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G proteins in *Ustilago maydis*: transmission of multiple signals?

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In the phytopathogenic fungus *Ustilago maydis*, cell fusion is governed by a pheromone signalling system. The pheromone receptors belong to the seven transmembrane class that are coupled to heterotrimeric G proteins. We have isolated four genes (*gpa1* to *gpa4*) encoding α subunits of G proteins. Gpa1, Gpa2 and Gpa3 have homologues in other fungal species, while Gpa4 is novel. Null mutants in individual genes were viable and only disruption of *gpa3* caused a discernible phenotype. *gpa3* mutant strains were unable to respond to pheromone and thus were mating-deficient. A constitutively active allele of *gpa3* (*gpa3*$_{Q206L}$) was generated by site-directed mutagenesis. Haploid strains harbouring *gpa3*$_{Q206L}$ were able to mate without pheromone stimulation, indicating that Gpa3 plays an active role in transmission of the pheromone signal. Surprisingly, Gpa3 is also required for pathogenic development, although pheromone signalling is not essential for this process.

Keywords: G proteins/pathogenicity/pheromone response/signal transduction/*Ustilago maydis*

Introduction

The Basidiomycete fungus *Ustilago maydis* causes corn smut disease. Pathogenic development is initiated when haploid cells of opposite *a* mating type recognize each other, fuse and generate an infectious dikaryon (Banuett, 1995). Pathogenicity is controlled by the multiallelic *b* mating type locus which encodes the bE and bW homeodomain proteins. The bE and bW proteins form heterodimers only in such combinations where they are derived from different alleles (Kahmann and Bölker, 1996).

Each allele of the biallelic *a* locus contains the structural genes for a lipopeptide pheromone precursor and for a receptor that recognizes the pheromone secreted by cells of opposite *a* mating type (Bölker *et al.*, 1992; Spellig *et al.*, 1994). Upon pheromone signalling, a set of genes is induced, leading to the formation of conjugation tubes and mating competence. Among these genes are the pheromone (*mfa*) and receptor (*pra*) genes as well as the *b* genes (Urban *et al.*, 1996a). After cell fusion, autocrine pheromone signalling maintains a high level of *b* gene expression that is required for the transition from the yeast-like growth of haploid cells to the filamentous growth of the dikaryon (Bölker *et al.*, 1992; Urban *et al.*, 1996a). For pathogenic development, however, pheromone signalling is not essential: diploids homozygous for *a* and haploid strains that carry a single allele of the *a* locus are pathogenic if they can form an active bE–bW heterodimer (Banuett and Herskowitz, 1989; Bölker *et al.*, 1995).

The transcriptional regulation of pheromone-inducible genes is mediated by Prf1, a transcription factor of the HMG-box family (Hartmann *et al.*, 1996). Prf1 is thought to be phosphorylated through a MAP kinase cascade activated by pheromone signalling (Hartmann *et al.*, 1996). A putative component of this cascade, the MAP kinase kinase (MAPKK) homologue Fuz7, has already been described (Banuett and Herskowitz, 1994). Δ*fuz7* strains are affected in *a*-locus-dependent processes like conjugation tube formation, filament formation and maintenance of filamentous growth. In addition, *fuz7* mutants were shown to have defects in *a*-independent processes like tumour induction and teliospore germination (Banuett and Herskowitz, 1994).

The pheromone receptors in *U.maydis* belong to the seven transmembrane class that are coupled to heterotrimeric G proteins (Dohlman *et al.*, 1991). It is proposed that binding of ligand to the receptor induces the exchange of GDP for GTP in the α subunit, resulting in dissociation of Gα from the Gβγ heterodimer. In different systems, it is either the Gα subunit or the Gβγ heterodimer that transmits the signal (Hamm and Gilchrist, 1996). Pheromone signalling has been extensively studied in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, where most components of the cascade are now known (Herskowitz, 1995). One of the remarkable differences between these yeasts is that, in *S.cerevisiae* (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987), the βγ complex plays the active role in signalling while it is the α subunit in *S.pombe* (Obara *et al.*, 1991).

In this study we have cloned a total of four *U.maydis* genes encoding Gα subunits. We show that one of these Gα proteins plays an active role in transmitting the pheromone signal and, at the same time, is required for pathogenic development.

