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Geranylgeranyl diphosphate synthase genes in entomopathogenic fungi

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Abstract Based on comparative amino-acid sequence alignment of geranylgeranyl diphosphate (GGPP) synthase from filamentous fungi, degenerated oligonucleotide primers were designed for searching GGPP synthase gene (s) in entomopathogenic fungi. Polymerase chain reaction with the designed primers amplified GGPP synthase homologues from five representative entomopathogenic fungi: *Metarhizium anisopliae, Beauveria bassiana, Verticillium lecanii, Paecilomyces farinosus*, and *Nomuraea rileyi*. Sequence comparison of the amplified of GGPP synthase homologue fragments revealed that *M. anisopliae* and *B. bassiana* have at least two different types of the GGPP synthase gene homologues. The first type (designated as *ggs1*), which is highly conserved among the five strains, has a unique Ser-rich region, SSXSSVSGSSS (X refers to L, A, V, or S), and is constitutively expressed throughout growth. In contrast, the second type of GGPP synthase gene homologue (*ggs2*) was discovered only in some strains, and genes of this type possessed high similarity to each other but showed relatively weak similarity to the *ggs1* genes, with no detectable transcription under the cultivation conditions applied in this experiment. The *ggs1* cloned from *M. anisopliae*, which encoded a putative protein of 359 amino acid residues, was heterologously expressed in *E. coli*. The recombinant protein showed activity to synthesize GGPP from farnesyl diphosphate and isopentenyl diphosphate. These results strongly suggested that the *ggs1* gene encodes a GGPP synthase involved in primary metabolism.

Keywords Geranylgeranyl diphosphate synthase · *Metarhizium anisopliae*

Introduction

In recent years, considerable attention has been paid to isoprenoid compounds, which include not only molecules essential for cell survival but various bioactive compounds produced through secondary metabolism (Scchettini and Poulter 1997). Isoprenoids (also referred to as terpenoids) are synthesized by a consecutive head-to-tail condensation of C_5 isoprene units, such as dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), and are classified by the number of five-carbon units present in their structures (Wang and Ohnuma 2000). During the elongation process of the isoprene unit, geranylgeranyl diphosphate (GGPP) synthase catalyzes the third condensation reaction of IPP to produce GGPP (C20). GGPP is the precursor of diterpenoids and locates at the branching point to many important isoprenoids for both primary metabolites including geranylgeranylation of G proteins for their function, the formation of ubiquinone in mitochondria and

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dolichols in polysaccharide biosynthesis, and secondary metabolites, such as carotenoids, gibberellins (Chappell 1995). In filamentous fungi, multiple GGPP synthase genes have been discovered by genome sequencing, and the encoded GGPP synthases seem to have distinctive functions. In *Gibberella fujikuroi* and *Penicillium paxilli*, two different types of GGPP synthase genes have been isolated: genes of the first type (*ggs1* from *G. fujikuroi* and *ggs1* from *P. paxilli*) are considered to encode GGPP synthases responsible for primary metabolism, while genes of the second type (*ggs2* from *G. fujikuroi* and *paxG* from *P. paxilli*) have been shown to work specifically in secondary metabolism, based on their location in the middle of the biosynthetic gene cluster and the loss of gibberellin/paxilline production in the disruptants (Tudzynski 2005; Young et al. 2001). The fact that the presence of the first type of GGPP synthase (*ggs1* of *G. fugikuroi* or *ggs1* of *P. paxilli*) could not compensate for the defect in the disruptants strongly suggested that each GGPP synthase of the second type has specific intracellular localization or other supplementary proteins to dictate the particular pathway in which it should participate.

Entomopathogenic fungi are classified as fungi that infect, invade into, and eventually kill their host insects. Several studies have investigated their entomopathogenicity and/or the toxins they emit against insects, such as the destruxins from *Metarhizium anisopliae* (Gupta et al. 1989), or the beauvericin and bassianolide isolated from *Beauveria bassiana* (Grove and Pople 1980), from the standpoint of their usefulness as pest control agents. Moreover, because complexes consisting of the entomopathogenic fungi and their host insects have been used for more than 600 years as herbal medicines to promote overall vitality and human longevity by strengthening the immune system (Liu et al. 2002), the entomopathogenic fungi have been considered promising sources for the discovery of novel bioactive compounds. Although both primary metabolites and several bioactive secondary metabolites of isoprenoidal nature, such as tenuipesines from *Paecilomyces tenuipes* (Kikuchi et al. 2004) and helvolic acid from *Metarhizium anisopliae* (Lee SY et al. 2008), have been identified in entomopathogenic fungi, no sequence of the GGPP synthase gene of entomopathogenic fungi has been reported due to the lack of adequate methods and tools for molecular genetic manipulation in these species.

In this report, two types of GGPP synthase gene homologues were amplified from entomopathogenic *M. anisopliae* and *B. bassiana* genome. The first GGPP synthase gene, named *ggs1*, is universally conserved among entomopathogenic fungi, but the second one was isolated specifically from *M. anisopliae and B. bassiana*, indicating that this gene played a unique role. The entire cDNA of the *ggs1* gene from *M. anisopliae* was overexpressed in *E. coli*, and the product was characterized enzymatically and identified as GGPP synthase.

