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Heterotrimeric G protein β subunit GPB1 and MAP kinase MPK1 regulate hyphal growth and female fertility in *Fusarium sacchari*

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ABSTRACT

Heterotrimeric GTP-binding proteins (G proteins) and mitogen-activated protein kinase (MAPK) cascades involve vegetative hyphal growth, development of infection-related structure, colonization in host plant and female fertility in phytopathogenic ascomycete fungi. In this study, a heterotrimeric G protein β subunit (Gβ), GPB1, and MAPK, MPK1, were characterized from *Fusarium sacchari* (= *Gibberella sacchari*; mating population B of the *G. fujikuroi*-species complex). GPB1 and MPK1 showed high homology to known Gβ and Fus3/Kss1 MAP kinases of other filamentous ascomycetes, respectively. Disruption (Δ) of *gpb1* suppressed hyphal branching and accelerated aerial hyphae formation in *F. sacchari*. Oppositely, disruption of *mpk1* caused delayed aerial hyphae formation. These indicated that GPB1 regulates vegetative hyphal growth negatively, and MPK1 does positively in *F. sacchari*. Both Δ*gpb1* and Δ*mpk1* showed female sterility. Level of intracellular cAMP in Δ*gpb1* was lower than wild type. Exogenous cyclic AMP (cAMP) partially restored enhanced aerial hyphae formation. These suggested that abnormal hyphal growth was caused by depletion of intracellular cAMP in Δ*gpb1*. cAMP has been reported to suppress development of perithecia in crossing between wild type strains. Thus, precise regulation of intracellular cAMP level via Gβ/MAPK is essential for normal hyphal growth and fertility.

1. Introduction

Heterotrimeric GTP-binding protein (G protein) in eukaryotic cells is a central component in signal transduction pathways involved in multiple biological processes. The heterotrimeric G protein/mitogen-activated protein kinase (MAPK) pathway in pheromone signalling is well characterized in budding yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Dohlman and Thorner 2001). The pheromone signal received in a receptor (STE2/STE3) is transmitted to a MAPK cascade (STE11-STE7-FUS3) by a Gβγ hetero dimer, resulting in expression of mating-related genes. The budding yeast has five MAPKs, Fus3,

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Kss1, Hog1, Slt2 and Smk1, involved in pheromone response, filamentous growth, adaptation to high osmolarity, cell wall integrity and sporulation, respectively (Krisak et al. 1994; Herskowitz 1995; Schwartz and Madhani 2004). To date, the subunits of heterotrimeric G protein and MAPK genes have been cloned and characterized in a number of filamentous fungi. The heterotrimeric G proteins and MAPKs in ascomycetes pathogenic to mammal and plant show high similarity in amino acid sequences and often play important roles in pathogenicity (Lengeler et al. 2000; Xu 2000). One of best-characterized plant pathogenic ascomycetes on the signal transduction pathway is the rice blast fungus *Pyricularia oryzae* Cavara (synonym, *Magnaporthe oryzae* (T.T. Hebert) M.E. Barr). Three G protein α subunit genes (*magA*, *magB* and *magC*), a β subunit gene (*MGB1*) and three MAPK genes (*PMK1*, *OSM1* and *MPS1*) have been cloned from *P. oryzae*. Gene disruption of *magB*, *MGB1*, *PMK1* or *MPS1* generated reduction of virulence (Xu and Hamer 1996; Liu and Dean 1997; Xu et al. 1998; Xu 2000; Nishimura et al. 2003). The reduced virulence depended on impaired development of appressorium, which is essential for infection of *P. oryzae* to host plant. In *Helminthosporium maydis* Y. Nisik. & C. Miyake (synonym, *Cochliobolus heterostrophus* (Drechsler) Drechsler), the corn southern blight fungus, and *Colletotrichum orbiculare* (Berk.) Arx (synonym, *Colletotrichum lagenaria* (Pass.) Ellis & Halst.), the anthracnose fungus, deficient mutants of *PMK1* homologue failed to form appressorium, resulting in reduced virulence (Lev et al. 1999; Takano et al. 2000).

The genus *Fusarium* includes a number of plant pathogens that cause destructive diseases in a variety of crops. In spite of the close phylogenetic relationship, the species in the complex carry various characters in conidiation, mating and infection manner. Two G protein α subunits (FGA1 and FGA2) and a β subunit (FGB1) have been characterized in asexual *F. oxysporum* Schlecht. f. sp. *cucumerinum* J.H. Owen, the cucumber wilt fungus (Bolker 1998; Jain et al. 2002, 2005). Deletion of these α or β subunit gene (Δ*fga1* or Δ*fgb1*) caused reduced virulence to cucumber seedlings, level of intracellular cAMP and heat sensitivity in *F. oxysporum* f. sp. *cucumerinum*. In addition, the Δ*fga1* and Δ*fgb1* showed abnormal hyphal morphology and reduced conidiation. The Δ*fga2* completely failed to develop symptom, although no altered morphological phenotype was observed (Jain et al. 2005). The *fgb1* from *F. oxysporum* f. sp. *lycopersici* W.C. Snyder & H.N. Hansen, the tomato wilt fungus, has also been cloned (Delgado-Jarana et al. 2005). The Δ*fgb1* of *F. oxysporum* f. sp. *lycopersici* showed increased vegetative growth on solid medium. It, however, showed reduced invasive growth on tomato fruits by injecting the microconidial suspension. A Gβ subunit, GBB1, in heterothallic *F. verticillioides* (Sacc.) Nirenberg (synonym, *G. moniliformis* Wineland) was involved in pathogenicity and mycotoxin (fumonisin B_1) production, but not in vegetative growth or sexual reproduction (Sagaram and Shim 2007). The genes encoding three Gα subunits, *GzGPA1*, *GzGPA2* and *GzGPA3* and a Gβ subunit, *GzGPB1*, were characterized in homothallic *F. graminearum* Schawabe (synonym, *G. zeae* (Schwein.) Petch) (Yu et al. 2008), where *GzGPA1* was involved in sexual reproduction and negative regulation of mycotoxin (deoxynivalenol and zearalenone) production; *GzGPB1* in the negative regulation of the mycotoxin production; *GzGPA2* and *GzGPB1* in normal hyphal growth and pathogenicity.