Results

Isolation of U.maydis genes encoding Gα subunits

Based on sequence conservation between Gα proteins, degenerate primers were used for PCR amplification of *U.maydis* genomic DNA. By this method, two genes (*gpa1* and *gpa3*) could be identified which contain open reading frames (ORF) that are highly similar to other Gα proteins (Figure 1). A third gene (*gpa2*) was isolated as a cosmid clone that hybridizes to the coding region of *S.cerevisiae*

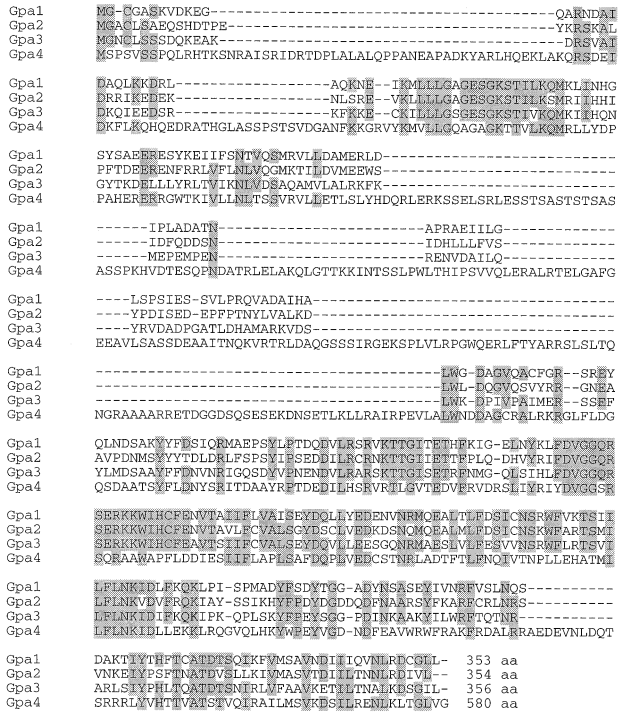

Fig. 1. Amino acid sequence alignments of the four Gα genes of *Ustilago maydis* (*gpa1*, *gpa2*, *gpa3* and *gpa4*).The multiple amino acid sequence alignment was performed using the program CLUSTAL W (Thompson *et al.*, 1994). Shading indicates positions at which at least three amino acids are identical.

