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The Gene *fpk1*, Encoding a cAMP-dependent Protein Kinase Catalytic Subunit Homolog, is Required for Hyphal Growth, Spore Germination, and Plant Infection in *Fusarium verticillioides*

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***Fusarium verticillioides* is an important pathogen of maize, being responsible for ear rots, stalk rots, and seedling blight worldwide. During the past decade, *F. verticillioides* has caused several severe epidemics of maize seedling blight in many areas of China, which lead to significant losses. In order to understand the molecular mechanisms regulating fungal development and pathogenicity in this pathogen, we isolated and characterized the gene *fpk1* (GenBank Accession No. EF405959) encoding a homolog of the cAMP-dependent protein kinase catalytic subunit, which included a 1,854-bp DNA sequence from ATG to TAA, with a 1,680-bp coding region, and three introns (lengths: 66 bp, 54 bp, and 54 bp), and the predicated protein precursor had 559 aa. The mutant *Δfpk1*, which was disrupted of the *fpk1* gene, showed reduced vegetative growth, fewer and shorter aerial mycelia, strongly impaired conidiation, and reduced spore germination rate. After germinating, the fresh hypha was stubby and lacking of branch. When inoculated in susceptible maize varieties, the infection of the mutant *Δfpk1* was delayed and the infection efficiency was reduced compared with that of the wild-type strain. All this indicated that gene *fpk1* participated in hyphal growth, conidiophore production, spore germination, and virulence in *F. verticillioides*.**

Keywords: *Fusarium verticillioides*, serine–threonine protein kinase, gene disruption, pathogenicity

Fusarium verticillioides (telemorph *Gibberella moniliformis*) is an important pathogen in many areas of the world and more than 10 plant species can be infected, such as maize, cotton, broomcorn, banana, *ect.* [14]. Maize can be infected by *F. verticillioides* through the root, spike, and seed in various developmental stages of healthy plants. Significantly, the pathogen can exist as an asymptomatic, intercellular endophyte; once maize experiences abiotic stress and gets weak, it can perceive this variety, take advantage of the weak host, and colonize the vulnerable tissues [21]. Specifically, infected grains are often contaminated with mycotoxins called fumonisins, harmful to humans and animals [12]. However, except for some isolated and analyzed genes associated with fumonisins, which were generally considered to be disrelated with pathogenicity [17], to date almost nothing about the molecular pathogenic mechanism in the fungus is known. The studies on the relation among hyphal growth, morphogenesis, and pathogenicity in *F. verticillioides* should be strengthened.

The extracellular environment plays an important role in fungal growth and differentiation. Signal transduction cascades mediate communication between environmental signals and the cellular components [4]. In fungi, two main transduction cascades, the cAMP/PKA and MAPK pathway, are at the heart of this network, integrating input signals regulating fungi growth, differentiation, and the interaction with plants [10].

The cAMP/PKA signalling cascade contains G-proteins, adenylyl cyclase, cAMP, and cAMP-dependent protein kinase (PKA), *ect.* [22]. As a ubiquitous signaling molecule, PKA is the first purified protein kinase and has been best understood. PKA is regulated by cAMP, a second messenger. When cAMP levels are low, the PKA holoenzyme is an inactive tetramer composed of two regulatory subunits and two catalytic subunits; when cAMP levels increase, cAMP binds to the regulatory subunits, causing conformational changes in the holoenzyme. As a result, two C subunits dissociate and the free C subunits are then catalytically active, and these are now able to carry out its function by phosphorylating target substrates including metabolic enzymes, kinases, and transcription factors [7]. Genes encoding the cAMP-dependent protein kinase have been cloned from a number of filamentous fungi and have been

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shown to be involved in multiple processes including hyphal growth, cell metabolism, differentiation and cycle progression, sporulation, stress resistance, *ect.* [4], and especially be related with pathogenicity in a number of phytopathogen fungi [9]. For instance, in *M. grisea*, disruption of the *cpkA* gene encoding the catalytic subunit of cAMP-dependent protein kinase resulted in a mutant defective in appressorium formation, and failed to cause lesions on a susceptible rice cultivar [13].

In order to determine the importance of signal pathways in the life cycle of *F. verticillioides* and to understand the molecular pathogenic mechanism of this non-appressorium-forming fungus, two protein kinases genes named *fpk1* and *fmk1* (GenBank Accession NO. EU417814), which belong to different signal pathways, were isolated respectively. In this paper, the gene *fpk1* encoding a cAMP-dependent protein kinase catalytic subunit was isolated and functionally characterized. Results of targeted gene disrupted mutants indicated that *fpk1* performs a critical regulatory function in fungal development, sporulation, as well as pathogenesis in *F. verticillioides*.

MATERIALS AND METHODS

Strains and Culture Condition

The wild-type strain FT1 was isolated from maize ears in Taian, Shandong, China. All the wild-type and mutant strains were routinely cultured at 25°C on PDA (potato dextrose agar) with a 12-h photo phase. For cloning purposes, the *E. coli* K12 derivative $DH_{5\alpha}$ was used. All chemicals used were of analytical grade.

DNA Manipulation and Sequence Analysis of Gene *fpk1*

Standard molecular techniques were performed according to Molecular Cloning (III). Fungal DNA and RNA were extracted by the CTAB protocol or Trizol protocol, respectively. Bacterial plasmid DNA was isolated using the plasmid mini kit (OMIGA), according to the manufacturer's suggestion. Phylogenetic tree generation, DNA and protein sequence alignment, editing, and analysis were performed using DNAman software, whereas homology searches were performed with the BLAST program. Primers were designed using Primer Premier 5.0 software.