Several MAPKs have been characterized in *Fusarium* species. The FMK1 of *F. oxysporum* f. sp. *lycopersici* and Gpmk1 of *F. graminearum* are homologues of Fus3/Kss1 in *S. cerevisiae* and PMK1 in *P. oryzae*. Whereas Δ*gpmk1* of *F. graminearum* showed defects of growth, hyphal morphology and conidiation, no morphological difference was observed from Δ*fmk1* of *F. oxysporum* f. sp. *lycopersici* during vegetative growth. The Δ*gpmk1* of *F. graminearum* and Δ*fmk1* of *F. oxysporum* both showed reduction of expression/secretion of cell wall degrading enzymes (CWDEs) and reduced invasive growth on host plant (Di Pietro et al. 2001). These reports suggested that Fus3/Kss1 homologues are related to the penetration into host tissues and the colonization by regulating CWDE activity in *Fusarium* species (Jenczmionka et al. 2003; Urban et al. 2003; Jenczmionka and Schäfer 2005). The MGV1 of *F. graminearum* was isolated as a homologue of MAPK Slt2 in *S. cerevisiae* (Hou et al. 2002). The Δ*mgv1* showed the reduced vegetative growth and virulence to wheat and hypersensitivity to Driselase (Kyowa-Kirin), a CWDE for fungus. Both Gmpk1 and MGV1 were involved in female fertility same as PMK1 and MPS1 in *P. oryzae* (Hou et al. 2002; Urban et al. 2003). Deletion of these MAPKs caused decrease of mycotoxin production on host plant (Hou et al. 2002; Urban et al. 2003). Introduction of dominant activating Gα of

Table 1 – Comparison of amino acid sequences of GPB1 and MPK1 with homologues in fungi.

Species	GPB1	MPK1
	Gene [accession number/identity (similarity)]	Gene [accession number/identity (similarity)]
Fusarium oxysporum f. sp. *lycopersici*	*fgb1* [AY219172/100 (100)]	*FMK1* [AF286533/100 (100)]
F. fujikuroi	–	*mk1* [AJ309177/98.6 (98.6)]
F. graminearum	*GzGPB1* [FG04104.1/91.4 (91.4)]	*pmk1* [AF448230/98.6 (98.6)]
F. solani	–	*FsMAPK* [Q00859/99.2 (99.2)]
Pyricularia oryzae	*MGB1* [AB086901/95.3 (96.9)]	*Pmk1* [U70134/97.5 (98.0)]
Neurospora crassa	*GNB-1* [AF491286/91.4 (94.7)]	*mak-2* [AF348490/95.5 (97.5)]
Aspergillus nidulans	*SfaD* [AF056182/81.9 (88.0)]	*makB* [AN3719.2/89.2 (92.3)]

Identity, the degree of correspondence between the two sequences; Similarity, the degree of resemblance (supposed to encode the identical amino acids) between the two sequences; –, not determined.

Aspergillus nidulans (Eidam) G. Winter, *fadA*^{G42R}, into *F. sporotrichioides* Sherb. enhanced T-2 toxin production (Tag et al. 2000).

Plant pathogenic ascomycete *G. fujikuroi*-species complex consists of at least nine mating populations (A–I), each of which is recognized as a species at present (Kerenyi et al. 1999; Zeller et al. 2003). They are distributed worldwide and cause diseases on diverse hosts, such as banana, maize, rice and sugarcane. In this study, we characterized Gβ and Fus3/Kss1 MAPK homologues in *F. sacchari* (E.J. Butler & Hafiz Khan) W. Gams (synonym *G. sacchari* Summerell & J.F. Leslie; mating population B in the species complex). We found out that the Gβ and the MAPK were involved in vegetative hyphal growth and female fertility, but not in pathogenicity in the species.

2. Materials and methods

2.1. *Fungal strains, culture and cross conditions*

Strains FGSC (Fungal Genetic Stock Center, Kansas City, MO, USA) 7610 (MAT1-2) and FGSC 7611 (MAT1-1) of *F. sacchari* were routinely maintained on PSA (Arie et al. 1999). CMC medium (1.5% (w/v) sodium carboxymethylcellulose, 0.1% (w/v) NH_4NO_3, 0.1% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4 \cdot H_2O$, 0.1% (w/v) yeast extract) was used for quantification of biomass. CMC was replaced with 1.5% (w/v) glucose to estimate the ability of utilization of CMC as the carbon source. V8-juice agar solid medium (VA) was used for other experiments. To elucidate the relationship between cAMP (cyclic adenosine monophosphate) and Gβ or MAPK, 10 or 30 mM cAMP (SIGMA) was added into VA.

Crossing of *F. sacchari* was performed by a method described by VanEtten (1978). Briefly, conidial suspension of a male strain was poured on a female strain cultured on VA at 26 °C. After 5 min, the conidial suspension was decanted and the fertilized cultures were incubated at 26 °C under 12 h light/12 h dark conditions.