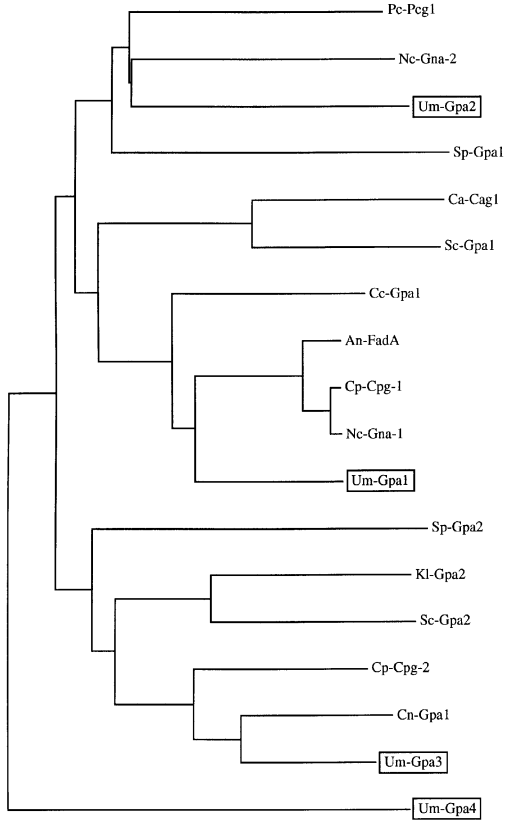

Fig. 2. Phylogenetic analysis of fungal G protein α subunits. The unrooted tree was constructed with the program CLUSTAL W (Thompson *et al.*, 1994) using Gpa4 as outgroup. Gpa4 is significantly less related to the other Gα subunits and has no fungal homologues. Abbreviations: Pc, *Pneumocystis carinii* f. sp. *carinii*; Ca, *Candida albicans*; Sc, *S.cerevisiae*; Nc, *N.crassa*; Cp, *C.parasitica*; Sp, *S.pombe*; Um, *U.maydis*; Cn, *C.neoformans*; An, *Aspergillus nidulans*; Kl, *Kluyveromyces lactis*; Cc, *Coprinus congregatus*. DDBJ/EMBL/GenBank accession numbers: Pc-*pcg1*, U307921; Ca-*cag1*, M88113; Sc-*gpa2*, U18778; Nc-*gna2*, L11453; Nc-*gna1*, L11452; Cp-*cpg2*, L32177; Cp-*cpg1*, L32176; Sp-*gpa1*, M64286; Sp-*gpa2*, D13366; Sc-*gpa1*, M15867; Cn-*gpa1*, U09372; An-*fadA*, U49917; Kl-*gpa2*, L47105 and Cc-*cgp1*, X68031.

gpa2. By coincidence, a fourth gene (*gpa4*) was isolated from a screen for differentially expressed genes (see Materials and methods). Genomic sequences were determined for all four genes, cDNAs were isolated for *gpa3* and *gpa4* only. The coding regions encompass 353 amino acids for Gpa1, 354 for Gpa2, 356 for Gpa3 and 580 amino acids for Gpa4, respectively (DDBJ/EMBL/GenBank accession numbers U85775, U85776, U85777 and U85778). Known fungal Gα subunits can be grouped into three distinct subfamilies (Figure 2). For each subfamily we have identified a corresponding *U.maydis* gene (*gpa1* to *gpa3*). *gpa4*, however, cannot be placed in any of these subgroups (Figure 2). The closest relatives to Gpa1 and Gpa2 of *U.maydis* are Gna-1 (73% identity) and Gna-2 (51% identity) of *Neurospora crassa*, respectively, while Gpa3 is most closely related to Gpa1 of *Cryptococcus neoformans* (75% identity). Gpa4 is very distantly related to other fungal Gα proteins and displays only 21% identity with Cpg2 of *Cryphonectria parasitica*.

gpa3 mutants are mating-deficient

To analyse the function of the identified Gα subunits, we have generated mutants in the haploid strains FB1 (*a1 b1*) and FB2 (*a2 b2*) for each of the four genes by deleting or disrupting the ORF (see Materials and methods). Compatible combinations (i.e. they carry different alleles of the *a* and *b* mating type loci) of mutant strains were tested for mating and pathogenicity. In plate mating assays *gpa1*, *gpa2* and *gpa4* mutants behaved like compatible wild-type strains and formed a white mycelium characteristic for the filamentous dikaryon (Holliday, 1974) (data not shown). When assayed for pathogenicity, such combinations of mutant strains were indistinguishable from wild-type combinations with respect to tumour development, spore production and spore germination (data not shown). This illustrates that individual mutations of these Gα genes do not affect essential steps in fungal development.

In contrast, strains deleted for *gpa3* exhibited a complex phenotype. Cells grown in liquid media were elongated and formed aggregates (Figure 3). On charcoal-containing plates, Δ*gpa3* colonies were covered by very short aerial filaments, giving the colonies a greyish appearance (Figure 4A). In plate mating assays, compatible Δ*gpa3* mutant strains or combinations of a wild-type strain with a compatible Δ*gpa3* strain failed to develop the white dikaryotic mycelium (Figure 4B). This could indicate that Δ*gpa3* mutants are unable to fuse; alternatively, they may have defects in the formation of long aerial hyphae. Upon infection of corn plants with compatible combinations of Δ*gpa3*, mutant strains did not induce disease symptoms (Table I). In combination with a compatible wild-type strain, Δ*gpa3* mutants showed attenuated tumour development (Table I). This indicates that Δ*gpa3* mutants are not