Isolation, Cloning, and Sequencing of the *fpk1* Gene

After four days of growth on PDA at 25°C, the mycelia of *F. verticillioides* FT1 strain were collected, and total RNA was extracted by the Trizol protocol. Two degenerate primers F_{DW} and F_{KM} were designed according to the conservative amino acid sequence of serine/threonine protein kinases from *Gibberella zeae* (XM-387427), *Neurospora crassa* (XM-957998), *Colletotrichum trifolii* (AAC04355), *Glomerella cingulata* (ABG89386), and *Aspergillus niger* (CAA64172). Two-step RT–PCR was performed using a RT–PCR kit (Takara), where the optimum conditions for degenerate PCR were: denaturing at 94°C for 5 min, followed by 31 cycles of amplification (94°C for 1 min, 51°C for 50 s, 72°C for 1 min), and followed by 10 min at 72°C. The PCR product was subcloned into the PMD-18-T vector (TaKaRa), and sequenced by Shenggong in Shanghai.

The special primers F_{I1}, F_{I2}, F_{GSP-R}, F_{GSP-1}, and F_{GSP-2} were designed based on the partial sequence obtained, and then 3' RACE and 5' RACE were performed using a 3' RACE kit (Takara) and 5' full RACE core set (Roche), according to the manufacturer's instructions. Finally, the full-length genes of cDNA and DNA were obtained by PCR using primer pair F_1–F_2. All the nucleotide sequences of primers used in this paper are shown in Table 1.

Construction of *fpk1* Gene Replacement Vector

Based on homologous combination theory, the *fpk1* gene-disruption vector pBS-K-H was constructed, which contains a hygromycin B resistance gene, *hph*, as a selectable marker. The 1.7-kb DNA fragment containing most of the *fpk1* gene was isolated from the PMD-18-T clone, digested with HindIII and XbaI, and cloned into pBS vector that was digested with the same restriction enzymes. The recombination

Table 1. All primers used in this study.

Name	Sequence (5'–3')	Location	Use
F_{DW}	CCACCARTCNACNGAYTTRTT	*fpk1* (NKSVDWW)	Degenerate PCR
F_{KM}	CARGTNGTNAARATGAARCA	*fpk1* (QVVKMKQ)	Degenerate PCR
F_{I1}	ACACCCTTTCCTTATTACC	*fpk1* (909–927)	3' RACE
F_{I2}	GGTGATGGATTTCGTTGA	*fpk1* (966–983)	3' RACE
F_{GSP-R}	GTGCCCCAGAGGGTAATAAGGA	*fpk1* (938–917)	5' RACE
F_{GSP-1}	GCATGCGACGTTCGTCGTCATTGGTAT	*fpk1* (899–872)	5' RACE
F_{GSP-2}	CGACTTGCTTCATCTTGACCACTTG	*fpk1* (868–846)	5' RACE
F_1	ATGCCTTCACTGGGGTTTC	*fpk1* (1–19)	Full-length gene
F_2	GCATCCATTACTCGCTCTG	Downstream of *fpk1* (1691–1709)	Full-length gene
F_{21}	TCATCTTTGGGTCCATTCTG	Upstream of *fpk1* (-138– -119)	Mutation screening
hph_{I2}	CTGCTTACAAGTGGGCTGAT	*hph*	Mutation screening
F_{t1}	GCTTTTCACCTGCCTCG	*fpk1* (121–137)	Probe
F_{t2}	GGAAAGGGTGTTTGAGCT	*fpk1* (919–902)	Probe
F_z	CGACAGATTTGTTGTAGCCTTTGT	*fpk1* (1247–1270)	Mutation screening
hph_1	ATGAAAAAGCCTGAACTC	*hph*	Transformant screening
hph_2	CTATTCCTTTGCCCTCGG	*hph*	Transformant screening