Materials and methods

Strains and cultivation conditions

Metarhizium anisopliae strain NAFF 635007 (The National Institute of Agrobiological Sciences Genebank) and other entomopathogenic fungi studied in this work were isolated from insect larvae bodies collected in several regions of Japan (Lee et al. 2005). The fungi were streaked on a potato dextrose agar (PDA; Difco, Detroit, MI) plate, incubated at 25°C for 5 days and stored at 4°C until use. For submerged cultivation, the stock culture was inoculated on a PDA plate at 25°C for 5 days. The grown mycelium was cut out as an 8-mm circle together with the agar, inoculated into 100 ml of Sabouraud maltose-yeast extract medium (SMY) consisting of 4% maltose, 1% Bacto peptone, and 1% yeast extract in a 500-ml baffled Erlenmeyer flask, and cultivated at 25°C at 180 rpm on a rotary shaker for 5–7 days.

Polymerase chain reaction amplification and sequence analysis of GGPP synthase genes

Genomic DNA of entomopathogenic fungi was prepared as described previously by Raeder and Broda (1985) with some modifications. TaKaRa Ex *Taq*™ (Takara Bio, Inc., Tokyo, Japan) polymerase was used for all amplifications of partial GGPP synthase genes, and polymerase chain reaction (PCR) amplification was performed in a 20-μl reaction mixture including 50 ng of genomic DNA as a template, 2 μl of 10× Ex *Taq* buffer, 2 μl of dNTP mixture (2.5 mM each), 10 pmol of each primer and 1 unit of polymerase. To amplify the larger part of the GGPP synthase gene from the five tested strains, a degenerate primer set (ggppF, 5′-CGAATTCTGCAGGNCARGGNATGGAYCTNTTYTGG-3′ and ggppR, 5′-CCGAATTCTGCAGTCNGTYAARTCYTCRCA-3′) was designed based on the amino-acid sequences of known fungal GGPP synthases for the conserved regions IV and VI proposed by Koyama (1999). A touch-down program composed of two separate PCR phases was adopted for the amplification: the first amplification phase was composed of an initial denaturation step at 95°C for 5 min and eight cycles consisting of denaturation (94°C for 30 s), annealing (an initial cycle of 60°C for 30 s followed by two cycles each at 58°C, 56°C, and 54°C), and extension (72°C for 1 min); the second phase was composed of 30 cycles consisting of denaturation (94°C for 30 s), annealing (54°C for 30 s), and extension (72°C for 1 min), followed by a final extension at 72°C for 7 min. Conserved regions suitable for designing

specific *ggs1* primers were identified based on the alignments of amplified nucleotide sequences of *ggs1*. The second primer set, ggsF (5′-CCGAATTCAGGCYGAG TCGTCSKYGTCGTCCGTG-3′) and ggsR (5′-CCGAATT CTCGTCSKYGTCGTCCGTGAGCGGCAGC-3′), was designed and used to amplify fragments containing a Ser-rich region from a wide range of entomopathogenic fungi (Table 1) using the following PCR protocol: initial denaturation (95°C for 5 min), followed by 30 cycles of denaturation (94°C for 30 s), annealing (64°C for 30 s), and extension (72°C for 1 min), with a final extension of 72°C for 7 min.

Each PCR product was subcloned into pUC19 (Takara) with *Escherichia coli* XL10-Gold Ultracompetent cells (Stratagene, La Jolla, CA) as the recipient for transformation. The subcloned fragment was sequenced using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems/ Hitachi, Foster City, CA) and a BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted using an RNeasy Plant Mini-Kit (QIAGEN, Tokyo, Japan) according to the procedures recommended by the manufacturer. First-strand cDNA was synthesized from 1 μg of total RNA by SuperScript™ III reverse transcriptase according to the manufacturer's protocol (Invitrogen, Paisley, Scotland) and was used as a template for amplification of *ggs1* using the ggsF/ggsR primer set. The primer set for actF (5′-CCGAATTCCACC GATCCAGACAGAGTACTTTCGC-3′) and actR (5′-CC GAATTCGACATCAAGGAGAAGCTCTGCTACGTC-3′) was used to detect the expression of the actin gene as an internal standard. PCR conditions were the same except for the cycle number: 95°C for 5 min; followed by 32 cycles for *ggs1* or 30 cycles for the actin gene at 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min; with a final extension step of 72°C for 7 min. The amplified cDNA was held at 4°C, and the PCR product was analyzed by electrophoresis on 2% agarose gel.

Northern blot analysis

Total RNA (7.5 μg) were electrophoresed on 1.2% agarose gel containing 16% formaldehyde in MOPS buffer and transferred to a Hybond-NX nylon membrane (Amersham Biosciences, Buckinghamshire, UK). Transferred RNA was crosslinked by UV exposure for 5 min. Hybridization was carried out at 65°C for 3 h in Rapid-hyb Buffer (Amersham Biosciences) using the ggsF/ggsR-amplified fragment from each strain labeled with [α-^{32}P]dCTP (Random Primer DNA Labeling kit version 2; Takara) as a probe. After several rounds of washing with 1X SSC buffer + 0.1% SDS and 0.5 SSC buffer +0.1% SDS (1X SSC containing 0.15 M NaCl and 0.015 M sodium citrate), the blot was exposed to X-ray film (Fuji, Tokyo, Japan) at −80°C. A ^{32}P-labeled actin probe was used as an internal standard.