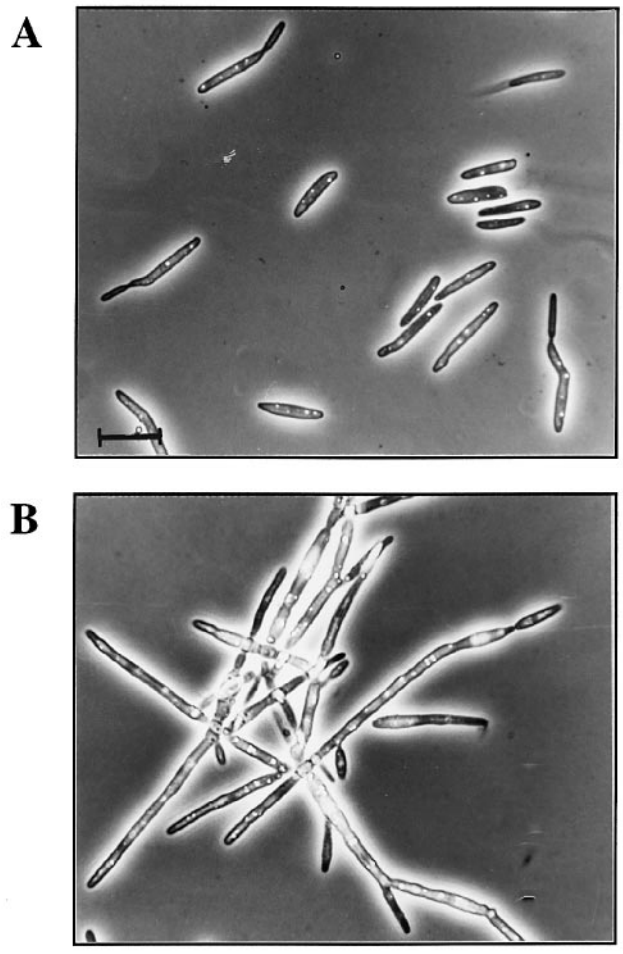

Fig. 3. Cell morphology of a Δ*gpa3* mutant strain. The wild-type strain FB2 (**A**) and FB2Δgpa3 (**B**) were cultivated in YEPS medium. Cells were photographed using a Zeiss Axiophot. The bar corresponds to 12 μm.

completely impaired in fusion with wild-type cells. The number of cell fusion events might be too low to be detected on plates, but must be sufficiently high for tumour induction.

Δgpa3 mutants cannot respond to pheromone

To investigate the mating defect of Δ*gpa3* mutants in more detail, we have analysed the expression levels of the pheromone-inducible *mfa1* and *mfa2* genes (Urban *et al.*, 1996a). In haploid Δ*gpa3* cells (*a1 b1* and *a2 b2*, respectively) the basal levels of *mfa1* and *mfa2* mRNA were reduced ~5-fold compared with levels in corresponding wild-type *a1 b1* and *a2 b2* strains (Figure 5A, lanes 1 and 2; Figure 5C, lanes 1 and 2). Despite the low level of pheromone gene expression in Δ*gpa3* mutants, such strains are still able to induce *mfa1* expression in wild-type strains of opposite *a* mating type (Figure 5B, lane 4 and not shown). In crosses of compatible wild-type strains, *mfa1* gene expression is strongly induced as a result of the activation of the pheromone signalling cascade, as can be seen in Figure 5A (lane 3). In crosses of compatible Δ*gpa3* mutants, however, no increase in *mfa1* expression was observed (Figure 5A, lane 4). To exclude a possible regulatory effect exerted by the bE–bW heterodimer which is formed after cell fusion (Urban *et al.*, 1996a), another set of crosses was performed with strains that differ only at *a* but carry identical *b* alleles (Figure 5B and C). Such strains can fuse but are unable to maintain a stable dikaryon (Snetselaar, 1993) because they lack the active bE–bW heterodimer. When the wild-type strains *a1 b1* and *a2 b1* were crossed, a dramatic increase in *mfa1* expression was observed due to pheromone stimulation (Figure 5B, lane 1). In the cross of the corresponding mutant strains *a1 b1* Δ*gpa3* and *a2 b1* Δ*gpa3*, however, no increase in *mfa1* expression could be detected (Figure 5B, lane 2). Also, in a cross between a haploid *a2 b1* wild-type strain with a *a1 b1* Δ*gpa3* mutant strain, no induction of *mfa1* expression could be observed

