermix (Bio-Rad, Hercules, CA, U.S.A). Genes encoding actin A (XP_001553368, primers 30&31), elongation factor 1-alpha (XP_001551786, primers 32&33) and tubulin (AA660307, primers 34&35) showed the same expression pattern in wild-type and mutant strains used in this work and were therefore used to normalize the cDNA amounts in the samples. To study the expression of bcpde1 and bcpde2, primer couples 15&16 and 28&29 were used. Annealing temperatures ranged from 58°C to 62°C, while extension times were fixed to 20 s. For each gene, the PCR efficiency was between 90 and 110%. The relative expression of bcpde1 and bcpde2 was calculated following the ΔΔCt (cycle threshold) Pfaffl method, from the mean of two different determinants of Ct values.
Statistical analyses were performed by independent comparison of the gene expression values in the mutant strains to those in the wild type in each condition by using two-sample t-tests in Excel (Microsoft).

**Generation of mutants**

The replacement constructs for single deletion of *bcpde1* and *bcpde2* were generated using the homologous recombination system in yeast according to Colot et al. [27]. Therefore, about 1 kb of the 5‘- and 3‘-non-coding regions of both genes were amplified from gDNA of *B. cinerea* wild-type strain B05.10 using primer pairs 1&4 and 3&4 for *bcpde1* and 17&18 and 19&20 for deletion of *bcpde2* (Fig. S1A). The hygromycin resistance (*hph*) cassette containing *hph* under control of the *tpyC* promoter of *A. nidulans* was amplified with primers 5&6 using pCSN44 [28] as template. After linearization of pRS426 [28] using EcoRI and XhoI, all fragments were co-transformed into uracil auxotrophic *B. cinerea* strain Characteristics Reference

**Table 1. Botrytis cinerea strains used in this study.**

| *B. cinerea* strain | Characteristics | Reference |
|---------------------|-----------------|-----------|
| WT: B05.10          | Isolate from Vitis vinifera (Germany); MATI-1 | [22,23] |
| Δ*bcpde1*           | B05.10, Δ*bcpde1:*hph, homokaryon | this study |
| Δ*bcpde2*           | B05.10, Δ*bcpde2:*hph, homokaryon | this study |
| ΔΔ*bcpde1/2*        | B05.10, ΔΔ*bcpde1/2:*nat, Δ*bcpde2:*nat, homokaryon | this study |
| Δ*bcpde1+gfp-bcpde1*| B05.10, Δ*bcpde1:*hph, gfp-*bcpde1:*nat, heterokaryon | this study |
| Δ*bcpde2+gfp-bcpde2*| B05.10, Δ*bcpde2:*hph, gfp-*bcpde2:*nat, heterokaryon | this study |
| Δ*bcpde2+gfp-bcpde1*| B05.10, Δ*bcpde2:*hph, gfp-*bcpde1:*nat, heterokaryon | this study |
| Δ*bac*              | B05.10, Δ*bac:*hph, homokaryon | [12] |
| Δ*bcpka1*           | B05.10, Δ*bcpka1:*hph, homokaryon | [11] |

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For Southern blot analysis, gDNA was restricted with *ScaI* and hybridized with a 70 bp DNA probe. Resistant colonies were transferred to GB5-plates (Werner-Bioagents, Jena, Germany). Single conidial isolates were obtained by spreading conidial suspensions on selection plates. Germinated conidia were transferred individually onto new plates until homokaryotic mutants were obtained.

**Transformation of *B. cinerea***

Protoplast transformation was performed as described previously [31]. Resistant colonies were transferred to GB5-plates containing 70 μg/ml of hygromycin B (Invitrogen, San Diego, CA, USA) or 70 μg/ml of nourseothricin (Werner-Bioagents, Jena, Germany). Single conidial isolates were obtained by spreading conidial suspensions on selection plates. Germinated conidia were transferred individually onto new plates until homokaryotic mutants were obtained.

**PKA activity assay**

Mycelia were grown for three days on CM- or GB5-agar plates with a cellophane overlay, harvested and ground under liquid nitrogen. Protein extraction was performed as described by...
Liebmann et al. [32]. 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 Healthcare 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.