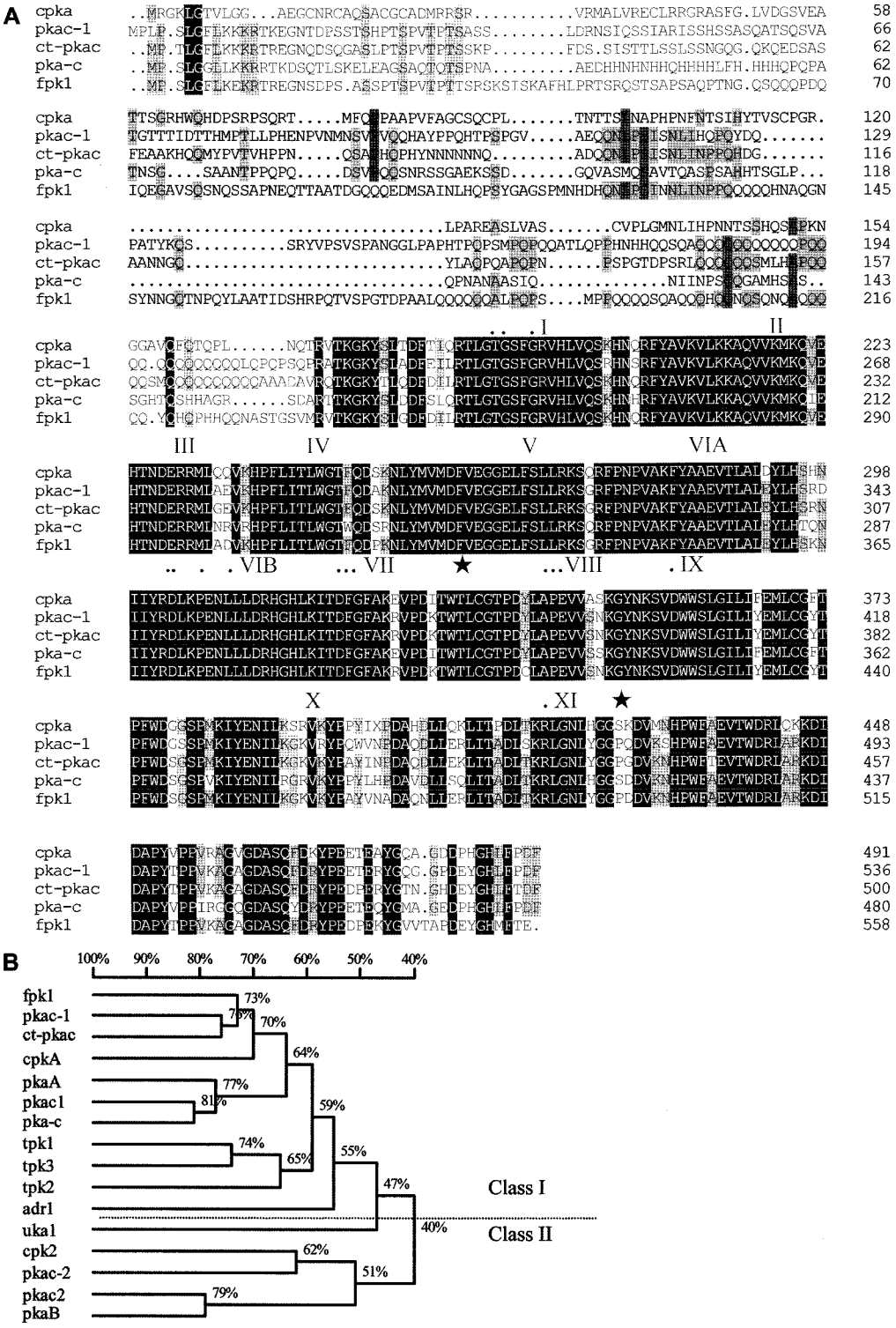

Fig. 1. *fpk1* encodes a cAMP-dependent serine–threonine protein kinase catalytic subunit in *F. verticillioides*.
A. Alignment of the predicted amino acid sequence encoded by *fpk1* with those of its homologs. The GenBank accession numbers and its corresponding fungus are as follows: cpka: *Magnaporthe grisea* (U12335); pkac-1: *Neurospora crassa* (AAF75276); ct-pkac: *Colletotrichum trifolii* (ABG89386); pka-c: *Aspergillus niger* (CAA64172). I–XI indicate the 11 subdomains of the cAMP-dependent protein kinases catalytic domains; Filled circles indicate the highly conserved amino acid or residues in the11 subdomains; Asterisks indicated two conserved autophosphorylation sites. **B**. Phylogenetic comparison of fungal PKAs.The phylogenetic tree was produced using the DNAMAN.The amino acid sequences used in this comparison were from *Aspergillus nidulans* (pkaA and pkaB), *Aspergillus fumigatus* (pkac1 and pkac2), *F. verticillioides* (*fpk1*), *Magnaporthe grisea* (cpkA and cpk2), *Neurospora crassa* (pkac-1 and pkac-2), *Saccharomyces cerevisiae* (Tpk1, Tpk2, and Tpk3), *Ustilago maydis* (adr1 and uka1), *Aspergillus niger* (pka-c), and *Colletotrichum trifolii* (ct-pkac).

Table 2. Comparison of the spores germination rate between wild-type and mutant strains.

Time (h)	2	3	4	5	6	7	8	9	10
FT1	20.1%	61.3%	75.8%	95.6%	99.3%	100%	100%	100%	100%
Δ*fpk1*	5.6%	40.5%	55.4%	65.7%	70.4%	72.6%	77.5%	81.3%	83.8%

The numbers of germinating conidia and total conidia were recorded in three microscopic fields at different times.

plasmid pBS-K and the vector pUCATPH were digested by BamHI, respectively, and then the linearization pBS-K was ligated with a 1.4-kb hygromycin phosphotransferase gene (*hph*) fragment by T_4 ligase, which was digested from pUCATPH and contained the *Aspergillus nidulans* PtrpC promoter but not the TtrpC terminator (Fig. 3).

Fungal Transformation and Mutant Selection

The protoplasts of the strain FT1 were prepared and transformed with the vector pBS-K-H digested by HindIII as described previously [25], but no restriction enzyme was added in the transformation system. Transformants were selected by hygromycin B at a concentration of 250 μg/ml on PDA plates. Monoconidial cultures resistant to hygromycin B were used for the next experiments. Homologous recombinations were screened by PCR using primer pair F_{21}–hph_{12}. Then, whether the gene *fpk1* was deleted and how the homologous combination and gene exchange occurred were verified using primer pairs F_{11}–F_Z, F_{12}–F_Z, and F_{12}–F_2, and further confirmed by Southern blotting analysis. DNA probes amplicated by primer pair F_{t1}–F_{t2} were DIG-labeled with a random primer labeling kit (Roche) according to the manufacturer's instructions. The genome DNA of the strains FT1 and Δ*fpk1* were digested by SacI and HindIII, respectively.

RT–PCR

The total RNA of the strains FT1 and Δ*fpk1* were extracted from the 4-day-old mycelia grown on PDA, by the Trizol protocol. Two-step RT–PCR was performed using a RT–PCR kit (Takara); the optimum conditions for RT–PCR were denaturing at 94°C for 5 min, followed by 31 cycles of amplification (94°C for 1 min, 49°C for