2.2. *Cloning of* gpb1 *and* mpk1

Fusarium sacchari was statically cultured on minimal liquid medium at 25 °C for 1 wk. Total cellular DNA and RNA were prepared from the powdered mycelia as described by Adachi et al. (1993) and Timberlake (1986), respectively. The total RNA was treated with RNase-free DNase I (Roche Diagnostics, Tokyo, Japan) to eliminate contaminated DNA. The mRNA was prepared from the total RNA by mRNA Separator Kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions and used for synthesis of a cDNA pool by Marathon cDNA Amplification Kit (Takara Bio). The primers B2F 5′-ATHTAYGCNATGCAYTGG-3′ and B1R 5′-AARTCRTCRTANCCNGC-3′ generated from the conserved region in Gβ genes in ascomycetes were used for amplification of a 0.8-kb internal region of *gpb1* cDNA from FGSC 7611. Primers designed from the sequence of *gpb1* internal region were used for 5′ and 3′-RACEs by Advantage 2 PCR Kit (Takara Bio). A primer GFGB4 5′-CCGTCAGGGAAGAATTGGATGGC-3′ and a nested primer GFGB3 5′-GGTTGGTAGGGTTAAGGCTGATG-3′ were used for 5′-RACE, and a primer GFGB1 5′-ACTTCGTTGCTTGCGGTGGTC TC-3′ and a nested primer GFGB2 5′-ATCGCAGCATCCTAA-CATCCTCG-3′ for 3′-RACE. The amplification was performed by Touchdown PCR according to the manufacturer's recommendations. Primers GFGB6 5′-CAATCCATCCACCAACCT CAGCC-3′ and GFGB7 5′-TGCTCGGTATAGCCGTCAACTCC-3′ were used for amplification of 2.2-kb genomic DNA fragment including *gpb1* coding region. To amplify a 0.4-kb internal region of *mpk1* from FGSC 7611 genomic DNA, the primers FMAPK1 5′-TACTTCAACCACGAGAACATCAT-3′ and FMAPK2 5′-TCAATGGCCTTGGTGTACTCCTT-3′ were designed from nucleotide sequence of *FsMAPK* in *F. solani* (Mart.) Sacc. f. sp. *pisi* W.C. Snyder & H.M. Hansen (Li et al. 1997).

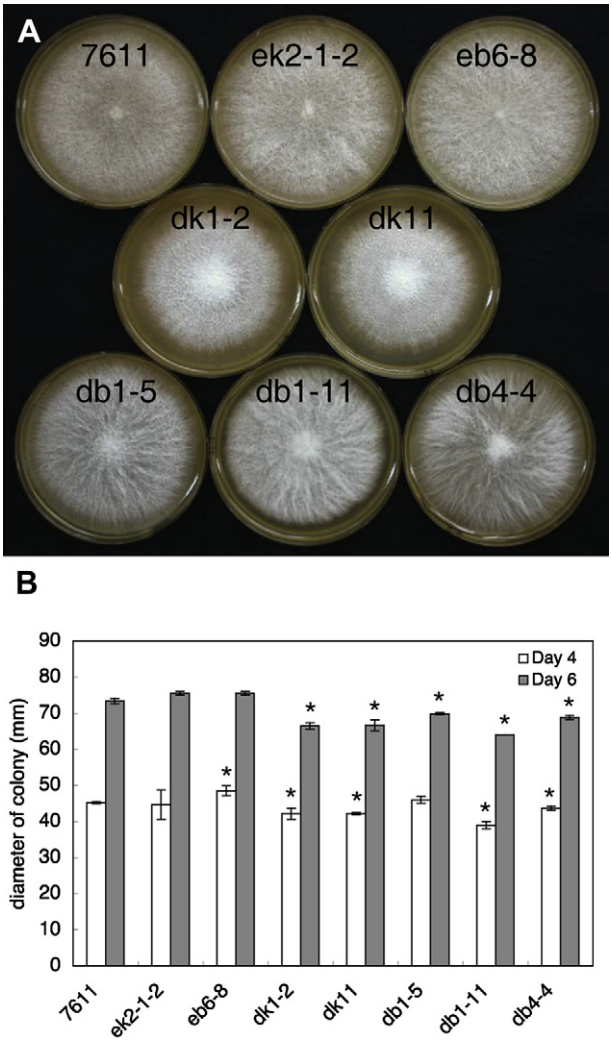

Fig. 1 – Vegetative growth of Δ*gpb1* and Δ*mpk1* on solid medium. A: Colony grown for 6 days. B: Size of the colony. Two micro litre of 10^2 conidia/μl suspension was inoculated on centre of VA and incubated at 26 °C. Diameter of colony was measured 4 and 6 days after inoculation. Mean value of 3 independent cultures was represented. 7611, FGSC 7611 (wild type); ek2-1-2 and eb6-8, ectopic transformants of FGSC 7611 carrying *mpk1* and *gpb1* disruption vectors, respectively; dk1-2 and dk11, Δ*mpk1* mutants; db1-5, db1-11 and db4-4, Δ*gpb1* mutants. Asterisk indicates significant differences by Tukey test ($P = 0.05$) between the control (FGSC 7611).

Full-length cDNA of *mpk1* was cloned by the same strategy as *gpb1* was done. The primer FMAPK2 and a nested primer FMAPK4 5′-AGACCGAAATCGCAGACCTTGAG-3′ were used for 5′-RACE, and the primer FMAPK1 and a nested primer FMAPK5 5′-CCAGGACCTTTCTGACGACCACT-3′ for 3′-RACE. Primers FMAPK8 5′-ACAGCCCAGCCCAGTTCAAGCCA-3′ and FMAPK10 5′-TCATCAAATCATCTTCAAGTCCC-3′ were used for amplification of 1.8-kb genomic DNA fragment including *mpk1* coding region. All PCR products were cloned in pGEM-T Easy vector (Promega). DNA sequences were determined using automated fluorescent DNA sequencer 377 (Life Technologies Japan, Tokyo, Japan) at Bioarchitect DNA Sequence Facility in RIKEN, Wako, Saitama, Japan.