Results

Identification of phosphodiesterases

By using the protein sequences of yeast Pde1 (CAAC4139) and Pde2 (CAAC49689) as queries for BlastP analyses, we identified two homologous proteins in the B. cinerea database (www.broadinstitute.org/annotation/genome/botrytis_cinerea): B0510_5711 and B0510_6935, respectively. The amino acid sequence of B0510_5711 revealed 55% identity (47% positives) to the yeast Pde1, whereas the B0510_6935 sequence revealed 26% identity (39% positives) to the yeast Pde2 protein. Exon-intron organizations of these genes (hereafter called B. cinerea pde1 and pde2) were confirmed by sequencing of cDNA clones. Both PDEs contain conserved phosphodiesterase domains and share sequence similarities with PDEs of other fungi.

For functional analysis, single and double deletion mutants of both genes were generated (Fig. S1, Table 1). As independent transformants exhibited identical phenotypes the results for one arbitrarily chosen mutant per construct (Δbcpde1-T1, Δbcpde2-T6, ΔΔbcpde1/2-T5) are shown.

Deletion of bcpde2 results in a drastic reduction of vegetative growth

To compare vegetative growth of the mutants and the wild type, all strains were grown on solid complete medium (CM) with or without different supplements (Fig. 1A). The bcpde1 deletion strain displayed a wild-type-like growth pattern on all media, whereas mutants lacking bcpde2 (Δbcpde2 and ΔΔbcpde1/2) showed drastically reduced growth rates. After one week the respective strains displayed colony diameters of about 3 cm, whereas the wild type and bcpde1 mutants exhibit growth rates of one cm per day. Osmotic stress generated by high sugar concentrations (1 M sorbitol) abolished conidia formation in Δbcpde2 and ΔΔbcpde1/2 mutants without affecting radial growth compared to basal CM. Addition of cAMP resulted in a decreased radial growth of all strains. The most striking effect was observed for the ΔΔbcpde1/2 mutant whose growth was completely blocked, whereas the single Δbcpde2 mutant was still able to grow though very slowly.

To investigate a possible involvement of the PDEs in conidiogenesis in more detail, we quantified the numbers of produced conidia on solid medium. While the Δbcpde1 mutant produced aerial mycelium and wild-type-like amounts of conidia, only about 20% of spores compared to the wild type were observed for the single and double bcpde2 deletion mutants (Fig. 1B, 1C). However, in contrast to the wild type and Δbcpde1 strains, which formed dark-pigmented sclerotia when incubated for three weeks in continuous darkness, the Δbcpde2 and ΔΔbcpde1/2 mutants formed abundant conidia instead of sclerotia in the dark (Fig. 1D).

Summarizing, BcPde2 plays an essential role in vegetative growth, conidiation and sclerotia formation. As the double deletion mutant was under some conditions even more affected than the single mutants, an additive effect of both PDEs is suggested.

BcPde2 has an impact on conidial shape, size and germination

Microscopic studies showed that conidia of Δbcpde2 and ΔΔbcpde1/2 mutants differed in shape and size from those of the wild-type and seemed to be highly vacuolized (Fig. 2A). While wild-type conidia displayed an average length of about 11.3 μm and width of 8.7 μm, the bcpde2 deletion resulted in a large variance of conidia size. In addition to the altered forms and diameters, Δbcpde2 conidia displayed delayed carbon source (10.5 mM glucose)-induced germination rates (Fig. 2B). However, after 24 hours, about 80% and 50% of the conidia from the single bcpde2 and double bcpde1/2 mutants, respectively, were able to germinate. Furthermore, the shape of the young germ tubes and hyphae was different in bcpde2 mutants (Fig. 2C). Usually, the wild type conidia germinate after a stage of swelling within the first four to six hours and form an appressoria-like structure. In most cases, a second germ tube at the opposite side of the sporid develops after a short break, and both hyphae grow in different directions and start branching. In contrast, to that, a majority of conidia of single and double bcpde2 mutants exhibit just one germ tube which grew straightforward without formation of appressoria-like structures or further lateral curves.

Germination of B. cinerea conidia can also be induced by hydrophobic surfaces in the absence of nutrients in a MAPK-dependent manner [9]. The conidia of the Δbcpde1 mutant developed the characteristic wild-type-like short germ tubes on polypropylene. In contrast, the Δbcpde2 strain formed longer germ tubes, while the conidia of the double knock-out strain generated either long and thin germ tubes, or did not germinate at all (Fig. 2D).

