

Complementation of the *Magnaporthe grisea* Δ *cpkA* Mutation by the *Blumeria graminis* PKA-c Gene: Functional Genetic Analysis of an Obligate Plant Pathogen

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Obligate plant-pathogenic fungi have proved extremely difficult to characterize with molecular genetics because they cannot be cultured away from host plants and only can be manipulated experimentally in limited circumstances. Previously, in order to characterize signal transduction processes during infection-related development of the powdery mildew fungus *Blumeria graminis* (syn. *Erysiphe graminis*) f. sp. *hordei*, we described a gene similar to the catalytic subunit of cyclic AMP-dependent protein kinase A (here renamed *Bka1*). Functional characterization of this gene has been achieved by expression in a Δ *cpkA* mutant of the nonobligate pathogen *Magnaporthe grisea*. This nonpathogenic *M. grisea* Δ *cpkA* mutant displays delayed and incomplete appressorium development, suggesting a role for PKA-c in the signal transduction processes that control the maturation of infection cells. Transformation of the Δ *cpkA* mutant with the mildew *Bka1* open reading frame, controlled by the *M. grisea* *MPGI* promoter, restored pathogenicity and appressorium maturation kinetics. The results provide, to our knowledge, the first functional genetic analysis of pathogenicity in an obligate pathogen and highlight the remarkable conservation of signaling components regulating infection-related development in pathogenic fungi.

Molecular genetic analysis of phytopathogenic fungi is revealing the presence of conserved signal transduction pathways that regulate infection-related development and the production of plant disease symptoms (Lev et al. 1999; Xu and Hamer 1996). These studies have been restricted to species that are tractable to genetic manipulation, leaving the vast majority of pathogenic species, including many significant crop disease-causing fungi, unexplored and poorly understood. Among these species are important pathogenic fungi that live as obligate pathogens. These species, including powdery mil-

dew and rust fungi, cannot be cultured or maintained away from living plants and, therefore, present a very significant challenge to biological investigation. Obligate pathogens are arguably the most specialized phytopathogenic species; they are able to survive and proliferate only in living plant tissue; cause very limited systemic damage to plants; often have very restricted host ranges and conform to the gene-for-gene hypothesis; and produce persistent, polycyclic, and damaging infections (Giese et al. 1997).

Blumeria (syn. *Erysiphe*) *graminis* f. sp. *hordei* is the causal organism of barley (*Hordeum vulgare*) powdery mildew disease and is a prime example of an obligate pathogen that has been difficult to characterize at the molecular genetic level, possessing fascinating developmental biology. Losses resulting from this disease can be as high as 25% of the barley harvest (Parry 1990), ranking the fungus as one of the most serious diseases of temperate cereals. *B. graminis* infections begin when asexual spores (conidia) germinate and penetrate the host epidermis by undergoing a well-described developmental pathway (Kunoh 1982). Within 15 to 30 min of landing on the host surface, *B. graminis* conidia develop a primary germ tube. Surface features are then perceived by the germ tube which, if appropriate, lead to the emergence of an appressorial germ tube after 4 to 6 h. The appressorial germ tube then elongates, undergoes cell division, and forms an appressorium. The penetration of the host cell by the appressorium is believed to involve cell wall-degrading enzymes and generation of turgor pressure (Francis et al. 1996; Frick and Wolf 1994). The process is markedly different from that of most appressorium-forming fungi because it does not require free water and occurs on dry leaves. The complexity of appressorium morphogenesis and its dependence on the perception of the environment implies involvement of specialized signal transduction pathways.

So far, molecular genetic analysis of *B. graminis* has been limited by its obligate nature, but the advent of genome-level analysis has provided new means by which its biology can be explored. Recently, a large number of *B. graminis* expressed sequence tag (EST) sequences has been generated (Thomas et

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al. 2001) from germinating *B. graminis* conidia, and classification of these genes by means of similarity has revealed some that are likely to encode signal transduction components. The presence of these expressed genes is consistent with the operation of particular signaling pathways, although proving the involvement of individual genes and determining the details of their interactions requires the development of novel methods and approaches. One of the *B. graminis* ESTs shows sequence similarity to the cyclic AMP (cAMP)-dependent protein kinase A catalytic subunit (PKA-c; here renamed *Bka1*) (Hall et al. 1999), suggesting a role for a cAMP-dependent signaling pathway in *B. graminis* appressorium development.

Enzymatic determination of PKA activity changes in germinating conidia, measurement of cAMP concentration fluxes, and the effects of spore treatment with cAMP and analogs have together provided evidence that the cAMP-signaling pathway plays a key role in initiating germ tube emergence in *B. graminis* (Kinane et al. 2000). cAMP levels and PKA-c activity have been shown to have a biphasic pattern during conidial germination. cAMP accumulates after 15 min and 4 h, prior to the emergence of the primary and appressorial germ tubes, respectively.