Isolation of the 3′- and 5′- ends of the *ggs1* gene

The 3′- and 5′- ends of the cDNA were amplified by rapid amplification of cDNA ends (Gene Racer Kit; Invitrogen) as directed by the manufacturer. The cDNA templates were generated by reverse-transcription (RT) reaction with SuperScript™ III RT. Since the Ser-rich region of *ggs1* has a unique amino acid sequence, the primers for the rapid amplification of cDNA ends (RACE) method were designed from this conserved aerea. The 3′-RACE was performed with GeneRacer™ 3′ Primer as the reverse primer and 5′-AAGCTCATGCAGGCCGAGTCGTCCTCGTCG-3′ as the gene-specific forward primer. The cycling protocol was as follows: 94°C for 2 min; five cycles of 94°C for 30 s and 72°C for 2 min; five cycles of 94°C for 30 s and 70 °C for 2 min; 25 cycles of 94°C for 30 s, 68°C for 30 s and 72°C for 2 min; and a final elongation at 72°C for 10 min. The 5′-RACE was carried out with nested PCR. The first amplification was done using GeneRacer™ 5′ Primer as the forward primer, and 5′-CCGACTTTAAGTTGACGTAGTCGTCGC GGATCTG-3′ as the gene-specific reverse primer with a cycling protocol of 94°C for 2 min; five cycles of 94°C for

Table 1 Strains of entomopathogenic fungi used for analysis of the GGPP synthase genes

Fungal strain	Fungal strain
Akanthomyces cinereus HF490	*Metarhizium flavoviride* HF698
Akanthomyces pistillariaeformis HF286	*Nomuraea rileyi* NAFF635039
Akanthomyces novoguineensis HF749	*Paecilomyces* sp. HF747
Aschersonia aleyrodis HF724	*Paecilomyces* sp HF331
Beauveria bassiana NAFF635036	*Paecilomyces farinosus* NAFF635038
Cordyceps militarais HF696	*Paecilomyces fumosoroseus* HF254
Cordyceps ramosopulvinata HF746	*Paecilomyces tenuipes* HF535
Gibellula leiopus HF748	*Verticillium lecanii* NAFF635037
Metarhizium anisopliae NAFF635007	

30 s and 72°C for 2 min; five cycles of 94 °C for 30 sec and 70 °C for 2 min; 25 cycles of 94 °C for 30 sec, 60 °C for 30 sec and 72°C for 2 min; and a final elongation at 72°C for 10 min. Subsequently, the second round PCR was done using 1 μl of a tenfold dilution of the first round mixture with GeneRacer™ 5′ Nested primer as the forward primer and 5′-GCCGCTCACGGACGACGAGGACGACTCGGC-3′as the gene-specific reverse nested primer. The PCR cycling conditions were 94°C for 2 min; 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 2 min; followed by a final elongation at 72°C for 7 min. The amplified fragments were blunt-ended and subcloned into the pUC19 vector (Takara), and the nucleotide sequences were determined as previously described.