2.3. *Vectors and transformation of fungi*

The *gpb1* disruption vector, pGGBH1, was derived form pGEMGB containing the above-mentioned 2.2-kb *gpb1* fragment in pGEM-T Easy vector (Promega, Tokyo, Japan). A 2.4-kb *Xho* I-*Eco*R V fragment of hygromycin phosphotransferase gene (*hph*) with *TrpC* promoter and terminator was prepared from pCSN43 (Staben et al. 1989) and inserted into the *Bcl* I site in *gpb1*, which locates at 743-bp downstream of the initiation codon, in pGEMGB.

The *mpk1* disruption vector, pGMKH1, was derived from pGEMGMK containing the above-mentioned 1.8-kb *mpk1* fragment in pGEM-T Easy vector. The *hph* cassette was inserted into the *Eco*R V site in *mpk1*, which locates at 314-bp downstream of the initiation codon.

Plasmids pII99 (Namiki et al. 2001) and pTEF-EGFP (Vanden Wymelenberg et al. 1997) were kindly gifted from Dr. T. Tsuge, Nagoya University and used for co-transformation to label

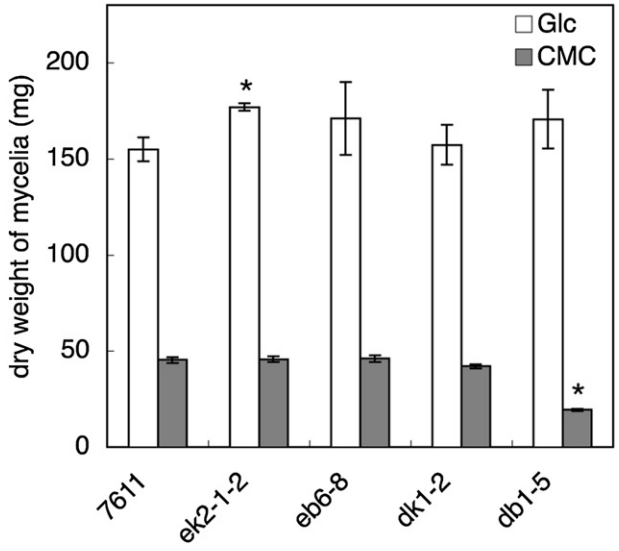

Fig. 2 – Biomass of Δ*gpb1* and Δ*mpk1* grown on liquid medium. Conidia of 10^7 were transplanted on 50 ml CMC medium (CMC; grey column) or glucose-containing medium (Glc; white column) and shaken at 26 °C for 7 days. Harvested mycelia were lyophilized and used for measuring dry weight. Mean values of 3 independent cultures were represented. 7611, FGSC 7611 (wild type); ek2-1-2 and eb6-8, ectopic transformants of FGSC 7611 carrying *mpk1* and *gpb1* disruption vectors, respectively; dk1-2, Δ*mpk1* mutant; db1-5, Δ*gpb1* mutant. Asterisk indicates significant differences by Tukey test ($P = 0.05$) between the control (FGSC 7611).

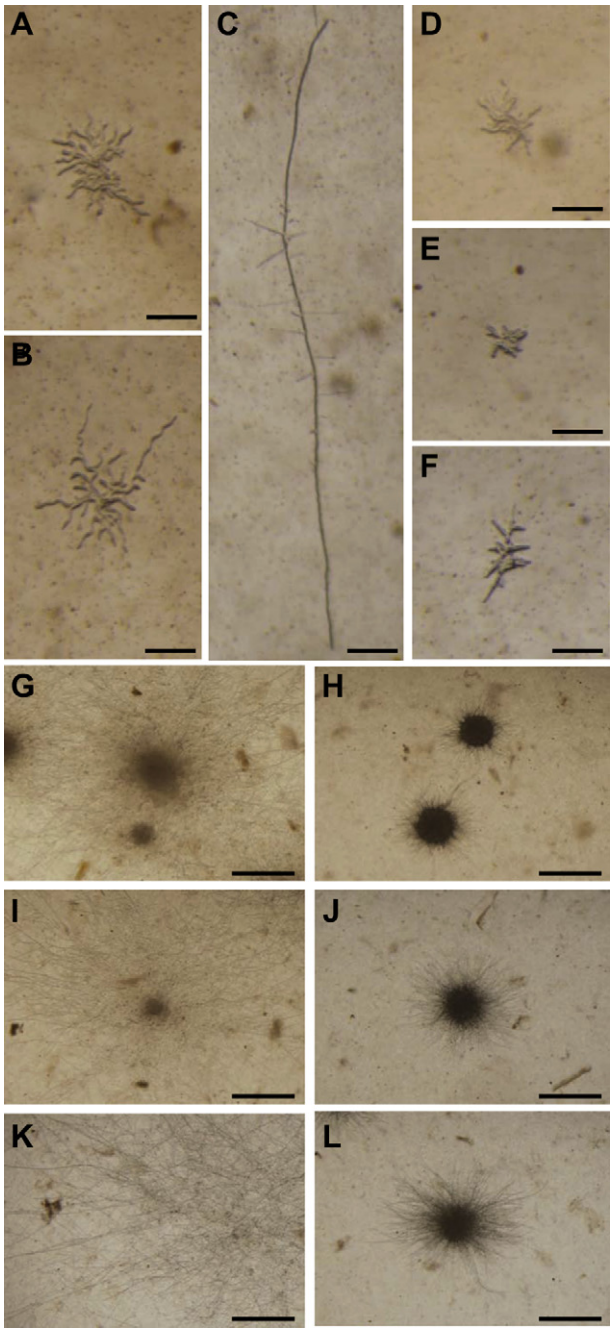

Fig. 3 – Morphology of hyphae of Δ*gpb1* and Δ*mpk1*. Conidia were spread on VA and incubated at 26 °C. A–F: Germinating conidia 20 h after inoculation. G–L: Colony 44 h after inoculation. A, D, G and H: FGSC 7611 (wild type); B, E, I and J: dk1-2 (Δ*mpk1*); C, F, K and L: db1-5 (Δ*gpb1*). D–F, H, J and L were grown on medium containing 30 mM cAMP. *Bars* 0.1 mm in A–F and 1 mm in G–L.