The cAMP signal transduction pathway has been implicated in developmental programs of a small number of nonobligate fungal pathogens, including *Ustilago maydis*, the corn smut fungus, in which mutants defective in PKA-c fail to produce symptomatic galls in host plants and are defective in cytokinesis (Gold et al. 1994). In *Colletotrichum trifolii*, PKA-c may be involved in germination and appressorial formation but more likely is involved in host penetration (Yang and Dickman 1997, 1999a, 1999b). The organism in which most studies have been carried out is the rice blast fungus *Magnaporthe grisea*, an ascomycete fungus pathogenic on rice and many other cereals, including barley (Kang et al. 1999). *M. grisea* germ tubes form appressoria that penetrate the host cell with mechanical force (de Jong et al. 1997; Howard et al. 1991). cAMP is required for appressorium morphogenesis because $\Delta mac1$ mutants, which are deficient in

adenylate cyclase activity, fail to make appressoria and are nonpathogenic (Choi and Dean 1997). This mutation is unstable in certain strains as a result of bypass suppressor mutations in the *SUM1* gene encoding the regulatory subunit of PKA (PKA-r) (Adachi and Hamer 1998). Furthermore, the addition of cAMP also can induce appressoria to form on normally noninductive hydrophilic surfaces or even from hyphal tips (Lee and Dean 1993). Targeted disruption of the *CPKA* gene encoding the PKA-c subunit gives rise to mutants that show a significant delay in appressorium formation, forming small nonfunctional cells (Mitchell and Dean 1995; Xu et al. 1997). The inability of $\Delta cpkA$ appressoria to bring about plant infection may be related to their abnormal carbohydrate and lipid mobilization in these cells and the consequent effects on turgor generation (Thines et al. 2000).

In this report, we describe the results of expressing the *B. graminis* gene *Bka1* in a $\Delta cpkA$ mutant of *M. grisea*. Expression of *Bka1* complemented the dysfunction in appressorium morphogenesis and partially restored its virulence phenotype. This provides, to our knowledge, the first functional gene analysis of *B. graminis* and shows that a gene from an obligate pathogenic species is able to act as a pathogenicity determinant in *M. grisea*.

RESULTS

Functional complementation of a *M. grisea* $\Delta cpkA$ mutant.

In order to express the *B. graminis* *Bka1* gene in *M. grisea*, two transformation vectors were constructed (Fig. 1A and B). Both constructs utilized a 1.28-kb *PstI*-*NcoI* fragment of the *MPG1* promoter, which has been shown previously to lead to high gene expression during conidial germination and appressorium development (Kershaw et al. 1998; Talbot et al. 1993). The *MPG1* promoter fragment was subcloned upstream of a 1.57-kb full-length genomic *Bka1* sequence in pLB2 and of a 1.4-kb *Bka1* cDNA sequence in pLB3. Both constructs were made in pCB1625 (Sweigard et al. 1998), comprising the *bar*

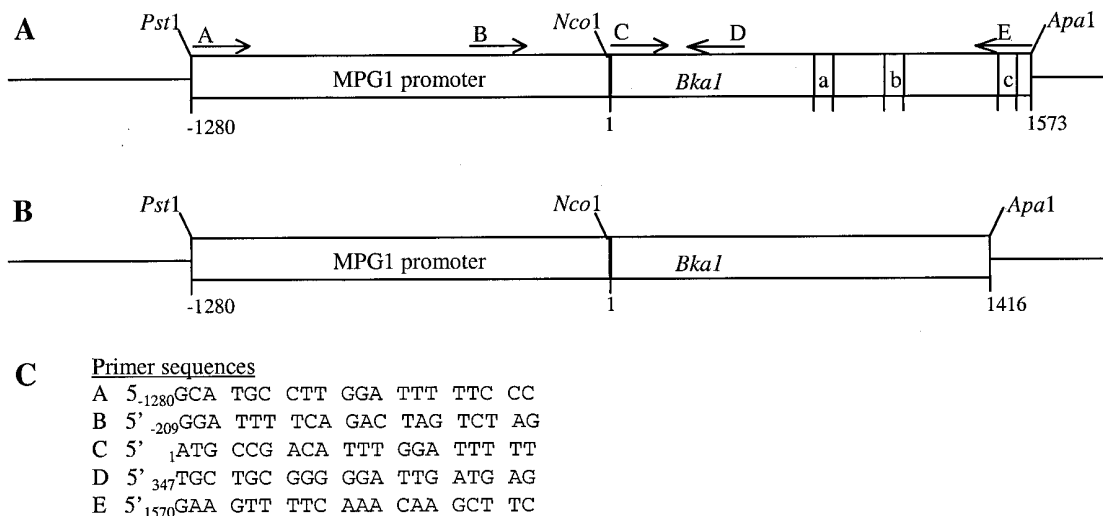


Fig. 1. Constructs used for transformation of *Magnaporthe grisea*. **A**, Plasmid pLB2 containing the genomic *Bka1* controlled by the *MPG1* promoter. **B**, Plasmid pLB3 containing *Bka1* without introns, which also are controlled by the *MPG1* promoter. **C**, Primer sequences, shown in **A**, indicated by arrows A to E.

selectable marker, which bestows bialaphos resistance in *M. grisea*, and were introduced into *M. grisea* strain DF51 (Xu et al. 1997), a *ΔcpkA:HPH* transformant derived from parent strain 4091-5-8 (Valent et al. 1986) with standard DNA-mediated

transformation (Talbot et al. 1993). After 6 days, bialaphos-resistant colonies were picked and transferred to new Basta-containing plates. A total of 30 and 28 positive transformants was generated with pLB2 and pLB3, respectively. A number