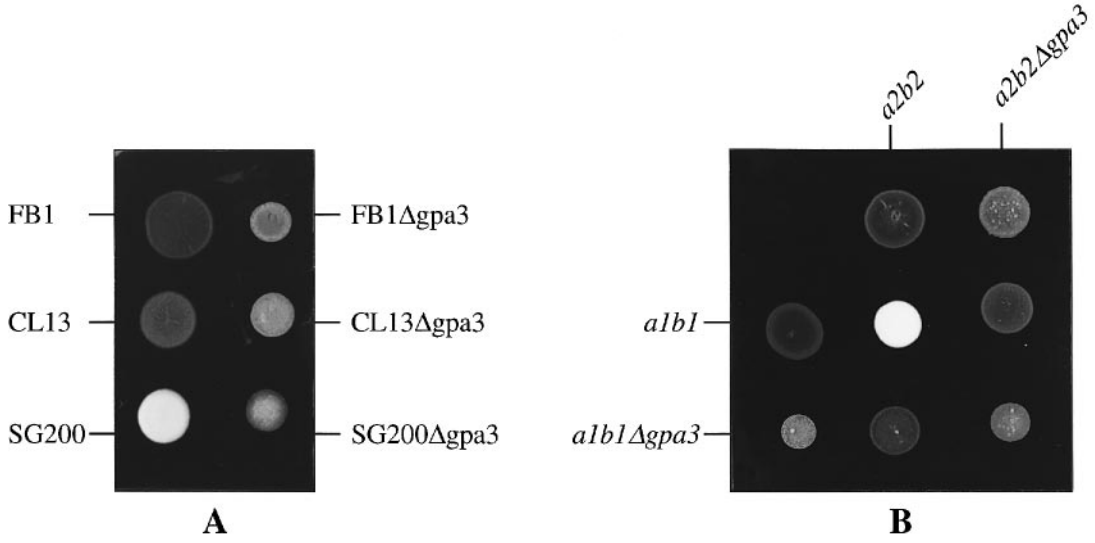

Fig. 4. Phenotype of Δ*gpa3* strains. All strains were spotted either alone or in combination on PD charcoal plates and incubated for 48 h. (**A**) The Δ*gpa3* allele was introduced in FB1 (*a1 b1*), CL13 (*a1 bE1 bW2*) and SG200 (*a1 mfa2 bE1 bW2*). Shown are the parental strains (left) and the corresponding mutant strains carrying the Δ*gpa3* allele (right). Of the three parental strains, only SG200 is filamentous. All three Δ*gpa3* mutant derivatives display the same colony morphology. (**B**) Strains indicated were spotted alone and in the combinations shown. The occurrence of white mycelium indicates the formation of dikaryotic hyphae. The genotype of the strains used are: FB1 (*a1 b1*), FB1Δgpa3 (*a1 b1 Δgpa3*), FB2 (*a2 b2*) and FB2Δgpa3 (*a2 b2 Δgpa3*).

(Figure 5B, lane 3). In an analogous set of crosses, we analysed the influence of the Δ*gpa3* mutation on the expression of the *mfa2* gene (Figure 5C). The results obtained here are fully consistent with those for *mfa1*. Taken together, these data show that the induction of the *mfa* genes upon exposure to pheromone is impaired in Δ*gpa3* mutants.

A constitutively active gpa3 allele allows pheromone-independent mating

To substantiate our assertion that Gpa3 is directly involved in transmission of the pheromone signal, we have constructed a mutant *gpa3* allele whose product is locked in the active GTP-bound state. To this end, a glutamine to leucine substitution (Q206L) was introduced at amino acid position 206 by site-directed mutagenesis. Analogous mutations in mammalian $G\alpha_s$ and $G\alpha_i$ subunits (Masters *et al.*, 1989; Wong *et al.*, 1991) as well as in the *goa-1* Gα subunit of *Caenorhabditis elegans* (Mendel *et al.*, 1995) have been shown to lower the GTPase activity and thus prevent the turnover to the inactive GDP bound state. The mutation Q206L was introduced into the *gpa3* gene of FB1 (*a1 b1*) by a two-step gene replacement procedure (see Materials and methods). The resulting strain FB1gpa3$_{Q206L}$ differs from FB1 only in this mutation. On plates, colonies of the mutant strain FB1gpa3$_{Q206L}$ exhibited a distinct glossy surface (Figure 6, panel A2). To permit genetic analyses, the *gpa3*$_{Q206L}$ allele was crossed into strains of different *a* and *b* background to generate the haploid strains ER12gpa3$_{Q206L}$ (*a1 b2 gpa3*$_{Q206L}$) and ER22gpa3$_{Q206L}$ (*a2 b2 gpa3*$_{Q206L}$) (see Materials and methods for details). Colonies of both strains were glossy, indicating that this phenotype is caused by the *gpa3*$_{Q206L}$ allele (not shown).