In conclusion, BcPde2 has an impact on number and size of macroconidia and nutrient- and hydrophobicity-induced conidial germination. Since the ability to germinate and the mode of germination are important steps in the early stages of infection we studied the role BcPde1 and BcPde2 may play in virulence.

BcPde2 is essential for full virulence

First, we investigated the mutants’ ability to penetrate onion epidermis cells (Fig. 3A). The Δbcpde1 strain developed one wild-type-like short germ tube (blue-stained) directly penetrating the onion cells. Some conidia derived from Δbcpde2 and ΔΔbcpde1/2 strains also formed germ tubes and penetrated the epidermis cells. However, many conidia of the double deletion mutant were either not able to germinate at all (similar to the observations in axenic culture), or they formed long germ tubes which grew on the surface without penetrating. In the rare cases, when hyphae penetrated the onion epidermis cells, a drastic effect was noticed: the hyphae of the Δbcpde2 single mutant thickened up to six fold of the normal hyphal diameter (Fig. 3A, asterisks). These hyphae were still able to branch and grew further in a wild type-like manner. The hyphae of the ΔΔbcpde1/2 strain also drastically thickened after penetration, but no further spreading was observed in any case.

When leaves of young bean plants were inoculated with conidia suspensions of the strains (Fig. 3B), both, the wild type and the Δbcpde1 mutant developed primary lesions (2 days post infection, dpi), followed by secondary spreading lesions (3 dpi) and full maceration of the plant tissue accompanied by formation of
conidia (6 dpi). On the contrary, ΔbcPde2 and ΔΔbcPde1/2 mutants were retarded in the infection process. Progression of infection stopped finally before reaching the soft rot stage. The ΔΔbcPde1/2 strain was even more delayed as primary lesions became only visible at 3 dpi. In accordance with the reduced virulence both ΔbcPde2 and ΔΔbcPde1/2 mutants lost the ability to produce conidia during infection.

Summarizing these results, BcPde2 is essential for full virulence. Both, the onion epidermis and bean leave pathogenicity tests revealed no defect in host penetration, but a retardation of invasive growth within the plant tissue and loss of in planta conidiation.
GFP-BcPde2 is localized in the cytoplasm and the plasma membrane

In order to visualize the intracellular localization of the two PDEs, we generated GFP fusion constructs of both proteins under control of the constitutively expressed oliC promoter. The gfp-bcpde1 fusion construct was expressed in two genomic backgrounds: wild type and Δbcpde1. Both strains expressing the GFP-BcPde1 construct displayed a wild-type-like phenotype. Fluorescence was microscopically examined in germinating conidia on glass slides in the presence of glucose. Strong fluorescent signals were observed in small undefined vesicles in hyphae and conidia, while only weak signals were seen in the cytosol (Fig. 4A–C). Often just one strong, punctual fluorescent signal per compartment was visible, but sometimes also several smaller ones were present (Fig. 4B and 4C). Time-lapse analyses showed that the GFP-BcPde1 fusion protein was highly dynamic and probably associated to mobile vesicle-like bodies (data not shown).

Similarly, a gfp-bcpde2 fusion construct was expressed in the wild type and the Δbcpde2 strain. All described phenotypes caused by
deletion of bcpe2, e.g. the significantly reduced virulence on bean plants, were restored by expressing the fusion construct in the mutant background indicating its full functionality (Fig. S2). Expression of the gfp-bcpe2 fusion construct in the wild-type background did not lead to phenotypic differences (data not shown). Localization of the fluorescent protein was examined in germinating conidia on glass slides as described above (Fig. 4D), and in water drops on onion epidermis strips (Fig. 4E). Invasion growing hyphae which have penetrated the onion epidermis cells showed wild-type-like growth. The GFP-BcPde2 fusion protein was evenly distributed throughout the cytosol in both conditions. However, fluorescence signals were enriched at the plasma membrane and septa.

Thus, BcPde1 and BcPde2 were found to be localized mainly in unknown, mobile vesicles, and to the plasma membrane and septa, respectively, suggesting a spatial distribution and function of both proteins.