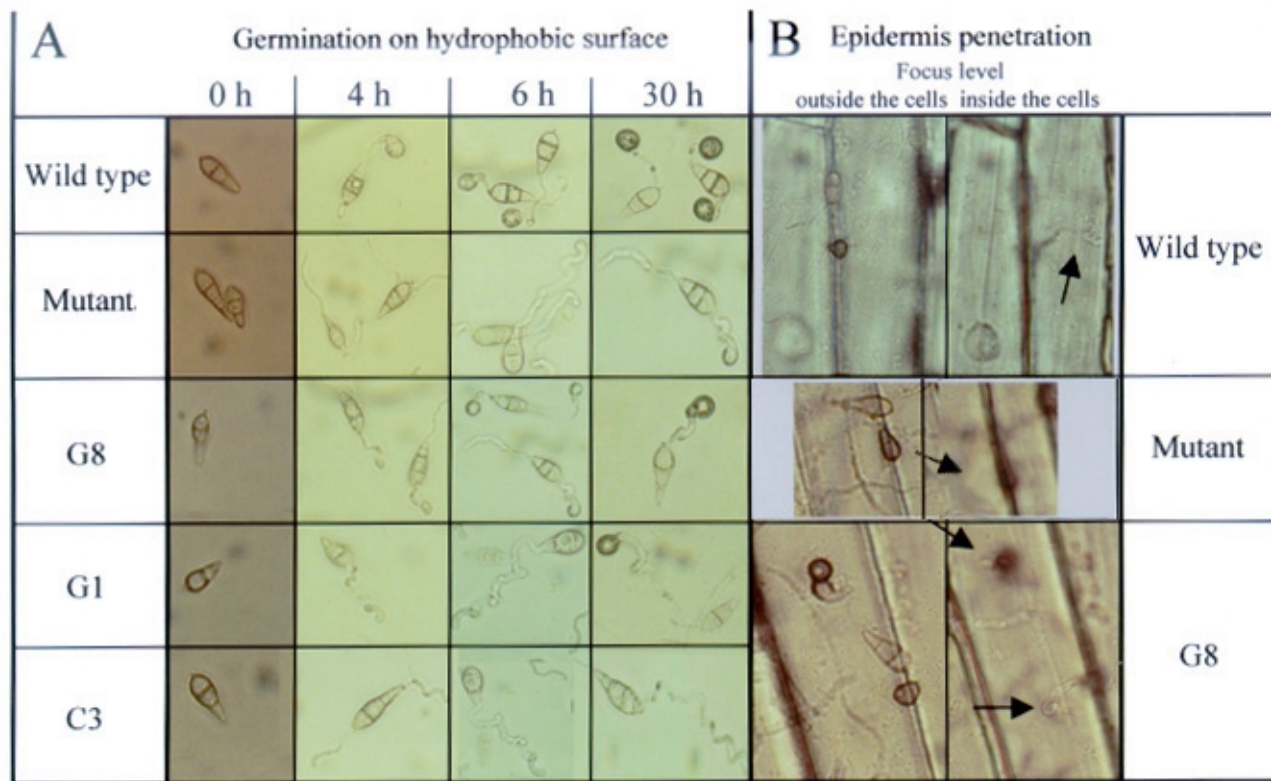


Fig. 2. Microscopic analysis of *Magnaporthe grisea* conidia. **A**, Germination of conidia on hydrophobic, plastic coverslips. Conidia were germinated in water droplets and photographed after 0, 4, 6, and 30 h. **B**, Appressorial penetration of leek epidermis 30 h after inoculation. Arrows indicate penetration hyphae (wild type and G8) or where the hyphae is expected (mutant).