mycelia with GFP. The transformation was performed as described by Kawabe et al. (2004).

2.4. *Quantification of intracellular cAMP*

cAMP was extracted from the mycelia grown on VA for 5 d at 26 °C. Harvested mycelia were ground in liquid nitrogen and suspended in distilled water. Quantity of soluble protein in each suspension was measured and used for normalization of extraction efficiency. Protein in the suspension was precipitated with equal volume of 12% (w/v) TCA. Assay was performed by cAMP Biotrak Enzyme immunoassay (EIA) System (GE Healthcare Japan, Tokyo, Japan) according to the manufacturer's instructions.

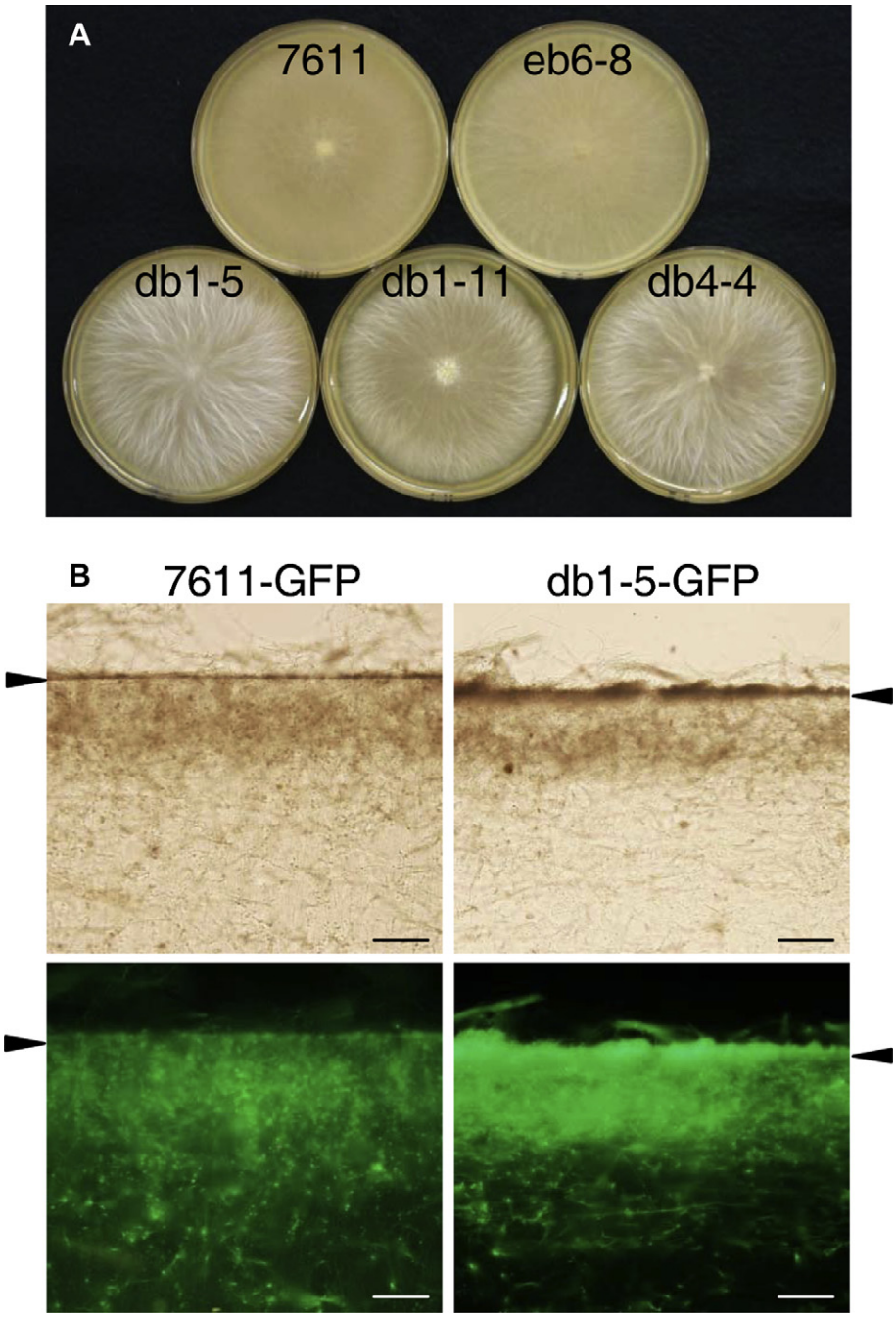

Fig. 4 – Morphology of substrate hyphae Δ*gpb1*. A: Substrate hyphae grown on VA. Distilled water was poured into 5-day-old culture, and aerial hyphae were scraped out using glass rod. 7611, FGSC 7611 (wild type); eb6-8, an ectopic transformant of FGSC 7611 carrying *gpb1* disruption vector; db1-5, db1-11 and db4-4, Δ*gpb1* mutants. B: Hyphae penetrating solid medium. FGSC 7611 and db1-5 expressing eGFP were grown on VA for 1 wk. Media with mycelia were sliced vertically and observed under normal light (upper panels) and UV (lower panels). Arrowhead indicates the surface of the medium. *Bars* 200 μm.