If Gpa3 is actively involved in pheromone signal transduction, we expected increased expression levels of pheromone-inducible genes in strains carrying the mutant *gpa3*$_{Q206L}$ allele. We have therefore compared *mfa1* gene expression in the haploid strain *a1 b1 gpa3*$_{Q206L}$ and its progenitor strain FB1 (*a1 b1*) under conditions without pheromone stimulation (Figure 7A). Compared with the low level of basal *mfa1* gene expression in the *a1 b1* strain, *mfa1* levels in the *a1 b1 gpa3*$_{Q206L}$ strain were increased at least 50-fold. This shows that *gpa3*$_{Q206L}$ behaves as a constitutively active allele of *gpa3*.

In mating assays, compatible combinations of strains carrying the *gpa3*$_{Q206L}$ allele produced dikaryotic filaments as efficiently as the corresponding combination of wild-type strains (Figure 6, compare panels B1 and B2). Next, we have analysed whether the apparent activation of the pheromone pathway in the *a1 b1 gpa3*$_{Q206L}$ strain is sufficient to allow mating with a strain which carries the same *a* allele and thus produces the same pheromone. A mixture of respective wild-type strains FB1(*a1 b1*) and FB6b(*a1 b2*) remained non-filamentous (Figure 6, panel C1). However, when *a1 b1 gpa3*$_{Q206L}$ was co-spotted with *a1 b2 gpa3*$_{Q206L}$, weak filament formation was observed after 3–4 days of incubation (Figure 6, panel C2), indicating that cell fusion had occurred independent of different

Table I. Pathogenicity of *gpa3* mutant strains

Strain	No. of infected plants	No. with tumours	Percentage with tumours
a1 b1 × *a2 b2*	28	26	93
a1 b1 Δ*gpa3* × *a2 b2* Δ*gpa3*	41	0	0
a1 b1 Δ*gpa3* × *a2 b2*	93	15	16
a1 b1 × *a2 b2* Δ*gpa3*	36	5	14
a1 b1 gpa3$_{Q206L}$ × *a2 b2 gpa3*$_{Q206L}$	74	38	51
a1 b1 × *a1 b2*	20	0	0
a1 b1 gpa3$_{Q206L}$ × *a1 b2 gpa3*$_{Q206L}$	85	30	35
CL13	18	14	78
CL13Δgpa3	22	0	0
CL13pra1::Tn5H#15	32	27	84
SG200	20	20	100
SG200Δgpa3	37	0	0
SG200Δgpa3 b^{con}	214[a]	0	0

[a]Seven independent transformants were tested.