**Loss of bcpe2 activates bcpe1 transcription**

We examined the transcript levels of both PDE-encoding genes in the wild type and the deletion mutants by qRT-PCR under in vitro (CM agar with a cellophane overlay) and in planta conditions (Fig. 5A). For in planta expression studies infected bean leaves were harvested at equal infection stages (primary lesions). On CM agar, the transcript level of bcpe1 was significantly increased about twofold in the bcpe2 knock-out strain in both conditions, while bcpe2 was similarly expressed in both, wild type and the Δbcpe1 mutant.

To investigate whether the expression of the PDE-encoding genes is affected in mutants with altered cAMP levels, we further measured the transcript levels in the Δbac (reduced cAMP levels) and the Δbcpe1 (elevated cAMP level) mutants. RNA of both mutants and the wild type was extracted from mycelia grown on CM agar (Fig. 5B). Interestingly, the bcpe1 expression was drastically increased in the bcpe1 mutant, similarly to the situation in the Δbcpe2 mutant (Fig. 5A). In contrast, no alteration of bcpe1 and bcpe2 expression levels was observed in the Δbac mutant.

**Bcpe1-expression rescues bcpe2 deletion phenotype**

As both proteins are intended to possess PDE activity, we expected them to have redundant functions. To show whether Bc-Pde1 is able to take over some functions of Bc-Pde2, we transformed the gfp-bcpe1 construct (bcpe1 is driven by the constitutive olc promoter) into the Δbcpe2 mutant. The complemented strains (Δbcpe2+gfp-bcpe1) displayed a wild-type-like phenotype regarding vegetative growth, virulence and sclerotia formation, indicating that the GFP-BcPde1 protein was functional and able to rescue the loss of BcPde2 (data not shown).

Microscopic analyses revealed a similar fluorescence signal of GFP-Bc-Pde1 (cytosol and mobile vesicle structures) as in the wild type (WT+gfp-bcpe1) or Δbcpe1+gfp-bcpe1 genomic backgrounds (data not shown).

**Loss of BcPde2 results in reduced intracellular cAMP levels**

As the phenotype of the bcpe2 mutant deletion mutant might be due to altered cAMP levels, the cytosolic cAMP contents were quantified in vegetatively growing mycelium (Fig. 6A). Intracellular cAMP levels of the bcpe1 deletion strains were similar to those of the wild type, while Δbcpe2 and ΔΔbcpe1/2 mutants showed slightly decreased cAMP levels. The Δbac and Δbcpe1/2 strains served as controls exhibiting significantly decreased and increased cAMP contents, respectively (data not shown), as it was previously described [11]. To show, if the unexpected decrease of cAMP levels in the Δbcpe2 mutants affect the PKA activity, we performed a PKA activity assay. The test was performed under different conditions with mycelia grown on complete CM agar or synthetic GB5 agar. In both cases, no PKA activity was visible in the Δbcpe2 and ΔΔbcpe1/2 mutant strains, while the wild-type
and the Δbcpde1 strains displayed similar PKA activities (Fig. 6B). Surprisingly, the Δbac mutant showed significantly elevated PKA activity compared to the wild type independently of the growth conditions.

Discussion

In *B. cinerea*, the Bac/cAMP/BcPka1-mediated signaling pathway was shown to play an important but not essential role in
vegetative growth, differentiation and virulence [5,8,10–12]. However, there are still lots of unanswered questions regarding the regulation of cAMP levels in this fungus. Previously, we have shown that B. cinerea has some specific differences considering the cAMP signaling pathway compared to other fungi [11]. To further investigate how intracellular cAMP levels are regulated, we have previously shown that the regulation of cAMP levels in this fungus. Previously, we have investigated how intracellular cAMP levels are regulated, we have shown that BcPde2, the homolog to the yeast high-affinity PDEs was different. Our suggestion that BcPde1 partially fulfills the regulation of cAMP levels in this fungus. Previously, we have investigated how intracellular cAMP levels are regulated, we have shown that BcPde2, the homolog to the yeast high-affinity PDEs was different. Our suggestion that BcPde1 partially fulfills its role during infection. The data presented in this work clearly show that BcPde2 is involved in both, the regulation of nutrient- and hydrophobicity-induced germination. The fact that hydrophobicity-induced germination is heavily affected, suggests that BcPde2 is probably involved in surface sensing, similarly to PdeH in M. oryzae which regulates surface sensing and guides germ tube growth during the early infection stages [20].