Expression of the *ggs1* gene and purification of the product

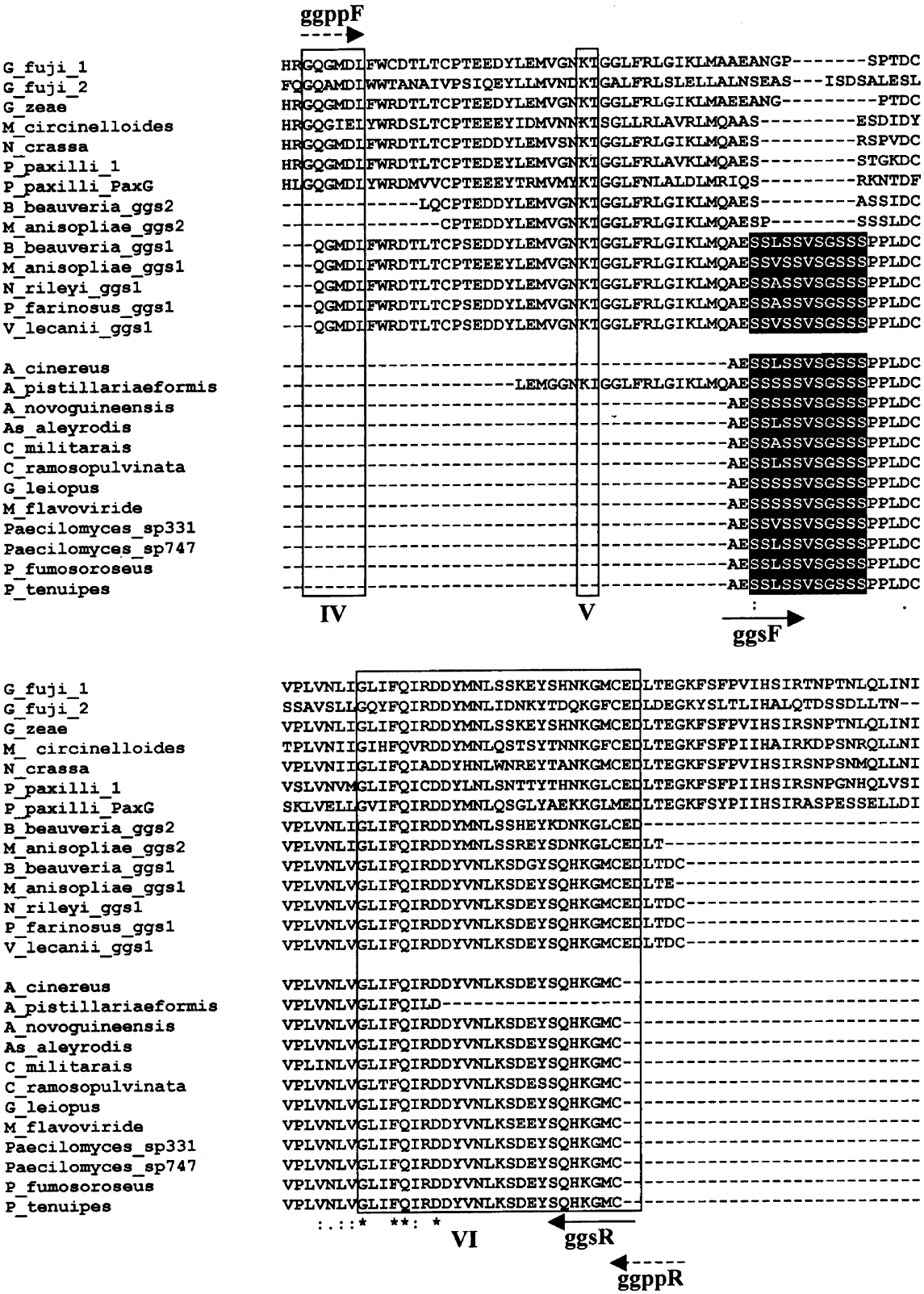
The full-length *ggs1* gene was amplified using GGS1F having a *Bam*HI site (5′-GAGAGGATCCATGTCGGAA GATGACGCACGC-3′) and GGS1R having a *Not*I site (5′-TACAGCGGCCGCTCACTCCTGGGTGGGGGCTGG-3′). The amplified fragment was inserted into the *Bam*HI and *Not*I sites in pET-32a (+), resulting in pET32a/ggs1. The expression vector was transformed into *E. coli* BL21 (DE3)/pLysS. The production of Ggs1-His-tag (Ggs1 containing six surplus histidines at the N terminus) was induced by addition of 1 mM IPTG to the cultures, followed by further incubation for 4 h at 30°C. Cells were harvested by centrifugation, suspended in 1 ml of buffer A containing (0.1M Tris-HCl pH 8.4 and 0.1 M KCl), and disrupted by sonication (30 s X 6 times) at 4°C. The soluble supernatant was obtained by centrifugation at 10,000 × *g* for 30 min at 4°C and was used as crude protein for further purification. The crude protein was loaded onto a HiTrap Blue HP column (GE Healthcare Bio-Sciences, Uppsala, Sweden) and the Ggs1-His-tag was purified to homogeneity by eluting with buffer A containing 400 mM of imidazole. The concentration of proteins was determined by using a Bio-Rad Protein Assay Kit (BioRad, Hercules, CA) according to the protocol from the manufacturer. The presence of recombinant protein was confirmed by Western blot analysis. Briefly, after 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent transfer onto nitrocellulose membrane, the proteins (30 μg) were exposed to a 1:10000 dilution of anti-His_6-antibody (Anti His-probe; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and detected with a 1:20000 dilution of peroxidase-linked secondary antibody of anti-rabbit of IgG (GE Healthcare). The membrane was then developed on medical X-ray film (Fuji). The molecular weight was estimated from the mobility on 12% SDS-PAGE. Gel staining was performed using Coomassie brilliant blue R-250.

Fig. 1 Multiple comparison of the homologues GGPP synthase genes from entomopathogenic fungi with those of fungal origin. The *ggs1* genes were amplified from 17 representative strains, and the *ggs2* genes were derived from *Beauveria bassiana* NAFF635036 and *Metarhizium anisopliae* NAFF635007. The Ser-rich region is *highlighted in shadow*. The conserved regions IV to VI discovered by Koyama (1999) are *boxed*. The CLUSTALW program was used for sequence alignment with the following data: G_fuji_1, the *ggs1* product from *Gibberella fujikuroi* (Mende et al. 1997); G_fuji_2, the *ggs2* product from *Gibberella fujikuroi* (Tudzynski and Hölter 1998); G_zeae, GGPPSASE from *Gibberella zeae* PH-1 [XM_390273]; M_circinelloides, the *carG* product from *Mucor circinelloides f. lusitanicus* (Velayos et al. 2003); P_pailli_1, the *ggs1* product from *Penicillium paxilli* (Young et al. 2001); P_paxilli_PaxG, the *paxG* product from *Penicillium paxilli* (Young et al. 2001); and N_crassa, the NCU01427.1 product from *Neurospora crassa* N150 (Galagan et al. 2003). The *solid* and *dashed arrows* indicate the position and direction of PCR primers designed to amplify the Ser-rich domain and generally conserved region of the GGPP synthase gene, respectively

Geranylgeranyl diphosphate synthase activity of recombinant protein

The method described by Chang et al. (2006) was used to determine GGPP synthase activity with some modifications. The reaction was conducted in 100 μl of solution containing 100 μg of protein sample, 50 mM Tris-HCl buffer (pH 7.5), 1 mM $MgCl_2$, 5 mM iodoacetamide (Sigma, St. Louis, MO), and 50 μM farnesyl diphosphate (FPP; Sigma). After preincubation at 37°C for 10 min, the reaction was started by adding 50 μM [1-^{14}C]-IPP (specific radioactivity 52 μCi/μmol; Sigma). The mixture was incubated at 37°C for 30 min and the reaction was terminated by the addition of distilled H_2O (100 μl) and NaCl-saturated water (200 μl). The radiolabeled products were then extracted with 1 ml of 1-butanol saturated with NaCl-saturated water, and the 1-butanol layer was recovered by centrifugation (13,000 rpm, 10 min). The radioactivity in 200 μl of the 1-butanol phase was quantitated by using a Beckman LS6500 multi-purpose scintillation counter (Beckman Dickinson and Co., Sparks, MD).