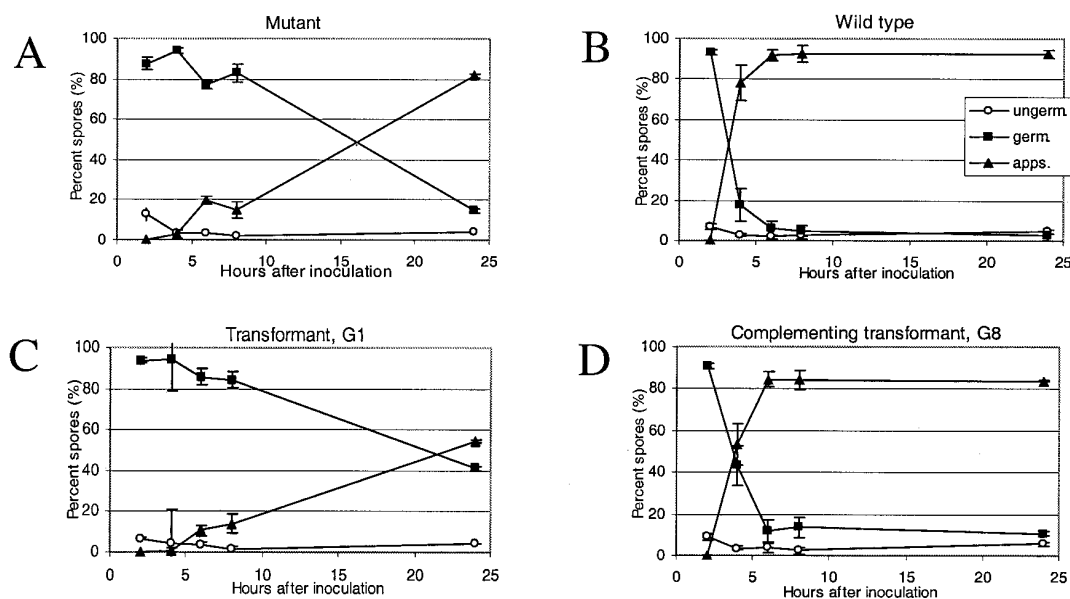


Fig. 3. Germination of *Magnaporthe grisea* conidia. Spores (2×10^3) of each strain were placed on plastic coverslips and observed 2, 4, 6, 8, and 24 h after inoculation. The number of spores that remained ungerminated (ungerm), germinated (germ), and developed appressoria (apps) was counted. **A**, Mutant DF51. **B**, Wild-type 4091-5-8. **C**, Transformant G1. **D**, Complementing transformant G8. Error bars indicate 95% confidence interval.

of control strains transformed with pCB1625 also were recovered. The transformants were all stable, as judged by their ability to grow on bialaphos plates after 7 to 8 weeks of sub-culturing on normal complete medium (CM) plates.

Infection-related development of *MPG1(p)::Bka1::Bar:: Δ cpkA::HPH* transformants.

The Δ cpkA mutants of *M. grisea* are delayed substantially in germ tube differentiation and produce small, nonfunctional appressoria (Mitchell and Dean 1995; Xu et al. 1997). In order to determine whether these phenotypes could be complemented by expression of the *B. graminis Bka1* gene, conidia of bialaphos-resistant transformants were allowed to germinate on hydrophobic plastic surfaces. Microscopic analysis showed that the timing and extent of germination and appressorium formation varied between the strains selected (Fig. 2A). Three bialaphos transformants, G1 and G8 (made with the genomic DNA vector pLB2) and C3 (made with the cDNA vector pLB3), were selected and examined. After 4 h, hooking of the wild-type (4091-5-8) germ tube began, and appressorium formation started shortly thereafter (Fig. 2A). At 6 h, most appres-

soria were fully formed. Conidia of the Δ cpkA mutant DF51 also had germinated by 4 h, but after 6 h, the germ tubes had only just started to hook, and it was not until after 30 h that all appressoria had formed. Conidial development in G8 was similar to wild-type strain 4091-5-8, whereas G1 developed similar to the Δ cpkA mutant (Fig. 2A). Another transformant, C3, had very slow and aberrant conidial development. It may be that the vector integrated into an important gene in this transformant.

To determine whether these morphological differences were reproducible, quantitative microscopic analysis was performed. Conidia (2,000) of each transformant were tracked during germination under appressorium-inducing conditions (Fig. 3). Wild-type strain 4091-5-8 (Fig. 3B) and transformant G8 (Fig. 3D) formed the maximum number of appressoria after 6 h, and 93% of the wild-type spores formed appressoria compared with 83% of those from G8. Development in the Δ cpkA mutant DF51 (Fig. 3A) and transformant G1 (Fig. 3C) was much slower. After 25 h, only 81% of the Δ cpkA spores had formed appressoria in the mutant and as few as 58% in the G1 transformant.