BcPde2 controls differentiation in B. cinerea
Similarly to M. oryzae [20], BcPde1 is dispensable for vegetative growth, while BcPde2 is essential for colony extension and regulation of differentiation processes, such as conidiogenesis in light and sclerotia formation in darkness. Deletion of bcpde1 resulted in light-independent conidiation and total loss of sclerotia formation in constant darkness indicating that BcPde2 plays an important role in light response and light-dependent differentiation processes. In contrast, the N. crassa pde2 deletion mutant was completely blocked in conidial morphogenesis [19], while loss of PdeH in M. oryzae led to enhanced conidiation [20]. However, double deletion of both PDEs in M. oryzae resulted in total block of conidiation similarly to the B. cinerea ΔΔbcpde1/2 strain. As the conidia of the ΔΔbcpde2 and ΔΔbcpde1/2 strains also varied in shape and size from that of the wild type, we conclude that BcPde2 is probably involved in cell wall and membrane stabilization as it was shown for Pde2 in Candida albicans [33]. However, in contrast to C. albicans, no differences were observed in shape or thickness of the cell wall and septa of the B. cinerea mutants stained with Calcofluor white (data not shown).

In B. cinerea, germination can be induced either by the presence of nutrients (on hydrophilic surfaces) or by hydrophobic surfaces (in the absence of nutrients), respectively [9]. Under the first condition, conidia develop thick and fast growing germ tubes, while small nose-like germ tubes are formed on hydrophobic surfaces. While nutrient-induced germination is mainly mediated via the cAMP signaling pathway through activation of Bcg3 and Bac, hydrophobicity-induced germination is regulated by the MAP kinase Bmp1 in a CAMP-independent manner [9,11]. Here, we clearly demonstrate that BcPde2 is involved in both, the regulation of nutrient- and hydrophobicity-induced germination. The fact that hydrophobicity-induced germination is heavily affected, suggests that BcPde2 is probably involved in surface sensing, similarly to PdeH in M. oryzae which regulates surface sensing and guides germ tube growth during the early infection stages [20].

BcPde2 plays an important role during infection
The data presented in this work clearly show that BcPde2 is required for full virulence. While the ΔΔbcpde2 mutant seems to penetrate in a wild-type-like manner, invasive growth is affected in onion epidermis cells as well as on living bean plants. Delayed lesion formation and retardation of the whole infection process has already been demonstrated for other B. cinerea strains with mutations in the cAMP pathway, such as Δbac, ΔbcpkaR and Δbcpka1 [11,12].

Furthermore, in the bcpde2 deletion strains, conidiogenesis was not only abolished under in vitro conditions but also completely inhibited during infection as it was also shown for the Δbac mutant probably due to the reduced cAMP level in both mutants [11,12]. In M. oryzae PdeH was shown to be needed during two critical steps of pathogenesis: infection-structure (appressoria) formation and invasive growth. However, in contrast to B. cinerea bcpde2 deletion mutants, deletion of pdeH resulted in elevated and not reduced cAMP levels [20].

In planta expression studies of both PDE-encoding genes in the wild type revealed expression of both genes already in early infection states (6 to 24 hpi) as well as during primary lesion formation (data not shown).
BcPde2 is responsible for maintenance of normal cAMP levels

Our speculation was that all the altered characteristics of the bcpde2 mutants are due to an abnormal intracellular cAMP level as the mutants displayed an intermediate phenotype between the Δbac (low cAMP levels) and the Δbcpka1 (high cAMP levels) mutant. Unexpectedly, the cAMP content was not increased in any bcpde deletion strain in contrast to other fungi. We observed wild-type-like concentrations for the Δbcpde1 strain, but reduced levels for Δbcpde2 and ΔΔbcpde1/2 mutants in three independent cAMP measurement approaches. This might be caused by an agonist-induced feedback loop in the cAMP signaling cascade due to a deregulated PKA activity in these mutants. A PKA activity assay with B. cinerea strains revealed no PKA activity at all in the Δbcpde2 and ΔΔbcpde1/2 strains. Thus, deletion of bcpde2 resulted in a decrease of the intracellular cAMP level and in accordance to this, absence of PKA activity. These contrary effects on cAMP level compared to the pdeH mutant in M. oryzae cannot be explained so far.