To identify the product specificity, the remaining 1-butanol phase was transferred to a 10-ml capped glass tube. Subsequently, 1 ml of acid phosphatase solution containing 16 units acid phosphatase from potato (Sigma) in 0.5 M acetate buffer (pH 4.7) was added to the sample together with 700 μl of methanol, and the mixture was kept at 37°C for 20 h. The hydrolyzed products were extracted with n-pentane. The products were separated by reverse-phase TLC using an RP-18 Plate F_{254S} (Merck, Darmstadt, Germany) with acetone/water (9/1) as solvent. The radiolabeled products were detected by autoradiography using a FujiFilm FLA-7000 bioimaging analyzer (FujiFilm, Tokyo, Japan). The positions of authentic standards were visualized with iodine vapor.


ggppF
G_fuji_1
G_fuji_2
G_zeae
M_circinelloides
N_crassa
P_paxilli_1
P_paxilli_PaxG
B_beauveria_ggs2
M_anisopliae_ggs2
B_beauveria_ggs1
M_anisopliae_ggs1
N_rileyi_ggs1
P_farinosus_ggs1
V_lecanii_ggs1
A_cinereus
A_pistillariaeformis
A_novoguineensis
As_aleyrodis
C_militarais
C_ramosopulvinata
G_leiopus
M_flavoviride
Paecilomyces_sp331
Paecilomyces_sp747
P_fumosoroseus
P_tenuipes
IV
V
ggsF
VI
ggsR
ggppR

Results

Amplification and sequence analysis of GGPP synthase gene homologues from entomopathogenic fungi

To search for GGPP synthase genes in entomopathogenic fungi, ggppF and ggppR primers were designed from the sequences of the conserved regions IV and VI proposed by Koyama (1999) for known prenyltransferases, respectively (Fig. 1) because both the sequences used for primer designation conserved among GGPP synthase but not among FPP synthase. PCR was performed using genomic DNA from the following five representative strains of entomopathogenic fungi as a template: *Metarhizium anisopliae* NAFF635007, *Beauveria bassiana* NAFF635036 *Verticillium lecanii* NAFF635037, *Paecilomyces farinosus* NAFF635038, and *Nomuraea rileyi* NAFF635039. The amplified fragments having an appropriate size (258 bp) were cloned and sequenced, and their sequences were analyzed by a BLAST search using the GenBank database. All the amplified fragments showed high overall similarity at amino acid level (70–77%) to the reported fungal GGPP synthase genes, and also possessed the conserved regions IV, V, and VI discovered by Koyama (1999), while the final D in the DDXXD (region VI) was replaced with N.

Sequence comparison of the amplified fragments revealed that *M. anisopliae* and *B. bassiana* have two different types of GGPP synthase gene homologues. Genes of the first homologue (*ggs1*) were obtained from all five species tested and showed extremely high similarity at amino acid level (92–97%) to each other. Genes of the second type homologue (*ggs2*), however, were obtained only from *M. anisopliae* and *B. bassiana.* The two *ggs2* sequences showed high similarity to each other (93%), but their similarity to the *ggs1*-type sequences was relatively lower (identity with the *ggs1* sequences: 80–83% for *ggs2* from *M. anisopliae* and 76–79% for *ggs2* from *B. bassiana*; Fig. 1).

Alignment of the deduced amino acid sequence with known fungal GGPP synthases revealed that the *ggs1* gene of entomopathogenic fungi contained a Ser-rich region, SSXSSVSGSSS (X refers to L, A, V, or S; Fig. 1), which is not found in other GGPP synthases of various origins and seems to be a unique feature of the GGPP synthase from entomopathogenic fungi. To determine whether entomopathogenic fungi have the GGPP synthase containing the Ser-rich region universally, PCR analysis was expanded to 12 more species of entomopathogenic fungi (Table 1) using a primer ggsF designed for the Ser-rich region, and a primer ggsR that was set inside of ggppR (Fig. 1). From the total 17 species, including the five species described previously, fragments having the expected size (150 bp) were amplified (data not shown), and the alignment of the deduced amino-acid sequences of the amplified 150-bp fragments indicated that they were almost identical to each other (93–98%), suggesting that the *ggs1* gene containing the Ser-rich region exists universally among entomopathogenic fungi, and that the sequence is highly conserved among genera or species (Fig. 1).