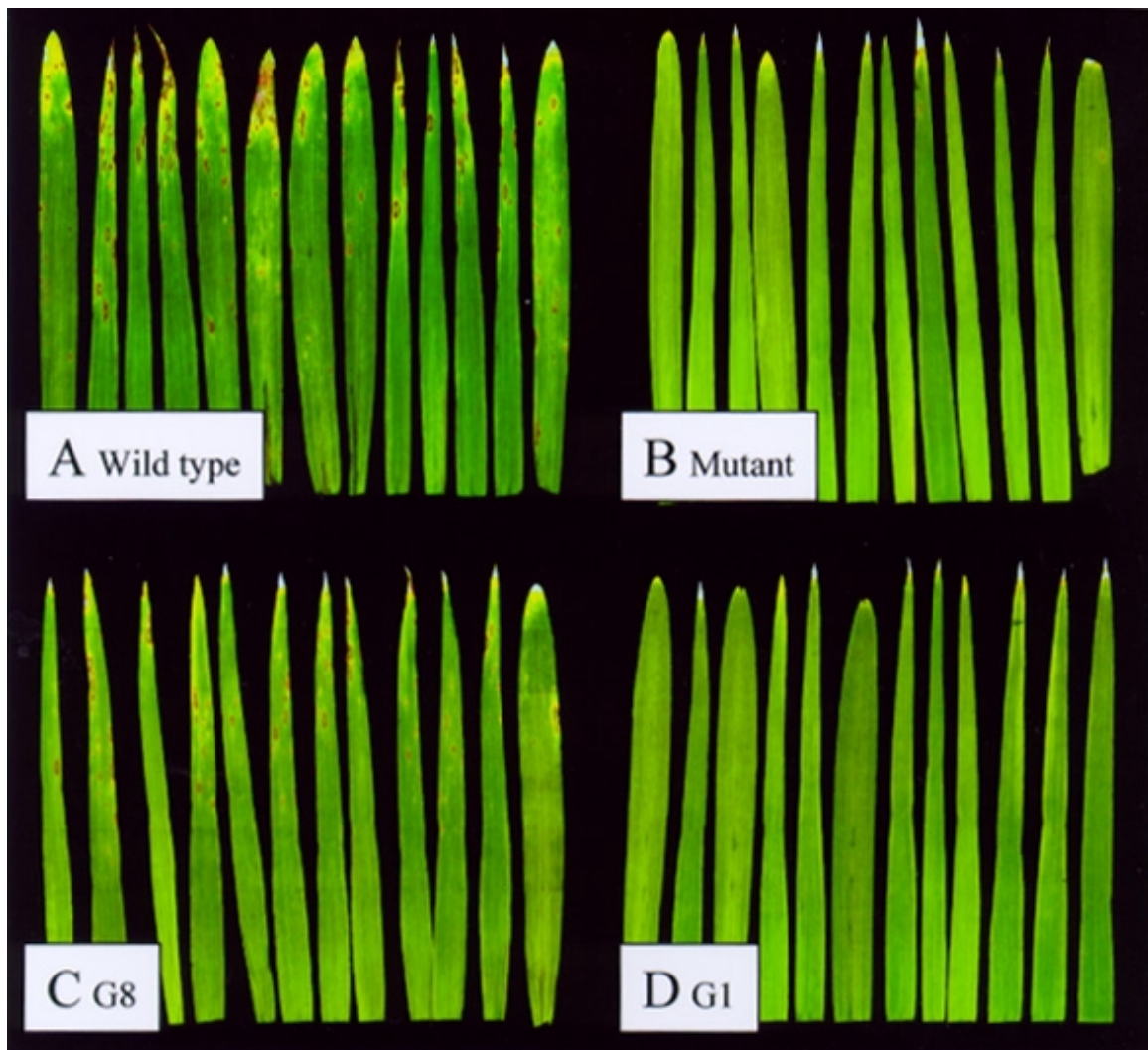


Fig. 4. Pathogenicity assay of *Magnaporthe grisea* spores on barley leaves. Spores were sprayed onto intact plants and photographed 5 days later. The wild type and the G8 transformant infected the leaves, whereas the mutant and the G1 transformant did not form lesions.

Appressorium-mediated cuticle penetration.

The $\Delta cpkA$ mutation results in appressoria that are non-functional and incapable of penetrating plant cuticles (Xu et al. 1997). The ability of appressoria to penetrate plant cuticles was, therefore, assessed on leek epidermis in an adaptation of the methods described by Chida and Sisler (1987) (Fig. 2B). Conidia were inoculated onto the epidermis in a drop of water and analyzed microscopically 30 h later. Wild-type strain 4091-5-8 and transformant G8 produced penetration pegs that had breached the cuticle and proliferated in the epidermis, and penetration hyphae could be seen clearly within the cells (Fig. 2B). In contrast, the $\Delta cpkA$ mutant DF51 had not produced penetration pegs, even after 30 h.

Pathogenicity of *M. grisea* transformants.

Deficiencies in appressorium development and function mean that $\Delta cpkA$ mutants are completely nonpathogenic on barley and rice (Mitchell and Dean 1995; Xu et al. 1997). In order to determine whether expression of *Bka1* could complement this phenotype, pathogenicity to susceptible barley cultivar Golden Promise was assessed. Spores from all of the transformants, $\Delta cpkA$ mutant DF51, and wild-type 4091-5-8 were sprayed onto 1-week-old seedlings, and the disease was allowed to progress for 5 days. The leaves were then examined for lesions (Fig. 4). As expected, the wild-type strain infected at a high frequency and mutant DF51 produced no lesions. G1 also produced no symptoms. Transformant G8, containing vector pLB2, infected barley, and the lesions were normal in appearance and size (Fig. 4). G8 produced 4.6 ± 2.2 lesions per leaf, whereas the wild type produced 10.2 ± 1.2 (Table 1). Fungal mycelium emanating from the disease lesions of the G8 and wild-type infections was tested for resistance to Basta (discussed below). Mycelium from the G8 lesions was Basta resistant, whereas the mycelium from the wild-type infections was sensitive, as expected.