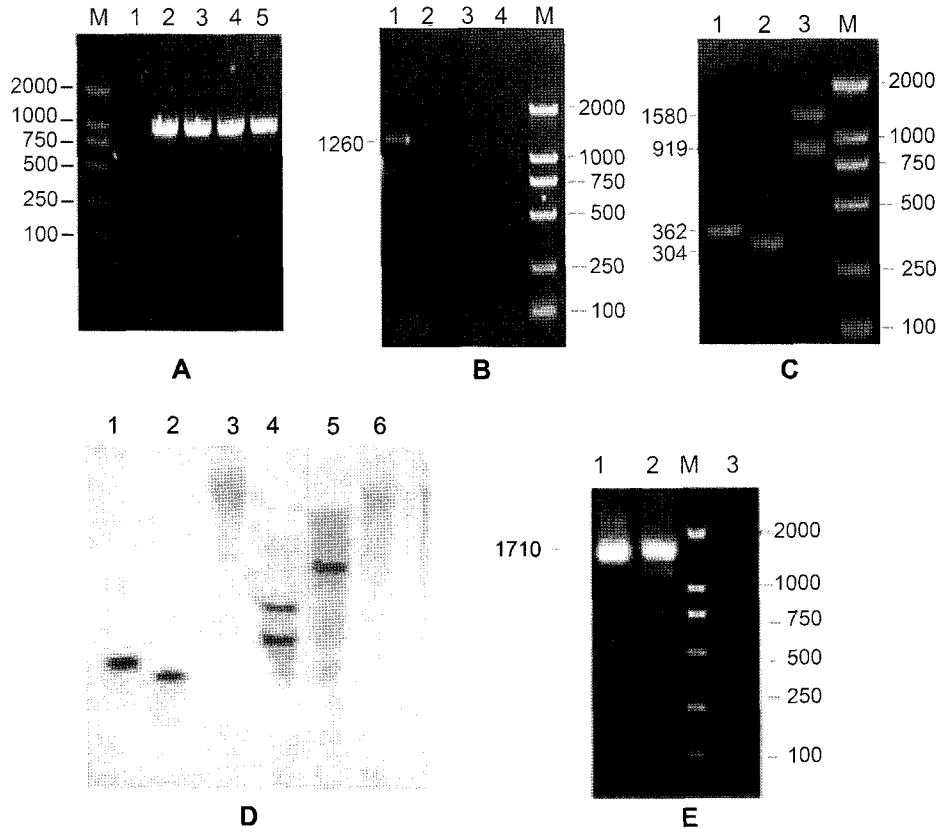

Fig. 2. Screening and confirmation of the mutant targeted disruption of *fpk1* gene in *F. verticillioides*.

A. PCR amplification for *hph* gene with primer pairs hph_1–hph_2 from the genome DNA of strains. (1) Wild-type; (2)–(4) ectopic transformants; (5) Δ*fpk1*. **B.** Screening the homologous recombination between the endogenous *fpk1* gene and the vector PBS-K-H with primer pairs F_{21}–hph_{12}. (1) Δ*fpk1*; (2)–(4) ectopic transformants. **C.** Confirming if the *fpk1* gene was deleted in the mutant Δ*fpk1* with primer pairs (1) F_{11}–F_Z; (2) F_{12}–F_Z; (3) F_{12}–F_2. **D.** Southern blotting. (1–3): the genomic DNA of FT1 digested by SacI, HindIII, and not digested; (4–6): the genomic DNA of Δ*fpk1* digested by SacI, HindIII, and not digested. **E.** RT–PCR product of *fpk1* gene in the genomic DNA of strain (1) wild-type; (2) ectopic transformant; (3) mutant Δ*fpk1* (M: Marker DL2000).

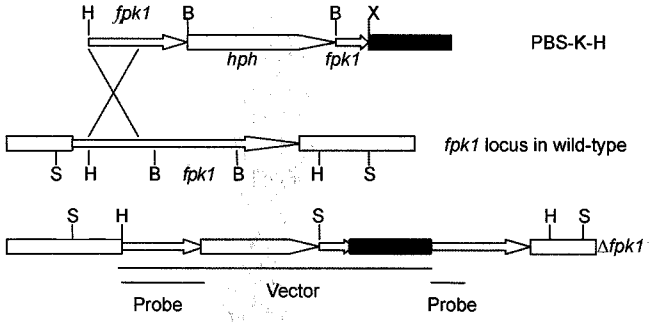

Fig. 3. Diagram of the process of the vector construction and the pattern of homologous recombinant in mutant Δ*fpk1*.
pBS-K-H: through two steps of recombination, the disrupted vector was successfully constructed, which contained a hygromycin B resistance gene (*hph*) as a selectable marker and two parts of the *fpk1* gene as recombination arms; Locus of *fpk1* gene: *fpk1* locus in genome of the wild-type strain and its restrictive enzyme sites; Δ*fpk1*: pattern of the homologous recombinant in mutant Δ*fpk1*. (S: *Sac*I; H: *Hin*dIII; B: *Bam*HI; X: *Xba*I; *hph*: hygromycin phosphotransferase gene).

1 min, 72°C for 2 min), and followed by 10 min at 72°C; the primer set for cloning the gene *fpk1* was F_1–F_2 (Table 1).

Assays for Vegetative Growth

The mutant strain Δ*fpk1* and wild-type strain FT1 were cultivated on PDA plates at 25°C, and the colony morphology was observed, measured, and contrasted every day. To determine and contrast the biomass produced by Δ*fpk1* and FT1, the same size of colonies were inoculated in PDB (potato dextrose broth) and cultured in a rotary shaker with 180 rpm at 25°C; mycelia were then separated from the medium by filtration, and lyophilized and weighed.

Assays for Sporulation and Germination of Conidiophore

The same size colonies of FT1 and Δ*fpk1* were excavated using a 5-mm punch, rinsed with 1 ml of sterilized water, and diluted 10 times, and then the spores were counted and contrasted using hemocytometer measurement, where three independent experiments were conducted for each strain.

Hundreds of spores of FT1 and Δ*fpk1* were collected from 5-day-old PDA cultures by filtration through two layers of Miracloth, and then were incubated in sterilized water at 25°C, respectively. Germination of spore was observed from the second hour using hemocytometer measurement, and the germination rates were calculated.

Ten μl of diluted spores suspension was spotted on a drop of PDA medium and added to a glass slide, and then covered with a cover slide and incubated in an empty Petri dish at 25°C. The germ tubes and the hyphal elongation were observed with a microscope after 5 h .