Quantification of the bcpka1 transcript level by qRT-PCR (data not shown) revealed no differences between the Δbcpde1, Δbcpde2, ΔΔbcpde1/2 and Δbac strains compared to the wild type indicating that the reduced PKA activity in bcpde2 mutants cannot be explained by reduced transcript levels. An interesting result was the significantly increased PKA activity in the Δbac mutant which was in contrary to our expectation for a mutant with very low cAMP level (Fig. 6B).

In S. cerevisiae and C. neoformans, Pde1 is a target of PKA phosphorylation and is therefore involved in PKA-mediated feedback-regulation of cAMP level [34]. Sequence analyses distinguished a putative PKA-phosphorylation site at a threonine residue (KRGT363) in the BcPde1 protein sequence according to predicted phosphorylation sites for yeast PKA substrates [35]. However, a split-ubiquitin based yeast-two hybrid assay with BcPka1 and BcPde1/BcPde2 did not reveal any indication for an interaction between BcPka1 and BcPde1 or BcPka1 and BcPde2 (data not shown). Yet, we cannot exclude that maybe the second subunit BcPka2 is crucial for the proposed feedback regulation. Furthermore, Hicks et al. [34] proposed that in addition to the feedback regulation via cAMP degradation another system involves the cAMP production through repression of the AC. This potential dual regulation for maintenance of transient cAMP spikes is still not well understood in fungi, in general. Therefore, future studies may focus on investigations in the temporal regulation of the cytosolic cAMP concentration in B. cinerea.

Localization studies revealed putative compartmentalization of cAMP response

Localization studies on the two PDE proteins revealed first hints for a compartmentalized cAMP distribution in B. cinerea, as shown

Figure 6. Quantification of cytosolic cAMP contents and PKA activity assay. A: Intracellular cAMP was determined in three day old mycelia cultures derived from GB5+ cellophane cultures. Two independent primary transformants were tested for each deletion strain. Represented are mean values and standard deviations of technical duplicates in one experiment. Relative cAMP contents are indicated in numbers compared to the WT (100%). Three independent experiments revealed approximately analogue results. B: PKA activities from three day old mycelium cultures grown on CM+ or GB5+ cellophane agar plates were monitored by gel electrophoresis according to Schumacher et al. [11]. The phosphorylated PKA substrate moved toward the anode (bottom). Samples with activator (+) contained cAMP.

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for mammals [36]. This allows spatially distinct pools of PKA to be differentially activated. The basis for this is that PKA isoforms are anchored at specific intracellular sites by A-kinase anchoring proteins (AKAPs) [37,30]. Therefore, discrete PKA populations could respond to gradients of cAMP and by this modify the activities of localized target proteins. The source for these gradients depends largely on PDE activities which can control intracellular diffusion of cAMP by their localization to specific subcellular compartments. PDE localization occurs through different mechanisms involving direct binding to membrane lipids or protein-protein interactions [36,39].

We expressed gfp fusion constructs of both B. cinerea PDEs to gain information about their intracellular localization and proposed function. BcPde2 was clearly shown to be distributed in the cytosol, but also accumulates in the plasma membrane. The GFP-BcPde2 fusion protein fully restored wild-type-like growth in the cytosol, but also accumulates in the plasma membrane. The GFP-BcPde1 fusion protein fully restored wild-type-like growth in the cytosol, but also accumulates in the plasma membrane. The GFP-BcPde2 fusion protein fully restored wild-type-like growth in the cytosol, but also accumulates in the plasma membrane.

Localization studies of both PDEs (PdeH and PdeL) in M. oryzae showed cytosolic distribution of PdeH with a dynamical association to the plasma membrane and vesicular compartments, whereas PdeL seems to be localized predominantly in the nucleus [20]. These data demonstrate a differential compartmentalization of both PDEs. Compartmentalized cAMP signaling may be important for polarized growth of germ tubes, appressoria and infection hyphae of M. oryzae. In this kind of cells, relevant changes in cAMP levels could be controlled in space and time and infection hyphae of B. cinerea of wild-type-like phenotype indicating that both PDEs have at least expressed promoter into the anchored within subcellular compartments [20].