The in vitro growth rate and sporulation of the wild type, mutant DF51, and transformants G8 and G1 also were measured (Table 1). For both parameters, the mutant was significantly impaired compared with the wild type. The wild type grew 7.3 ± 0.3 mm per day, whereas the mutant, G8, and G1 all grew 6.6 to 6.8 ± 0.3 mm per day. This difference is statistically significant ($P < 0.001$). The production of conidia varied as well. The wild type produced 5.3×10^7 spores per plate and the mutant produced 2.3×10^7 spores per plate. G8 (3.7×10^7) and G1 (1.5×10^7) produced similar numbers of spores as the mutant. A Mann-Whitney *U* test showed that the production of wild-type spores was significantly higher than in other strains

Table 1. Phenotypic analysis of *Magnaporthe grisea* strains, including the wild type, mutant, G8, and G1^a

Strain	Wild type	Mutant	G8	G1
Pathogenicity ^b	10.2 ± 1.2	$0.0 \pm 0.0^{**}$	$4.6 \pm 2.2^{**}$	$0.0 \pm 0.0^{**}$
Growth rate ^c	7.3 ± 0.3	$6.6 \pm 0.3^*$	$6.8 \pm 0.2^*$	$6.7 \pm 0.3^*$
Spore production ^d	5.3 ± 0.6	$2.3 \pm 0.6^{**}$	$3.7 \pm 0.6^{**}$	$1.5 \pm 0.7^{**}$

^a * and ** Indicate statistical difference from the wild type at $P < 0.05$ and $P < 0.001$, respectively. The Kruskal-Wallis test was used for growth rate and the Mann-Whitney *U* tests were used for spore production (Fowler et al. 1998).

^b Lesions per plant. Eighty plants were infected and analyzed.

^c Radial growth rate: mm/day.

^d Spores produced per plate in 6 days ($\times 10^7$ spores per ml).

($P < 0.05$). It also showed that G8 produced significantly more spores than G1 and the mutant ($P < 0.001$). Together, the expression of *Bka1* in transformant G8 appears to have resulted in at least partial complementation of these secondary $\Delta cpkA$ mutant phenotypes.

MPG1(p)::PKA-c is intact and transcribed only in the active transformant.

The complementation of the mutant phenotype in transformant G8 suggests that the introduced *Bka1* gene encoded PKA activity. It was surprising, however, that only 1 out of 58 transformants tested displayed complementation. Polymerase chain reaction (PCR) and Southern blotting were therefore used to determine whether intact copies of the transgene had integrated into the *M. grisea* genome. Genomic DNA was recovered from G8 and five inactive transformants (three cDNA and two genomic DNA transformants, selected randomly), and PCR was performed with primers from within the *Bka1* and *MPG1* sequences (Fig. 5). Primers C and E from *Bka1* (Fig. 1) amplified bands of 1,570 bp were taken from genomic clone transformants G8, G3, and G1 (Fig. 5, lanes 4 to 6, respectively) and of 1,415 bp were taken from cDNA transformants C1, C2, and C3 (Fig. 5, lanes 7 to 9, respectively). These amplicons were of identical size to those produced from the source plasmids (Fig. 5, lanes 10 and 11). The predicted 2.7- or 2.9-kb bands corresponding to *MPG1(p)::Bka1* were shown, by Southern blotting, to be absent in strains C1, C2, C3, and G3, but present in G1 and G8 (blot not shown). To discriminate the integration events in G1 and G8, PCR with additional primers was performed. Primers A and D and B and D produced the expected size amplicons in G1 and G8. In con-

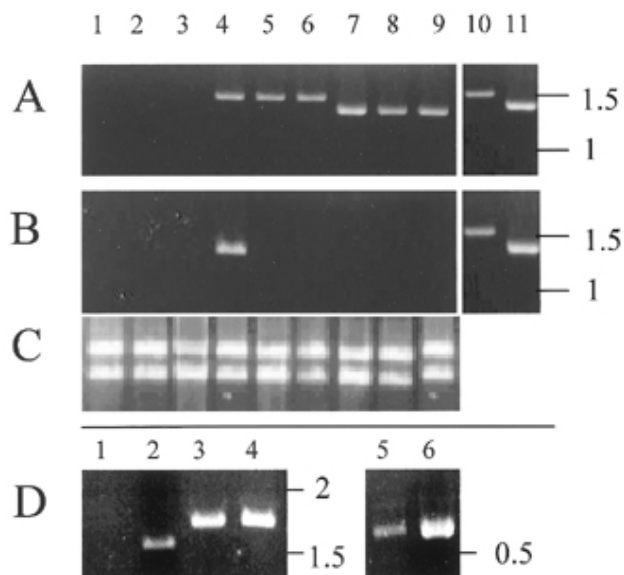


Fig. 5. Polymerase chain reaction (PCR) and reverse-transcription (RT)-PCR assays of the presence and expression of *Bka1* in *Magnaporthe grisea* (primer locations in Fig. 1). **A to C**, Lanes: 1, wild type; 2, mutant; 3, empty vector; 4, G8; 5, G3; 6, G1; 7, C3; 8, C2; 9, C1; 10, genomic DNA plasmid control; and 11, cDNA plasmid control. **D**, Lanes 1, 3, and 5, G1; and 2, 4, and 6, G8. **A**, PCR with primers C and E. **B**, RT-PCR with primers C and E. **C**, RNA used for RT-PCR in **B**. **D**, PCR with primers A to D (lanes 1 and 2), B to E (lanes 3 and 4), and B to D (lanes 5 and 6).

trast, primers B and E produced a fragment only from the active transformant G8 (Fig. 5D). Together, the PCR and hybridization show that only G8 contained the intact *MPGI(p)::Bkal* construct.