Pathogenicity Assays on Maize Seedling

Maize seeds (variety: YeDan12) were planted in a nutritious plate plug and incubated in a culture dish with the filter paper kept moist. Spores of the Δ*fpk1* and FT1 were collected from 5-day-old cultures by filtering through two layers of Miracloth, diluted with sterile water to a concentration of 0.5×10^5–1×10^5 spores/ml, and then inoculated on the maize seeds two times by spraying before and after maize seeds germinating. The nutritious plate plug and the culture dish were all placed in a phytotron at 28°C, 85% r.h., with a 14-h light/10-h dark cycle. Lesion formation was investigated from 2 days, and disease severity was quantified by calculating the disease index (disease grades: 0, no symptom; 1, 1~2 leaves tips showed small chorotic lesions; 2, 3~5 leaves tips showed chorotic lesions; 3, some leaves marginally withered; 4, large area etiolation or whole leaf yellow and withered).

Results

Isolation and Characterization of the Gene *fpk1* Encoding a cAMP-dependent Serine–Threonine Protein Kinase Catalytic Subunit

Using degenerate primers F_{DW} and F_{KM}, a 420-bp DNA fragment was amplified. BLAST and sequence analysis showed that this fragment was a part of the Ser/Thr protein kinase gene and contained the typical subdomain of protein kinase. The transcriptional initiation site and the polyadenylation site were determined by 5' and 3' RACE. The full-length cDNA sequence was assembled by overlapping the three nucleotide sequences and designated as *fpk1* (GenBank Accession No. EF405959); it contains a 1,680-bp open reading frame, coding a protein kinase precursor of 559 amino acid residues. The full length of the DNA sequence, which was amplified using primer pair F_1–F_2 designed from the obtained sequence, contains 1,854 bp and the open reading frame is interrupted by 3 introns. Phylogenetic analysis indicated that FPK1 is most similar to other cAMP-dependent serine–threonine protein kinase in fungi. Further analysis found that the deduced peptide sequence of *fpk1* contains all subdomains of protein kinase, the highly conserved amino acids, and characteristic motifs of PKA catalytic subunits, including the ATP binding motif GTGSFGRV (amino acids 187–194) in subdomain I; the invariant K209 in subdomain II, which is considered to be essential for maximal enzyme activity; the nearly invariant E228 in subdomain III; the catalytic loop motif RDLKPEN (amino acids 302–308) sequence in subdomain VIB; the magnesium ion-chelating loop-DFGF (amino acids 321–324) of subdomain VI; the highly conserved motif APE (amino acids 343–345) in subdomain VIII; and the conserved D357 in subdomain IX and K417 in subdomain XI. Two conserved autophosphorylation sites, T334 and S426, were also found in the sequence (Fig. 1A). These conserved amino acids and characteristic motifs were consistent with other PKAs reported previously [2]. The phylogenetic tree indicated that the gene *fpk1* belongs to class I of the PKA catalytic subunit in fungi (Fig. 1B), and may perform an important regulation function as similar genes like *adr1*, and *cpkA*.

Isolation of *fpk1* Disruption Mutants by Gene Replacement

By transforming protoplasts of the strain FT1 with the gene-disruption vector pBS-K-H, more than 200 transformants

were obtained. From these, a *fpk*1 gene-disruption isolate (named Δ*fpk1*) was successfully screened using the primer set F_{21}–hph_{12}. Only transformants with homologous recombination between pBS-K-H and the endogenous *fpk1* gene would have a 1,260-bp PCR product, and there were no PCR product in the ectopic transformants. Then, whether the gene *fpk1* was deleted and how the homologous combination and gene exchange occured were verified by primer pairs F_{11}–F_Z, F_{12}–F_Z and F_{12}–F_2, and further confirmed by Southern blotting analysis (Fig. 2).

As a result, it could be concluded from Fig. 2 that (1) the vector pBS-K-H was only inserted in the genome of mutant Δ*fpk1* with a single copy through homologous recombination; (2) in the mutant Δ*fpk1*, homologous recombination occured by single cross-over, and especially only part of the *fpk1* gene in the left arm was combined and exchanged; and (3) the integrated *fpk1* gene was interrupted by inserting the vector pBS-K-H, although no gene deletion occurred. Construction of the vector and the pattern of homologous recombinant in mutant Δ*fpk1* are shown in Fig. 3.

RT–PCR

In order to confirm whether the *fpk1* gene was disrupted, the presence of *fpk1* transcripts was determined by RT–PCR with the primer pair F_1–F_2. DNA fragments of 1,710 bp were obtained in the wild-type strain and ectopic transformant, but no such fragment was obtained in Δ*fpk1* (Fig. 2E). Thus, it could be determined that the *fpk1* gene could not be transcribed normally in mutant strain Δ*fpk1*.

Disruption of *fpk1* Reduced Vegetative Growth

Growth assays were carried out with the wild-type strain FT1 and mutant strain Δ*fpk1* on PDA and in PDB. The colony morphology of the mutant Δ*fpk1* differed drastically from the wild-type strain FT1 on PDA. It was smaller and more sparse than that of FT1, as it grew with very fewer and shorter aerial mycelia. Growth assays in PDB revealed that the hyphal biomass of the mutant strain Δ*fpk1* was reduced compared with that of the wild-type strain (Fig. 4).

Disruption of *fpk1* Affected Sporulation and Conidia Germination

Because producing dormant spores is an important characteristic, and the first essential step of infection is the spore germination and differentiation of germ tubes that develop into infectious hyphae on the suface of the host plant [14], we therefore compared the sporulation, germination pattern, and early hyphal development between mutant strain Δ*fpk1* and the wild-type strain FT1 of *F.*