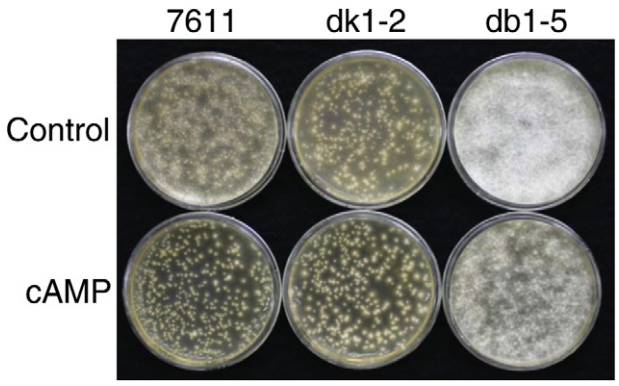

Fig. 5 – Aerial hyphae formation of Δ*gpb1* and Δ*mpk1*. Conidia of FGSC 7611 (7611), Δ*mpk1* (dk1-2) and Δ*gpb1* (db1-5) were spread on VA with or without cAMP (10 mM) and incubated for 3 d at 26 °C.

2.5. *Accession numbers*

The sequences of *gpb1* and *mpk1* have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AB242269 and AB242270, respectively.

3. Results and discussion

3.1. *Isolation and disruption of* gpb1 *and* mpk1 *in* F. sacchari

Full-length cDNAs and genomic DNA fragments including entire coding regions of Gβ and Fus3/Kss1 MAPK homologue genes were cloned from *F. sacchari* FGSC 7611 and designated *gpb1* and *mpk1*, respectively. Comparison between sequences of the genomic DNA and the cDNA of *gpb1* and *mpk1*, respectively, identified 4 and 3 introns all of which carried the conserved sequences of 5′ and 3′ splicing signals. Southern blot analysis revealed that *gpb1* and *mpk1* each was single-copy

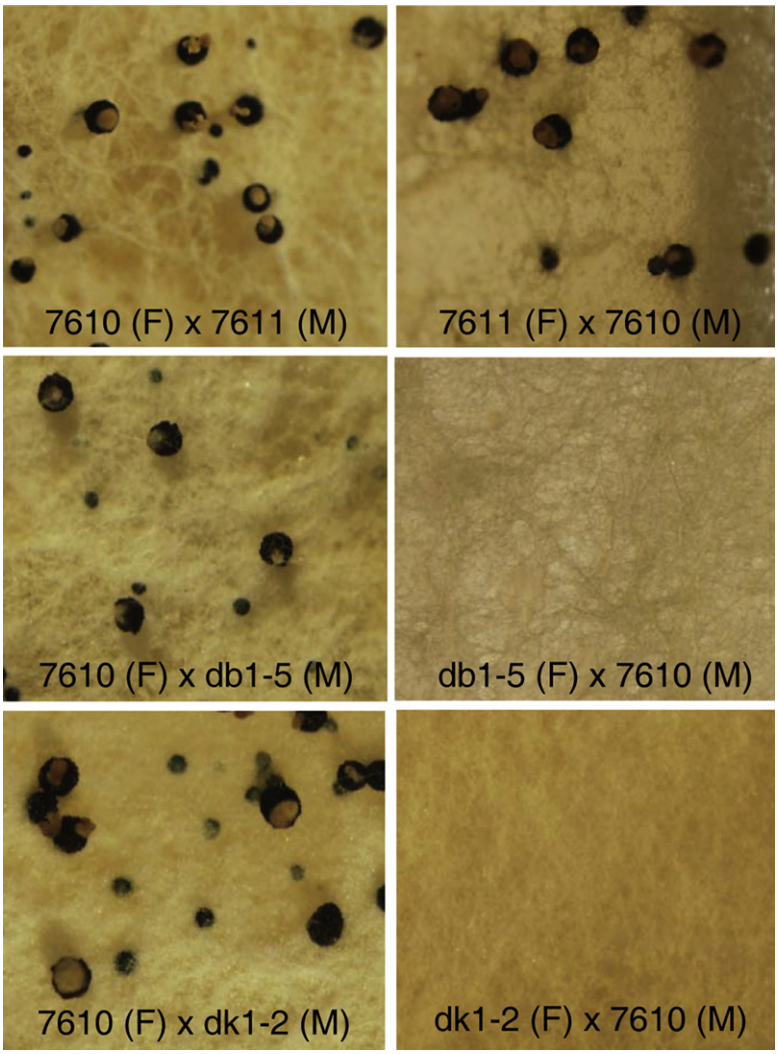

Fig. 6 – Sexual reproduction of Δ*gpb1* and Δ*mpk1*. Male and female fertilities of FGSC 7611 (7611), Δ*mpk1* (dk1-2) and Δ*gpb1* (db1-5) were tested using FGSC 7610 (MAT1-2) as a tester. F and M indicate the strains used as female and male, respectively.

gene in *F. sacchari* (data not shown). GPB1 and MPK1 consisted of 359 and 355 amino acids, respectively.

Deduced amino acid sequences of GPB1 and MPK1 were completely identical to the homologues in *F. oxysporum* f. sp. *lycopersici* (Table 1). The homology of the amino acid sequence of MPK1 to MK1 in *F. fujikuroi* Nirenberg (synonym, *G. fujikuroi* (Sawada) Wollenw.; *G. fujikuroi*-species complex MP-C) was lower than FMK1 in *F. oxysporum* (Table 1). The difference in homology is caused by the amino acid exchanges at the position 279–283, from KTSDL in FMK1 to QDLGS in MK1. A nucleotide insertion and a deletion result in partial frame shift at the region coding the amino acids in MK1. Except for the site, the amino acid sequence of MK1 is identical to MPK1 and FMK1.