To determine whether the *MPGI(p)::Bkal* construct led to expression of *Bkal* in *M. grisea*, total RNA was extracted from mycelia grown in liquid CM for 2 days at 30°C. Reverse-transcription (RT)-PCR was performed on the transformants, mutant, and wild type (Fig. 5B and C) with primers C and E. A 1,415-bp band was amplified only from strain G8 (Fig. 5, lane 4). This suggests that no or very low expression was achieved in the nonpathogenic transformants and is likely to explain their failure to complement. Furthermore, the size of the RT-PCR amplicon from G8 is consistent with correct splicing of the *B. graminis* genomic sequence by the *M. grisea* transcription apparatus.

DISCUSSION

Functional complementation of the $\Delta cpkA$ mutation from *M. grisea* with the *Bkal* gene from *B. graminis* restored pathogenicity to the mutant. This observation, combined with the sequence of the gene, implies that *Bkal* encodes an active PKA-c, which is sufficient to restore this activity to a *M. grisea* null mutant. The *Bkal* gene is thus a functional homolog of the *cpkA* gene.

The $\Delta cpkA$ mutant is nonpathogenic to healthy leaves, because appressoria fail to penetrate the host cells (Mitchell and Dean 1995; Xu et al. 1997). In this study, we have shown that a transformant strain, G8, expressing the *B. graminis Bkal* gene under control of high-level, appressorium-specific promoter *MPGI(p)*, could substantially complement the nonpathogenic phenotypes associated with $\Delta cpkA$ strains. Spores from this transformant were able to induce lesions on the host leaves at approximately half the frequency of an isogenic *M. grisea* wild-type strain, whereas the mutant was completely nonpathogenic (Fig. 4 and Table 1). The frequency of appressorium development on glass was almost as high in G8 as it was in the wild type and approximately 20 h faster than the mutant (Xu et al. 1997). The mutant also was impaired in growth rate and sporulation, and the phenotypes were partially restored in the pathogenic G8 transformant.

The failure of G8 to fully restore all of the mutant phenotypes associated with $\Delta cpkA$ mutations was intriguing. The most likely explanation for these deficiencies is that the promoter is inappropriate in timing or strength. A signaling component, such as *CPKA*, is likely to be required at a relatively low but sustained level throughout fungal growth and development (Thevelein 1994). The promoter used for expression of the *Bkal* transgene was from *MPGI*, which encodes a hydrophobin in *M. grisea* (Talbot et al. 1993). This promoter was chosen because of its high-level expression during germination and appressorium development (Kershaw et al. 1998). The repression of *MPGI* during growth in rich medium may explain the inability of the *Bkal* transgene to restore wild-type growth kinetics to transformant G8. Alternatively, the partial complementation may be because of amino acid sequence differences between the PKA-c from *B. graminis* and *M. grisea*, which plausibly could limit the effectiveness of the introduced protein. We also cannot exclude the possibility of pleiotropic effects being caused by the random insertion of transgenes in the *M. grisea* genome.

We chose to transform *M. grisea* with genomic and cDNA sequences. The pathogenic transformant (G8) was transformed with genomic DNA, and the expressed *Bkal* transcript was confirmed by RT-PCR to be the spliced version of the gene. Thus, it seems that *M. grisea* is able to splice this *B. graminis* gene. The ability to test genomic sequences from *B. graminis* for complementation in *M. grisea* greatly increases the experimental utility of the protocol.

A noteworthy aspect of this study is that only 1 out of 58 transformants expressed the *Bkal* gene. Each of the five inactive transformants that were examined in detail had truncated versions of *Bkal* and/or multiple integration events. Only one transformant expressed the gene, as determined by functional assays and confirmed by RT-PCR. This low frequency of complementation may reflect the toxicity of high-level expression of the *Bkal* gene. It may be that transgene expression was counter-selected during the transformation procedure. In considering how to adapt this procedure for the routine analysis of *B. graminis* genes, it would thus be desirable to produce vectors that express heterologous genes under control of a range of promoters or a controllable promoter.

MATERIALS AND METHODS

Strains and media.

M. grisea wild-type (4091-5-8) and mutant (DF51) strains were cultured at 24°C in a cycle of 18-h light–6-h dark. The fungi were grown in 19-mm petri dishes on CM plates containing 15 μ l per ml of Basta (150 mg per ml; Aventis, Lyon, France) (Talbot et al. 1993). Mutant DF51 (Xu et al. 1997) was generated by replacing the *EagI–EcoRV* fragment of the PKA-c gene with the HPH gene in 4091-5-8 (Valent et al. 1986).

Vectors for transformation of *M. grisea*.