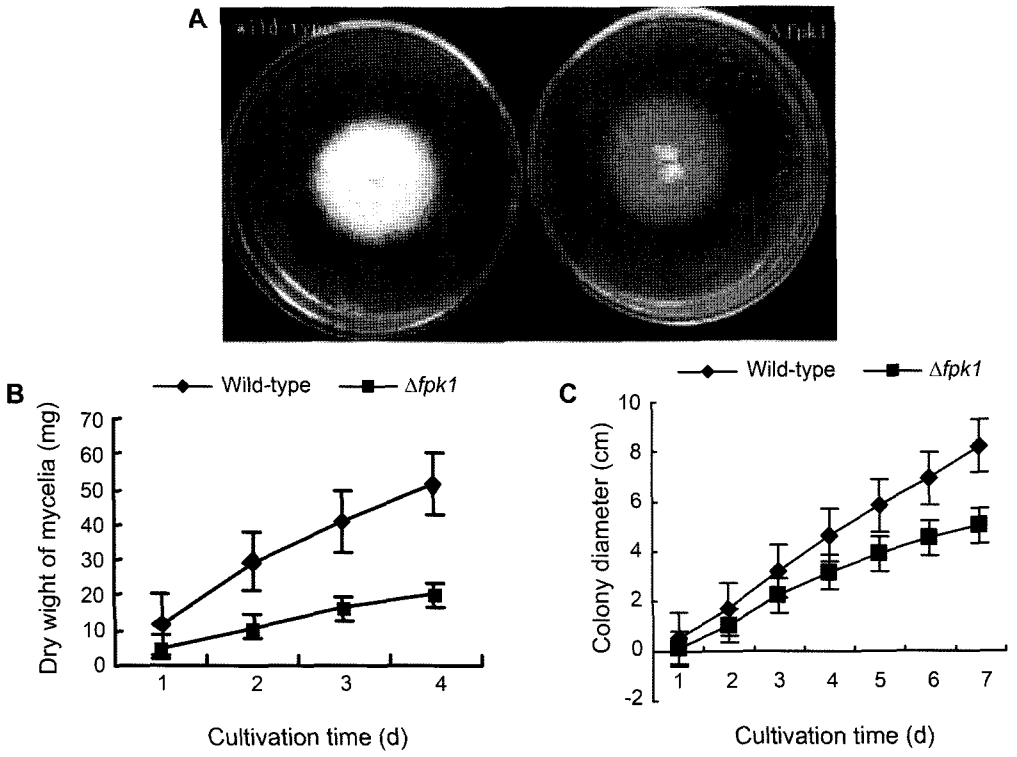

Fig. 4. Comparison of the vegetative growth between wild-type strain FT1 and the mutant strain Δ*fpk1*.
A. Colony morphology of FT1 and Δ*fpk1* cultured on PDA plates at 25°C at 3 days postinoculation (dpi). **B.** Comparison of the dry weight of mycelia cultured in PDB at 25°C, 180 rpm. **C.** Colony diameter of FT1 and Δ*fpk1* at different cultivation times (results are representative of three independent experiments).

verticillioides. As a result, conidia produced by Δ*fpk1* were normal in size and morphology compared with that of FT1, but the amounts were reduced. The spores amount of FT1 was 6.2×10^6/ml in average, whereas the amount of Δ*fpk1* was only 2.54×10^5/ml. The spore germination of Δ*fpk1* was delayed, and the germination rate was reduced, compared with that of FT1. After 6 h, the spores of the wild-type strain nearly all germinated, but the spore germination rate of Δ*fpk1* only reach 70% at the same time (Table 2). Specially, the germ tubes of Δ*fpk1* elongated very slowly, and the fresh hypha was stubby and lacked branch; correspondingly, the germ tubes of wild-type strain elongated very fast, and the extended hypha was gracile and full of divarications.

Disruption of *fpk1* Reduced Pathogenicity

In order to assess the pathogenicity of the mutant strain Δ*fpk1*, maize seeds of susceptible maize variety YeDan 12 were cultured in nutritious plate plugs and culture dishes, respectively, and then were inoculated with the conidia suspension of FT1 and Δ*fpk1*. In the nutritious plate plug assays, the maize seedlings were inoculated with conidia suspension (10^5 spores/μl) by spraying onto the seeds and basal culm of maize seeding two times in turn. The seedlings inoculated by the wild-type strain grew weakly, and showed leaf marginal withering at 7 days postinoculation (dpi) that expanded into large area setiolation at 10 dpi and eventually developed into the whole leaf being yellowed and withered at 14 dpi; in contrast, the Δ*fpk1*-inoculated maize seedlings did not develop any symptoms until 12 dpi when the outer leaves tips showed small chorotic lesions at 12–14 dpi. The maize seedlings grew as strong as the control, which was inoculated with sterile water, except several leaves tips showed withering (Fig. 5A). In the nutritious plate plug assays, the disease severity and disease incidence were investigated (Table 3). From Table 3, we could say that the symptoms in plates inoculated with the mutant strain were delayed and alleviated compared with that of the wild-type strain. In culture dish assays, the maize seedlings inoculated by the wild-type strain showed small brown flecks at 3 dpi that expanded into large necrotic areas at 5 dpi and eventually rotted away at 7 dpi; but only a few small brown spot emerged on leaf sheathes or leaves in the Δ*fpk1*-inoculated maize seedling at 5–7 dpi (Fig. 5B). All this indicated that the mutant strain was significantly reduced in pathogenicity.

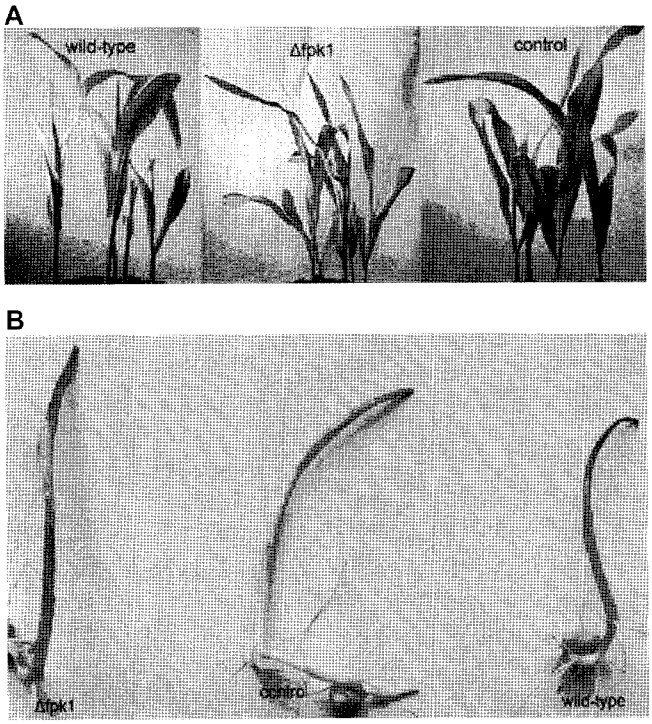

Fig. 5. Infection assay on maize seeding of susceptible variety YeDan 12.
A. The symptoms of maize seedling blight were observed at 12 dpi, which were inoculated in nutritious plate plugs. **B.** The photograph was taken at 7 dpi, which were of culture in culture dish. Control: inoculated with sterile water; all were cultured at 28°C, 85% r.h., with a 14-h light/10-h dark cycle.