To analyze the function of GPB1 and MPK1, each of them was disrupted by insertion of an *hph* cassette into the coding region in FGSC 7611. Nine and four disruptants for *gpb1* and *mpk1* were identified by PCR and Southern blot analysis from 61 and 118 transformants, respectively. Three *gpb1* disruptants (Δ*gpb1*), db1-5, db1-11 and db4-4, and two *mpk1* disruptants (Δ*mpk1*), dk1-2 and dk11, were mainly used for the following experiments. Transcripts of *gpb1* and *mpk1* were not detected form Δ*gpb1* and Δ*mpk1*, respectively, by RT-PCR. Three transformants eb6-8, eb2-4 and ek2-1-2 carrying ectopically inserted disruption vector were used as controls.

3.2. *Vegetative growth of Δgpb1 and Δmpk1*

Both Δ*gpb1* and Δ*mpk1* showed slightly slower radial growth on VA than FGSC 7611 (Fig. 1A and B). The conidial production of Δ*gpb1* and Δ*mpk1* was comparable to FGSC 7611 on solid VA medium, and liquid PSB and CMC media. The biomass of Δ*gpb1* was significantly reduced to 42.6% of wild type FGSC 7611 in CMC liquid media, and the suppression of growth was relieved by addition of glucose (Fig. 2). These results suggested that GPB1 was involved in utilization of cellulose as carbon source. When Δ*gpb1* and Δ*mpk1* were cultured on the solid VA medium overlaid with the cellophanes, the degradation of cellophanes by them was slower than wild type (data not sown), suggesting that both GPB1 and MPK1 are involved in cellulose degradation. CWDEs, such as cellulases, pectinases and xylanases, have been considered as important factors in invasion and colonization of *Fusarium* species in host plant (Kang and Buchenauer 2000, 2003; Wanjiru et al. 2002; Roncero et al. 2003). Disruption of MAPK gene decreased an activity of extracellular polygalacturonase and an expression level of *endo*-pectate lyase gene in *F. oxysporum* f. sp. *lycopersici*, and decreased the activities of secreted CWDEs including xylanolytic and proteolytic enzymes in *F. graminearum* (Di Pietro et al. 2001; Delgado-Jarana et al. 2005; Jenczmionka and Schäfer 2005). Δ*gpb1* and Δ*mpk1*, however, maintained pathogenicity to sugarcane (*Saccharum* sp.; cvs. F177 and NiF8) in this study (data not shown).

Although no significant difference was observed in the frequency of conidial germination in Δ*gpb1* and Δ*mpk1*, Δ*gpb1* showed abnormal hyphal growth after germination. The Δ*gpb1* hyphae had few branches and grew straight by predominate apical extension of primary hyphae (Fig. 3C). Similar abnormal hyphal growth was reported in ΔGβ of *F. oxysporum*, *F. verticillioides*, *C. heterostrophus* and *Cryphonectria parasitica* (Murrill) M.E. Barr (Kasahara and Nuss 1997; Ganem et al. 2004; Delgado-Jarana et al. 2005). Kasahara and Nuss (1997) described the formation of highly dense mycelial mat on solid media by ΔGβ of *C. parasitica*. When eGFP-labelled Δ*gpb1* was grown on VA, Δ*gpb1* formed more profuse hyphae and thicker hyphal layer over and at around the surface of the medium than FGSC 7611 (Figs. 3K and 4A). Vertical extension of hyphae into VA tended to be reduced in Δ*gpb1* (Fig. 4B). These phenotypes were not observed in Δ*mpk1* (Fig. 3B and I). The Δ*gpb1* formed profuse aerial hyphae in 3 days after spreading conidia on VA (Fig. 5). Oppositely, Δ*mpk1* produced less aerial hyphae than wild type (Fig. 5). Thus, GPB1 regulates aerial hyphae formation negatively, and MPK1 does positively. Reduced aerial hyphae in Δ*gpmk1* in *F. graminearum* has been reported (Jenczmionka et al. 2003).

Jain et al. (2003) and Jain et al. (2005) reported that the disruptants of the genes (*fga1* and *fga2*) of heterotrimeric G protein α subunit and β subunit *fgb1*) showed increased heat tolerance in conidia germination of *F. oxysporum* f. sp. *cucumerinum*. But the phenotype was not observed in Δ*gpb1* or Δ*mpk1* of *F. sacchari* (data not shown).

3.3. *Fertility of Δgpb1 and Δmpk1*

In homothallic *F. graminearum*, two MAPKs, Gpmk1 (a Fus3/Kss1 homologue) and MGV1 (a Slt2 homologue), are involved in formation of perithecia (Hou et al. 2002; Urban et al. 2003). A Gα, GzGPA1, is involved in sexual reproduction in *F. graminearum*, but Gβ, GzGPB1, is not (Yu et al. 2008). The perithecia and the ascospores were produced when a wild type strain FGSC 7610 was used as the female for crossing with Δ*gpb1* or Δ*mpk1* of *F. sacchari* (Fig. 6). But no perithecium was observed