Interestingly, inactivation of BcPde2 causes a transcriptional up-regulation of bcpde1 (Fig. 5), and BcPde1 is able to restore wild-type-like growth and differentiation of the bcpde2 mutant, when the bcpde1 gene is overexpressed in the bcpde2 background indicating that both PDEs are functionally related. Furthermore, bcpka1 deletion caused an increased bcpde1 transcript level (9).

Botrytis-specific differences in the cAMP pathway have already been observed before. As mentioned above, Δbcpka1 and ΔbcpkaR strains displayed almost identical phenotypes regarding the elevated cAMP levels, and ΔbcpkaR deletion did not result in a strain with a constitutively active PKA [11,12]. Furthermore, the Δbcpka1 mutant differs from Δlac regarding light-regulated conidiation and in planta conidiation suggesting that additional cAMP binding proteins must exist. Recently, we deleted two genes encoding proteins with highly conserved cAMP-binding domains which could act in a BcPka1-independent cAMP-dependent signaling pathway (unpublished data). The functional analysis of these two mutant strains is in progress. Furthermore, nothing is known so far, about an influence of compartmentalization of the cAMP signal in filamentous fungi. Of course, distinct spikes of the second messenger can lead to various effects dependent on the intracellular localization.

Supporting Information

Figure S1 Gene replacement strategies and Southern blot analyses of B. cinerea strains Δbcpde1, Δbcpde2 and Δbcpde1/2. A: Strategies for generation of Δbcpde1 (top), Δbcpde2 (bottom) and Δbcpde1/2 (middle) strains. All primers used for cloning of the replacement vectors and the diagnostic PCR analyses for proving homologous integration are indicated with numbers (1–25) and further described in the materials and methods section. Introns are depicted as white bars in arrows illustrating the genes. Restriction sites for Southern blot analyses are depicted. Top: Physical maps of bcpde1 wild type (WT: B05.10) and Δbcpde1 locus. The wild type B. cinerea B05.10 was transformed with the bcpde1 knock-out fragment (consisting of both flanking regions and the hphR resistance cassette derived from vector pCSN44) resulting in Δbcpde1 mutants via homologous recombination and insertion of hphR. Bottom: Physical maps of bcpde2 in
WT: B05.10 and replacement of bcpde2 by hphR resistance cassette yielding Δbcpde2. Middle: Replacement of bcpde2 by the natR resistance cassette in strain Δbcpde1 resulted in ΔΔbcpde1/2 mutants. Three independent mutants were tested for additional ectopic integrations of the replacement fragments. The wild type (WT), Δbcpde1 T1, T2, T3, all Δbcpde2 mutants T1, T6, T19 and ΔΔbcpde1/2 strains T5 and T6 displayed each just one hybridizing fragment with the expected size after hybridization with the probe (see 1A and materials and methods section). (TIF)

**Figure S2** Infection of living beans and analyses of in planta development. Primary leaves were inoculated with droplets of conidial suspensions of the indicated strains. The gfp fusion constructs were able to restore the wild-type phenotype of the Δbcpde2 mutant strain. Images were taken after 2 to 7 days post infection (dpi).

(TIF)

**Table S1** All primers used in this study.

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: KH BT. Performed the experiments: KH BB MK. Analyzed the data: KH. Contributed reagents/materials/analysis tools: BT. Wrote the paper: KH BT.

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**Figure 7. Schematic model of the postulated cAMP signaling network in B. cinerea.** Activation of the adenylate cyclase Bac through the Gx subunits Bcg1 and Bcg3 leads to cAMP synthesis, which is suggested to further transduce incoming signals to the protein kinase A, composed of the regulatory subunit BcPkaR and the major catalytic subunit BcPka1. Different mutations in the mentioned signaling components were shown to have impact on the listed differentiation processes [11,12]. Characterization of the phosphodiesterase BcPde2 led to this model, demonstrating positive or negative effects of this protein on cAMP signaling. An interconnection with BcPde1 on signal transduction and transcriptional regulation was further shown. Numbers in brackets are further described in the text.

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