Sequence analysis of the full-length *ggs1* from *M. anisopliae*

To analyze the structure of *ggs1* in more detail, we isolated the whole *ggs1* gene from the *M. anisopliae* genome by using the RACE method. The *ggs1* gene (1080 bp; DDBJ accession no. AB501284) codes for a protein of 359 amino acids and has no obvious intron. The corresponding protein sequence was analyzed using the Conserved Domain Database in NCBI, which revealed that *ggs1* of *M. anisopliae* contained all five domains highly conserved among prenyltransferases (domains I–V) as well as two aspartate-rich DDXXD motifs in domains II and V, which domains have been proven essential for prenyltransferase activity (Chen et al. 1994; Fig. 2). The sequence of the deduced *ggs1* product showed highest homology with the putative GGPP synthase of *Epichole festucae* (81% identity) and strong similarity with other fungal GGPP synthases from *Giberella fujikuroi*, *Giberellla zeae*, *Magnaporthe grisea*, and *Neurospora crassa* (74%, 72%, 70%, and 67% identity, respectively).

Transcription analysis of *ggs1* during cultivation

The transcription profile of *ggs1* was analyzed by Northern blot hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) to deduce the role of *ggs1*, using *M. anisopliae* and *B. bassiana,* because both of them possess at least two different types of GGPP synthase genes. Total RNA was isolated from the liquid culture at the 2nd, 4th, and 6th days, which correspond to the early log phase, transition phase, and stationary phase, respectively. RT-PCR with ggsF/ggsR primers and Northern blot analysis indicated that the *ggs1* genes in both strains were constitutively transcribed throughout cultivation, as shown in Fig. 3a, b, respectively, while the expression of the *ggs2* genes was not detected throughout the cultivation (data not shown).

Heterologous expression of *ggs1* in *E. coli* and enzymatic characterization of its product

To examine the actual function of the *ggs1* gene, the *ggs1* gene was introduced into the expression strain *E. coli* BL21 (DE3/pLysS) using the plasmid pET32a/ggs1. The transformant was cultivated in LB medium at 37°C and was induced to express the *ggs1* gene by cultivating for 4 h after adding 1 mM IPTG. A protein of about 55 kDa, corresponding to the size of the *ggs1* product with six