An expression vector with the promoter for *MPGI* (*M. grisea*) and *Bkal* was constructed. The *MPGI* promoter was moved from pNJ190 (*NcoI–PstI* digest) into expression vector pCB1265 (containing the Basta resistance gene). PCR with primers forward (1), TAT ACC A₁TG G_{NcoI}CG ACA TTT GGA T₁₆, and reverse (2), TAT AGGG CCC P_{stI}T₁₅₇₃C AGA AGT TTT CAA ACA AG₁₅₅₅, was performed with 25 cycles for 30 s at 94°C, 1 min at 54°C, and 1.5 min at 72°C. In addition, 10 U of *Taq*-polymerase (Promega, Bie and Berntsen, Rodovre, Denmark), 1 \times buffer (Promega, Bie and Berntsen), 2.5 mM MgCl₂, and 1 pmol of each primer, all in a 20- μ l volume, were used on genomic DNA and cDNA of the PKA-c gene from *B. graminis* (Hall et al. 1999). The fragments were digested with *NcoI* and *ApaI* and inserted into pCB1265 containing the *MPGI* promoter. To confirm that the gene was in frame and intact, the recombinants were sequenced by Dideoxy terminator sequencing (Sanger et al. 1977) with a PRISM BigDye terminator kit (PE Biosystems, Naerum, Denmark), and the reactions were analyzed on an ABI Prism 377 DNA sequencer (Perkin-Elmer, Allerod, Denmark). DNA restriction and agarose gel fractionation were performed according to standard methods (Sambrook et al. 1989).

Fungal transformation.

Protoplast isolation was carried out as described by Talbot et al. (1993). Protoplasts were made from mutant strain DF51.

Protoplasts were resuspended in STC buffer (0.6 M sucrose; 10 mM Tris-HCL, pH 7.5; and 10 mM CaCl₂), adjusted to a concentration of 5×10^6 per ml, and cooled slowly to -70°C for storage. An aliquot of 150 μl of protoplasts and 2 μg of DNA was used for each transformation. Protoplasts were regenerated overnight at 24°C in the dark and then overlaid with media containing Basta at a concentration of 15 μg of media per ml. Basta-resistant transformants were recovered after 7 days and subcultured to CM plates supplemented with 15 μg of Basta per ml. Transformants were stored at -20°C on sterile filter paper disks that were kept dry in 50-ml Falcon tubes with silica gel. The transformants were named with a G when genomic DNA was used for the transformation and a C when cDNA was used.

Pathogenicity assay.

M. grisea spores were collected in sterile water from 6-day-old plates. The spores were counted, and the concentration was adjusted to 1×10^6 per ml. Conidial suspension (3 ml) was sprayed onto 1-week-old 'Golden Promise' barley seedlings (SCRI, Invergowrie, Scotland) in a pot containing 15 to 20 plants. The seedlings have two leaves at this stage. After 5 days, the number of lesions per plant was scored. A total of 80 plants was infected for each *M. grisea* strain, and the experiment was repeated once. Thirty lesions from the G8 and wild-type infections were excised from the leaves with a sterile razor blade and transferred to CM plates. When a colony formed, it was subcultured to CM plates containing Basta.

Growth rate and spore production.

The growth rate of *M. grisea* strains was measured on plates as the radial increase in millimeter per day of the fungus. The measurements started 3 days after subculturing and ended 9 days later, when the fungus had grown to the edge of the petri dish. Twenty plates were used per strain, and the experiment was repeated three times. Harvesting spores in 3 ml of water from 6-day-old plates and counting the spores in a fraction of the water determined the spore production. Twenty plates were used per strain, and the experiment was repeated twice.

Conidia germination.

A sample of the spores collected for the pathogenicity test was used for the germination assays. Conidial concentration was adjusted to 2×10^4 per ml, and 90- μl drops were placed on plastic coverslips (Menzel-Glaser, Braunschweig, Germany) or epidermis from leek leaves floating on distilled water. The spores were germinated for 0 to 30 h in a moist chamber at 24°C . Germinating spores were counted and photographed with 160T film (Kodak, Rochester, NY, U.S.A.) at 400 \times magnification with an Axioplan light microscope (Carl Zeiss, Thornwood, NY, U.S.A.). Two hundred spores on plastic slides were counted per strain, and the experiment was carried out three times. A total of 200 spores was examined on the leek epidermis.

RNA and DNA extraction.

A modified cetyltrimethylammonium bromide extraction procedure was used to extract genomic DNA from resting conidia (Hall et al. 1999), and Trizol reagent was used to extract RNA from resting conidia (GIBCO-BRL, Taastrup, Den-

mark), following the manufacturer's protocol. An additional separation step was introduced (spin at $12,000 \times g$ for 10 min at 4°C) before the addition of chloroform to remove high-molecular DNA.

PCR and RT-PCR.

To examine integration events, PCR was performed using the primers listed in Figure 1C. PCR reactions were run, as reported above. For the creation of first-strand cDNA, 5 μg of total RNA was incubated at 70°C for 10 min with primer E, cooled on ice, and then incubated at 42°C for 50 min with 1 mM deoxynucleoside triphosphate, 5 mM dithiothreitol, 1 \times buffer (GIBCO-BRL), and 200 U of SuperScript II (GIBCO-BRL), followed by 15 min at 70°C . To amplify cDNA, 2 μl of the 20- μl RT-PCR mix was used in a 50- μl PCR reaction with the primers C and E.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Blumeria genomics database: www.crc.dk/phys/blumeria
 Plant protection database (Planteværnsdatabase vedr. plantesygdomme 2000): www.lr.dk/planteinfo/pcp/MeldugVa