Discussion

F. verticillioides is rapidly developing as a model corn phytopathogenic fungus. In contrast to other model fungi, such as *M. grisea* and *C. trifolii*, it could not form appressoria, an important infection structure, and how it perceives the

Table 3. Disease severity in nutritious plate plug.

Time	Strains	Total plants	Disease incidence (%)	Disease index	F test 2	
					0.05	0.01
7 dpi	FT1	45	97.8	52.2bB	**	**
	Δ*fpk1*	45	4.4	1.1aA	ns	ns
	CK	45	2.2	0.6aA		
14 dpi	FT1	45	100	79.4	**	**
	Δ*fpk1*	45	66.7	16.7cC	*	*
	CK	45	6.7	2.3aA		

**: Greatly significant difference; *: significant difference; ns: no significant difference.

host signaling and the function of the PKA signaling pathway in the process of penetration and colonization in *F. verticillioides* are still unknown. In this paper, the gene *fpk1* encoding the catalytic subunit of PKA was isolated and its biological functions were analyzed by observing a gene knock-out mutant.

In our study, one important phenomenon was that hyphal growth of mutant Δ*fpk1* was reduced both on solid medium and in liquid medium. The mutant strain produced fewer and shorter aerial hyphae, and formed sparse colonies on PDA plates. It indicated that the gene was related with the hyphal growth, as were the similar genes in yeast *Candida albicans* and some pathogenic fungi. In *Saccharomyces cerevisiae*, three catalytic subunits are encoded by the genes *TPK1*, *TPK2*, and *TPK3*; *TPK2* promotes filamentous growth, whereas *TPK1* and *TPK3* play negative roles, and triple mutants lacking the three genes are inviable [18]. In *C. albicans*, a PKA gene named TPK2 is shown to be required for hyphal growth and differentiation [3]. In *U. maydis*, the genes encoding two catalytic subunits of PKA are *uka1* and *adr1*, where *adr1* and *adr1 uka1* double mutations both undergo filamentous growth [5, 10].

Besides the colony morphogenetic variety, the mutant strain Δ*fpk1* was reduced both in sporulation and spore germination, and the germ tubes outgrowths were deviant. It indicated that the gene *fpk1* was related with sporulation and conidial germination in *F. verticillioides*. Filamentous fungi undergo distinct life-cycle phases of growth and reproduction, where switching between these two phases is highly regulated and initiation is governed by perception of a combination of physiological and environmental conditions [1]. The G protein/cAMP/PKA pathway has been proved to be related with these developmental changes and sporulation in a number of fungi. Shimizu and Keller [19] showed that a cAMP-dependent protein kinase A is involved in conidiation in *A. nidulans*. Targeted disruption of the gene encoding the G protein β subunit caused suppression of conidia production in *F. oxysporum* [6]. Recently, Zhao *et al.* [27] reported that an *A. fumigatus* mutant, Δ*pkaR*, showed reduced growth as well as germination defects. In phytopathogenic fungi *C. trifolii* and *M. grisea*, the PKA pathways have been proved to be essential for both appressorium formation and spore germination [13, 24, 25]. In *F. verticillioides*, it has been reported that the GBB1 gene encoding the heterotrimeric G protein β subunit regulates conidiation [21]. In our study, the result that the gene *fpk1* was required for conidiation is accordant with previous researches.

The most important phenomenon was that the mutant strain Δ*fpk1* showed less virulence, as it caused delayed infection, reduced infection efficiency, and limited chlorotic and necrotic symptoms when inoculated in susceptible maize varieties, so we could conclude that the *fpk1* gene is required for pathogenicity in *F. verticillioides*. The PKA signaling pathway has been proved to be required for plant infection in many pathogenic fungi. In *C. lagenarium* [20], *C. trifolii* [24, 25], and *M. grisea* [23], the PKA pathway is required for formation of appressoria, an important infection structure. Disruption of the PKA C subunit in *C. trifolii* and *M. grisea* resulted in an inability to penetrate and colonize intact host plants [24, 25]. In our study, the reduction in virulence of mutant Δ*fpk1* may be the consequence of the reduced hyphal growth and spore germination, as well as formation of deviant germ tubes. However, it was unknown how to change the biochemical factors such as pathogenic cell wall degrading enzymes and mycotoxins in the process, and further biochemical and physiological evidences for the reduced virulence should be established.

Although the structures and functions of the genes encoding protein kinase A in fungi are highly conserved, we should be cautious to not generalize the functions of the specific gene. Because the signaling net is complicated, cross-talk between the cAMP pathway and others occur at several gradations, where different pathways usually coordinately regulate multiple processes [10]; furthermore, not only one PKA catalytic subunit exists in one fungus, and until now, fewer direct substrates of PKA have been identified in fungus. Identification of the targets of the PKA signaling pathway and understanding how to accomplish the biological function of PKA will be a major challenge for future studies.