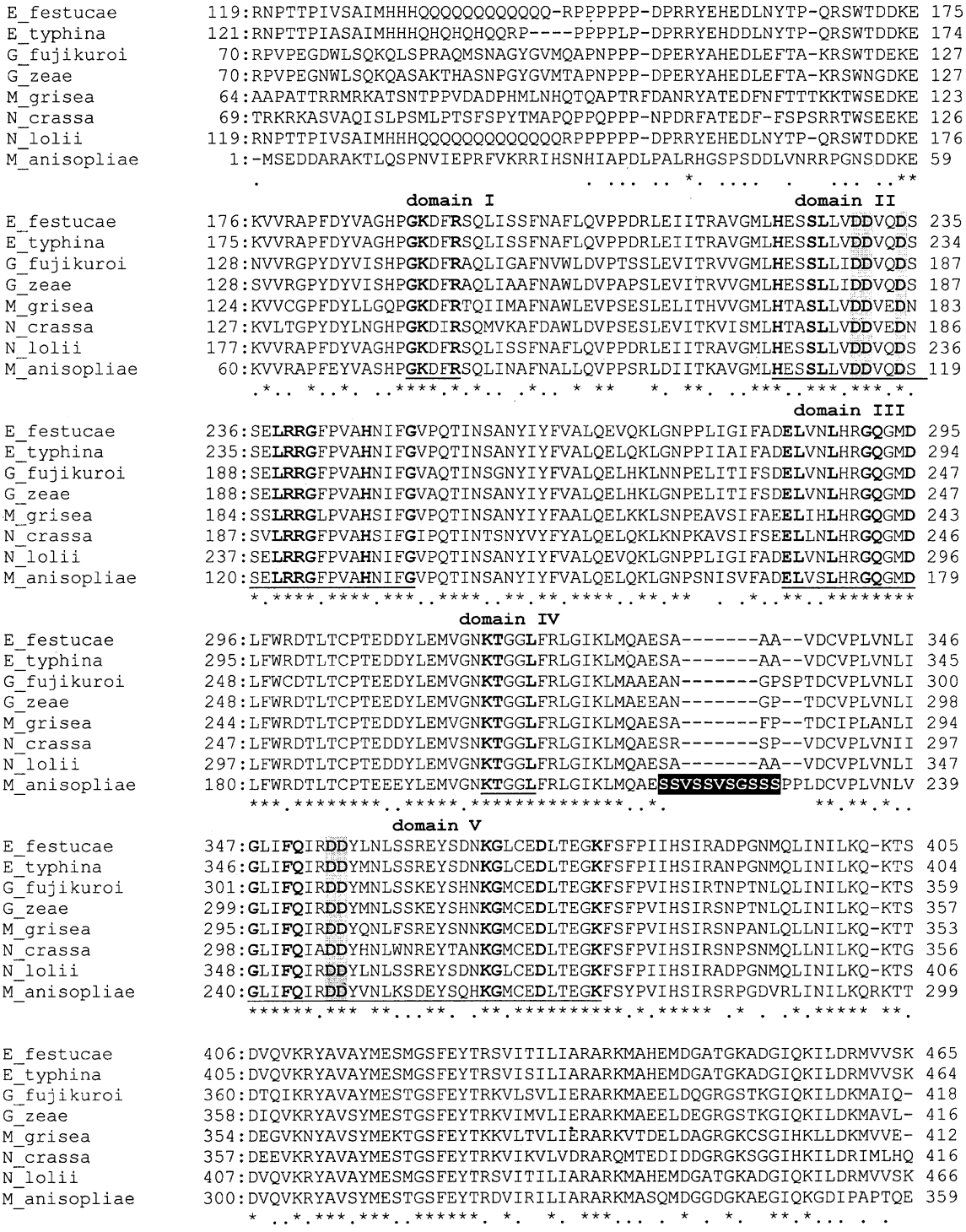


Fig. 2 Alignment of the deduced amino acid sequence of *ggs1* with the fungal GGPP synthases *Epichole festucae* (AAW88517), *Epichole typhina* (AAW88518), *Gibberella fujikuroi* (Q92236), *Gibberella zeae* (XP_390273), *Magnaporthe grisea* (XP_368486), *Neurospora crassa* (XP_960695), and *Neotyphodium lolii* (AAW88513). The consensus domains of all GGPP synthases classified according to Chen et al. (1994) are indicated with *bold letters*. The serine-rich domain is shown in the *black box*. The FARM (first aspartate rich-motif) and the SARM (second asparatate rich-motif) are *highlighted in gray*. The alignment was performed using the GENETYX program

surplus histidine residues, was detected only in the extract from the transformant harboring pET32a/ggs1 by both SDS-PAGE and subsequent Western blot analysis with anti His-tag antibody (Fig. 4a, b).

GGPP synthase is an enzyme which elongates the isoprenoid chain by catalyzing condensation reaction of FPP with IPP, yielding GGPP. To ensure that *ggs1* encodes GGPP synthase, the 55-kDa His-tagged protein was purified and

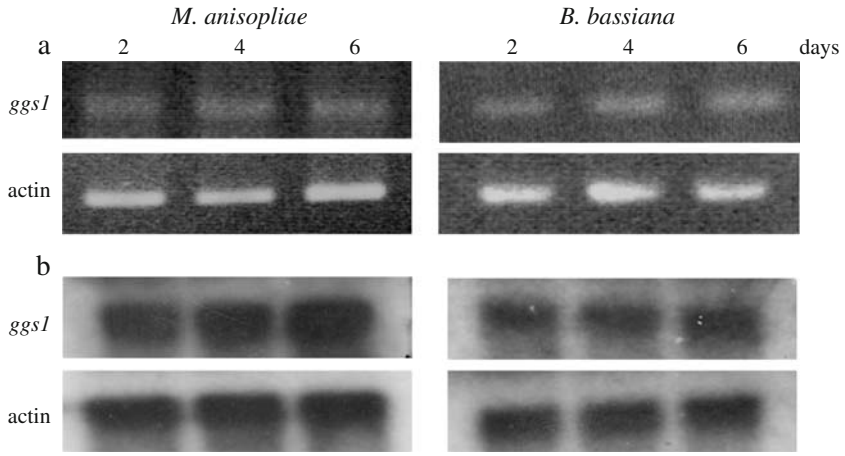


Fig. 3 The expression of *ggs1* in *Metarhizium anisopliae* and *Beauveria bassiana* during cultivation in SMY liquid medium at 28°C. **a** Relative RT-PCR analysis and **b** Northern blot analysis of the *ggs1* expression at early log phase (2nd day), transition phase (4th day) and stationary phase (6th day). RT-PCR on *ggs1* (190 bp) and actin (400 bp) were performed with 32 cycles (for amplification of *ggs1* from *M. anisopliae* and *ggs1* from *B. bassiana*) and 30 cycles (for amplification of the actin gene from both strains) after RT reaction, respectively

was assayed for its GGPP synthase activity by monitoring the incorporation of radio-labeled IPP in the reaction product. The purified 55-kDa recombinant protein showed definite IPP incorporation-specific activity at 337.94 nmol/mg of protein/min. The IPP-incorporated products were further examined by radio-TLC, revealing that the *ggs1* gene product dominantly synthesized GGPP (Fig 5).

Discussion

In living organisms, GGPP is regarded as an important molecule in terpenoid biosynthesis because it is an essential linear precursor for the biosynthesis of diterpenes, carotenoids, retinoids, and the side chains of chlorophylls. In filamentous fungi, a single FPP synthase is present and produces FPP by consecutive condensation of DMAPP with two molecules of IPP, and several different GGPP synthases are involved in the construction of GGPP by further condensation of FPP with IPP, as evidenced by the presence of many plausible GGPP synthase genes on the

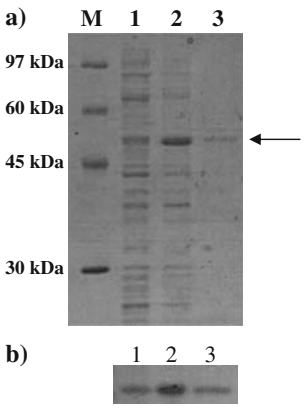


Fig. 4 Production in *E. coli* and purification of recombinant Ggs1 protein. **a** SDS-PAGE showing the expression of *ggs1* in *E. coli*. The protein profile of the *E. coli* cells before and after IPTG induction was checked on a 12% SDS-PAGE. *Lanes* represent the molecular weight marker (*lane M*), uninduced and induced *E. coli* cell lysate (*lanes 1*, and *2*), and purified recombinant Ggs1 (*lane 3*). The *arrow* indicates the position at which the anti-His tag antibodies reacted. **b** Western blot analysis of recombinant Ggs1