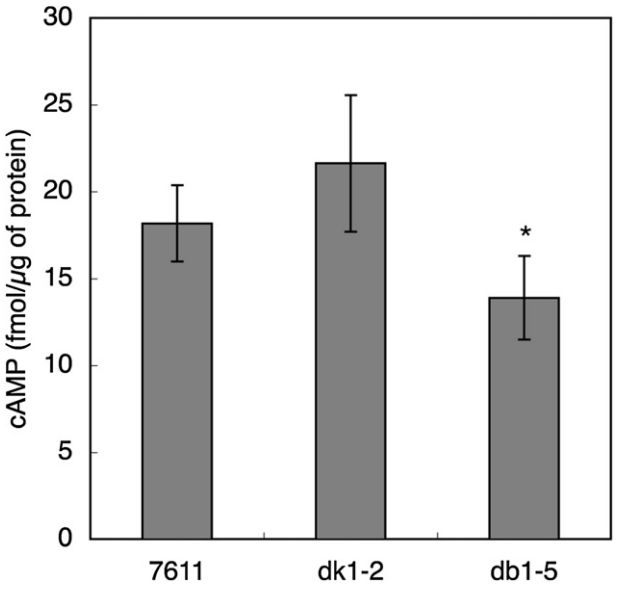

Fig. 7 – Intracellular cAMP level in Δ*gpb1* and Δ*mpk1*. Assay was repeated twice for each sample. The value represents the mean of three independent samples for each strain. FGSC 7611 (wild type); Δ*mpk1* (dk1-2); Δ*gpb1* (db1-5). Asterisk indicates significant differences by Tukey test ($P = 0.05$) between the control (FGSC 7611).

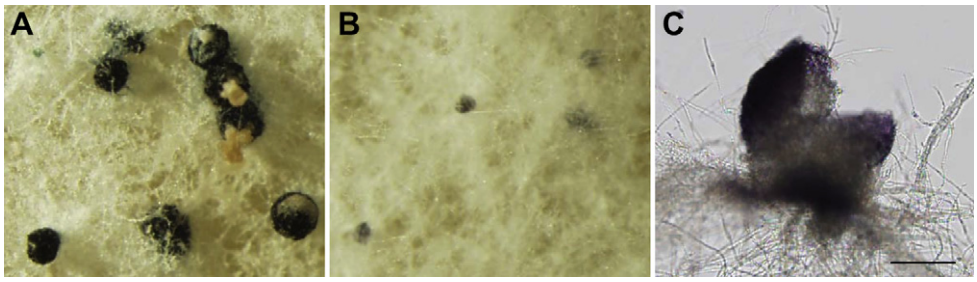

Fig. 8 – Effect of cAMP on female fertility. FGSC 7611 grown on VA without (A) or with (B) 10 mM cAMP for a week was fertilized with FGSC 7610. Perithecium-like structure from the crossing on cAMP-containing media was crashed to observe inside (C). Pictures were taken in 22 days after fertilization. Magnification of A and B is ×240. *Bar* 100 μm.

when Δ*gpb1* or Δ*mpk1* was used as the female for crossing with FGSC 7610 (Fig. 6). These results indicate that both GPB1 and MPK1 were essential for female fertility in *F. sacchari*.

3.4. *Effect of cAMP on Δ*gpb1 *and Δ*mpk1

The intracellular cAMP level in Δ*gpb1* was 76.4% of FGSC 7611; cAMP in Δ*mpk1* seemed more than FGSC 7611, although it was not significant (Fig. 7). The reduction of intracellular cAMP level has been observed in the disruptants of Gα gene *fga1* and Gβ gene *fgb1* in *F. oxysporum* f. sp. *cucumerinum* (Jain et al. 2002, 2003). The Gβ disruptants of *Fusarium* spp. form less branched hyphae (Fig. 3C; Delgado-Jarana et al. 2005). Oppositely, exogenous cAMP caused profuse hyphal branching in *F. graminearum* (Robson et al. 1991). These results suggested that the abnormal vegetative growth in Gβ disruptants of *Fusarium* spp. including *F. sacchari* was resulted from the depletion of intracellular cAMP. To elucidate the relationship between the morphological change and cAMP level, conidia of FGSC 7611, Δ*gpb1* and Δ*mpk1* were plated on cAMP-containing VA. The addition of 30 mM cAMP clearly suppressed hyphal growth in both FGSC 7611 and the disruptants (Fig. 3D–F). Wild type produced small round colony with short aerial hyphae in 44 h after plating (Fig. 3H). Accelerated apical extension in Δ*gpb1* was restored by exogenous cAMP, but it still showed abnormal hyphal extension and branching (Fig. 3F). When wild type strain was cultured on 10 mM cAMP-containing VA as female, perithecia formation was drastically suppressed (Fig. 8). In only one case of nine repeats, a few small perithecia-like structures were produced on cAMP-containing VA, but no ascospore was observed in these perithecia-like structures even after 4 wk-fertilization (Fig. 8). The female sterility in the Δ*gpb1* was not restored by addition of cAMP. Aerial hyphae formation of wild type on cAMP-containing VA was similar to Δ*mpk1*, indicating the possibility that the cAMP inhibits MPK1 pathway in *F. sacchari* (Fig. 5). In *Sclerotinia sclerotiorum* (Lib.) de Bary, exogenous cAMP suppresses the expression and the phosphorylation of MAPK, *smk1*, which regulates the growth and sclerotial development (Chen et al. 2004).

Our present results presented that GPB1 and MPK1 oppositely regulated hyphal branching and aerial hyphae formation. These indicated that appropriate regulation of both Gβ and MAPK was necessary to normal mycelial growth, which leads to initiate formation of protoperithecium. This might be the reason of female sterility in Gβ- and MAPK-disruptants. In this study we suggested that cAMP interferes MAPK pathway in both vegetative and sexual phases. Suppression of female fertility by cAMP supports this. Further experiments are required to clarify the function of cAMP in life cycle of *F. sacchari*.

Disclosure

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