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REFERENCE

1. Adams, T. H. and J. H. Yu. 1998. Coordinate control of secondary metabolite production and asexual sporulation in *Aspergillus nidulans*. *Microbiology.* **1:** 674–677.
2. Burghard, L., M. Meike, B. Armin, and A. Axel. 2004. The cyclic AMP-dependent protein kinase A network regulates development and virulence in *Aspergillus fumigatus*. *Infect. Immun.* **72:** 5193–5203.
3. Cloutier, M., R. Castilla, N. Bolduc, A. Zelada, P. Martineau, M. Bouillon, *et al.* 2003. The two isoforms of the cAMP-dependent protein kinase catalytic subunit are involved in the control of dimorphism in the human fungal pathogen *Candida albicans*. *Fungal Genet. Biol.* **38:** 133–141.
4. Cletus, A. and J. H. D'Souza. 2001. Conserved cAMP signaling cascades regulate fungal development and virulence. *Microbiology* **25:** 349–364.

5. Dürrenberger, F., K. Wong, and J. W. Kronstad. 1998. Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 5684–5689.
6. Jain, S., K. Akiyama, R. Takata, and T. Ohguchi. 2005. Signaling *via* the G protein β subunit FGA2 is necessary for pathogenesis in *Fusarium oxysporum*. *FEMS Microbiol. Lett.* **243:** 165–172.
7. Johnson, D. A., P. Akamine, E. Radzio-Andzelm, M. Madhusudan, and S. S. Taylor. 2001. Dynamics of cAMP-dependent protein kinase. *Chem. Rev.* **101:** 2243–2270.
8. Kronstad, J., A. D. De Maria, D. Funnell, R. D. Laidlaw, N. Lee, M. M. de Sá, and M. Ramesh. 1998. Signaling *via* cAMP in fungi: Interconnections with mitogen-activated protein kinase pathways. *Arch. Microbiol.* **170:** 395–404.
9. Lee, N., C. A. D'Souza, and J. W. Kronstad. 2003. Of smuts, blasts, mildews, and blights: cAMP signaling in phytopathogenic fungi. *Annu. Rev. Phytopathol.* **41:** 399–427.
10. Lengeler, K. B., R. C. Davidson, C. D'souza, T. Harashima, W.-C. Shen, P. Wang, X. Pan, M. Waugh, and J. Heitman. 2000. Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. Rev.* **64:** 746–785.
11. Martin, B. D. and Y. Oded. 1999. Serine/threonine protein kinases and phosphatases in filamentous fungi. *Fungal Genet. Biol.* **26:** 99–117.
12. Missmer, S. A., L. Suarez, M. Felkner, E. Wang, A. H. Merrill, K. J. Jr Rothman, and K. A. Hendricks. 2006. Exposure to fumonisins and the occurrence of neural tube defects along the Texas-Mexico border. *Environ Health Perspect* **114:** 237–241.
13. Mitchell, T. and R. A. Dean. 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* **7:** 1869–1878.
14. Munkvold, G. P. and A. E. Desjardins. 1997. Fumonisins in maize - Can we reduce their occurrence? *Plant Dis.* **81:** 556–565.
15. Osherov, N. and G. S. May. 2001. The molecular mechanisms of conidial germination. *FEMS Microbiol. Lett.* **199:** 153–160.
16. Proctor, R. H., T. M. Hohn, and S. P. McCormick. 1995. Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Mol. Plant Microbe Interact.* **8:** 593–601.
17. Proctor, R. H., D. W. Brown, R. D. Plattner, and A. E. Desjardins. 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genet. Biol.* **38:** 237–249.
18. Robertson, L. S. and G. R. Fink. 1998. The three yeast protein kinases A have specific signaling functions in pseudohyphal growth. *Proc. Natl. Acad. Sci. U.S.A.* **95:** 13783–13787.
19. Shimizu, K. and N. P. Keller. 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. *Genetics* **157:** 591–600.
20. Takano, Y., K. Komeda, K. Kojima, and T. Okuno. 2001. Proper regulation of cyclic AMP-dependent protein kinase is required for growth, conidiation, and appressorium function in the anthracnose fungus *Colletotrichum lagenarium*. *Mol. Plant Microbe Interact.* **14:** 1149–1157.
21. Uma, S. S. and W. B. Shim. 2007. *Fusarium verticillioides* GBB1, a gene encoding heterotrimeric G protein β subunit, is associated with fumonisin B biosynthesis and hyphal development but not with fungal virulence. *Molec. Plant Pathol.* **8:** 375–384.
22. Wang, P. and J. Heitman. 1999. Signal transduction cascades regulating mating, filamentation and virulence in *Cryptococcus neoformans*. *Curr. Opin. Microbiol.* **2:** 358–362.
23. Xu, J. R., M. Urban, J. A. Sweigard, and J. E. Hamer. 1997. The CPKA gene of *Magnaporthe grisea* is essential for appressorial penetration. *Mol. Plant Microbe Interact.* **10:** 187–194.
24. Yang, Z. H. and M. B. Dickman. 1999. Molecular cloning and characterization of Ct-PKAR, a gene encoding the regulatory subunit of cAMP-dependent protein kinase in *Colletotrichum trifolii*. *Arch. Microbiol.* **171:** 249–256.
25. Yang, Z. H. and M. B. Dickman. 1999. *Colletotrichum trifolii* mutants disrupted in the catalytic subunit of cAMP-dependent protein kinase are nonpathogenic. *Mol. Plant Microbe Interact.* **12:** 430–439.
26. Zhao, P. B., Q. X. Zhou, F. X. Guo, and D. C. Li. 2007. Inserter mutation by restriction enzyme-mediated integration in *Fusarium moniliforme* and isolation of pathogenicity mutant. *Acta. Phytopathol. Sinica* **37:** 545–548.
27. Zhao, W., J. C. Panepinto, J. R. Fortwendel, L. Fox, B. G. Oliver, D. S. Askew, and J. C. Rhodes. 2006. Deletion of the regulatory subunit of protein kinase A in *Aspergillus fumigatus* alters morphology, sensitivity to oxidative damage, and virulence. *Infect. Immun.* **74:** 4865–4874.