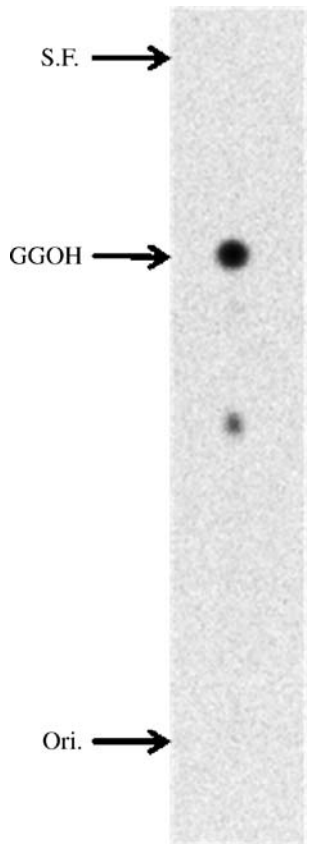


Fig. 5 TLC analysis of radiolabelled products by Ggs1-catalyzed reaction. Alcohol products (GGOH) derived from Ggs1-catalyzed products by acid phosphatase-treatment products was separated by reversed phase TLC with a solvent system of acetone /H_2O (9:1). *Ori* origin of development, *S. F.* solvent front. The position of authentic geranylgeranyl alcohol (GGOH) is indicated by an *arrow*

genome (Tudzynski and Hölter 1998; Young et al. 2001, 2005). This study has characterized a GGPP synthase gene from entomopathogenic fungi for the first time.

Initial PCR screening indicated the presence of two kinds of plausible GGPP synthase genes in entomopathogenic fungi. By redesigning the PCR primers and expanding the target strains, it was demonstrated that one class of GGPP synthase gene homologue (termed *ggs1*) containing the unique S-rich region is well conserved among the entomopathogenic fungi. Moreover, we were able to confirm that the *ggs1* gene was expressed throughout the cultivation, similar to the cases of the GGPP synthase genes required for primary metabolism in *G. fujikuroi* and *P. paxilli* (Mende et al. 1997; Young et al. 2001), suggesting that the *ggs1* of entomopathogenic fungi may also play an essential or at least a common role, such as for primary metabolism. In contrast, the second class of the GGPP synthase gene homologue (*ggs2*) was only obtained from two out of the five strains tested. Judging from the result that the *ggs2* transcription was observed only after late log-phase, the *ggs2* genes would seem to be not essential for cell growth or primary metabolism, suggesting that these genes would be correlated to the production of secondary metabolites, like other GGPP synthase genes (*ggs2* or *paxG*) in *G. fujikuroi* or *P. paxilli* (Tudzynski and Hölter 1998; Young et al. 2001).

The ggppF/R primer set newly designed for this experiment was able to amplify different types of GGPP synthase genes universally, while the primer set ggsF/R amplified *ggs1* specifically. The former primer set should be useful in searching for GGPP synthase genes in general, the latter primer set should be useful for specifically selecting the *ggs1* gene, and the two sets in combination should be useful for detecting the *ggs2* gene, which may be related to secondary metabolism.

Amino acid sequence comparison of *ggs1* with fungal GGPP synthases from the Genbank and Pfam databases revealed that all of them have five highly conserved regions related to enzymatic activity (Fig. 3). Interestingly, the unique S-rich region SSXSSVSGSSS (X refers to L, A, V, or S) was found in all the *ggs1* genes from the representative strains (Fig. 1), indicating that the S-rich region is a unique feature of the GGPP synthase genes of entomopathogenic fungi. Because a search of the PROSITE or Pfam databases did not show any similar sequences, it is not clear at present whether the S-rich region has any specific function or importance for the catalytic activity of GGPP synthase. Mutational analysis in vitro or in vivo might clarify its function in the future.

Although transcription was detected for *ggs1* of *M. anisopliae*, this does not reveal its actual function, because homologous genes can occasionally be nonfunctional. One such example is *ggsA* of *A. flavus*, which has been reported to be a pseudogene, probably due to its missing a methionine start codon and its having a frameshift mutation that results in a premature translation stop codon, despite the fact that the deduced product had the five conserved domains of prenyl diphosphate synthase and transcription was detected (Zhang et al. 2004).

To examine the possibility that *ggs1* might be nonfunctional, the *ggs1* gene from *M. anisopliae* was heterologously expressed in *E. coli* and the product was examined for GGPP synthase activity. The activity was confirmed, clearly demonstrating that *ggs1* is a functional gene of *M. anisopliae*. Although GGOH derived from GGPP was the major spot, a minor spot having much slower mobility was also detected on the radio TLC. Based on a comparison with authentic solanesol, the minor spot seems to have been derived from hexaprenol, implying that *ggs1* might encode HexPP synthase. However, sequence comparison with known GGPP synthases and HexPP synthases clearly indicated that the *ggs1*-encoded enzyme shares high similarity only with GGPP synthase, and not with HexPP synthases (supplementary data 1). Together with the low presence of the minor product (11% of GGPP), this indicated that the *ggs1*-encoded enzyme would better be classified into the family of GGPP synthases.

In summary, this is the first cloning and functional analysis of GGPP synthase genes from entomopathogenic fungi. Because entomopathogenic fungi are regarded as a precious resource producing various bioactive isoprenoid compounds by way of primary or secondary metabolism, it is hoped that the genetic information obtained in this study will be utilized for the development of strains that can produce useful isoprenoid compounds in large quantities.

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