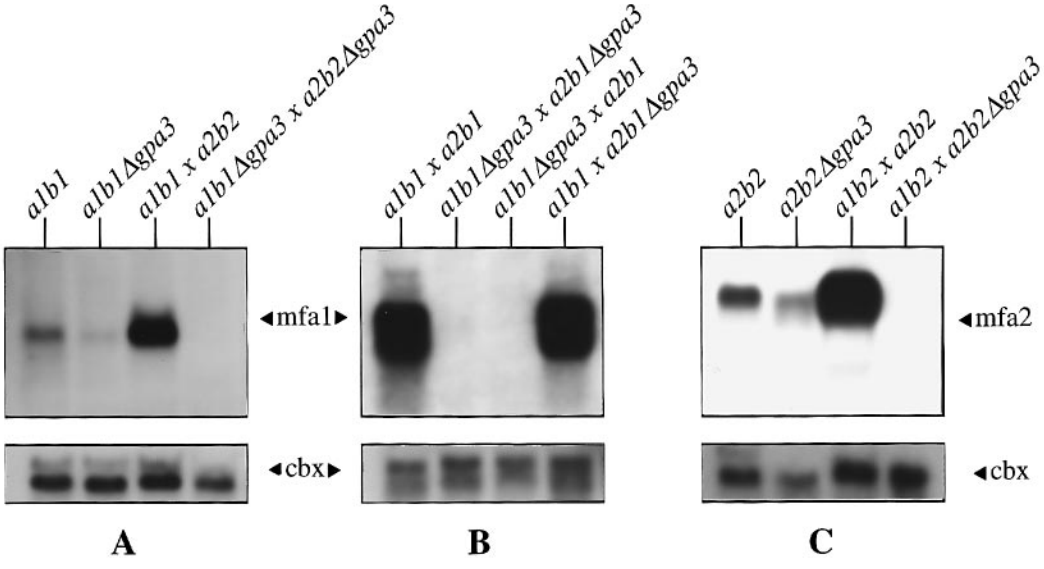

Fig. 5. Transcription of the pheromone-inducible *mfa* genes in Δ*gpa3* mutant and wild-type strains. Strains and strain combinations listed were grown on CM-charcoal plates for 48 h. Total RNA was prepared and subjected to Northern analysis using *mfa1* (**A** and **B**) or *mfa2* (**C**) as a probe. The *cbx* gene served as an internal control for loading.

a alleles. To substantiate this observation, the same strain combinations were co-injected into maize plants. Some 35% of the plants infected with the combination of strains carrying the mutant $gpa3_{Q206L}$ allele developed tumours, while the corresponding combination of wild-type strains failed to do so (Table I). This shows that the constitutively active form of Gpa3 allows mating without pheromone stimulation. Gpa3 thus plays an active role in transmitting the pheromone signal.

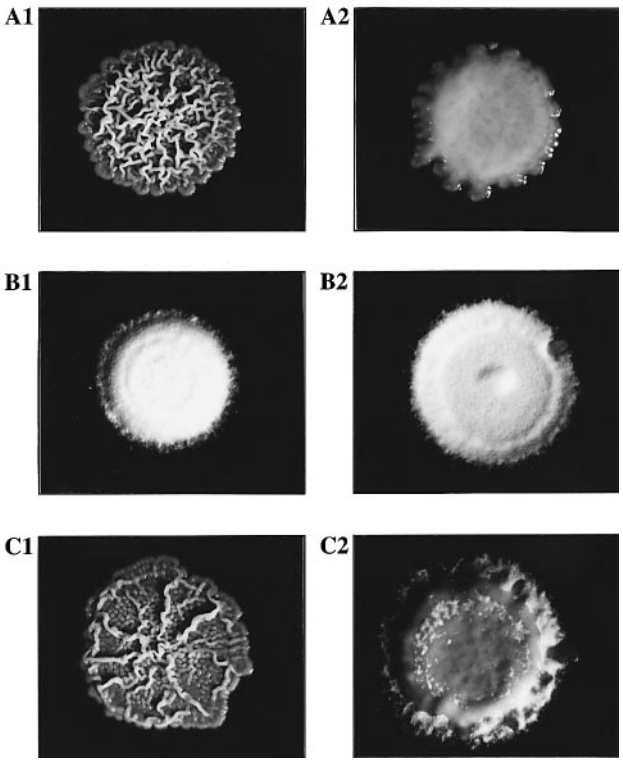

Fig. 6. Mating reactions of strains carrying the $gpa3_{Q206L}$ mutation. All strains and strain combinations were spotted on PD-charcoal medium and incubated for 4 days at room temperature. Colonies were photographed with an Olympus SZH stereo microscope camera system. The genotypes of strains were as follows: (**A1**) *a1 b1*; (**A2**) *a1 b1* $gpa3_{Q206L;}$ (**B1**) cross of strain *a1 b1* with the compatible strain *a2 b2*; (**B2**) cross of strain *a1 b1* $gpa3_{Q206L}$ with the compatible strain *a2 b2* $gpa3_{Q206L;}$ (**C1**) cross of strain *a1 b1* and the incompatible strain *a1 b2*; (**C2**) cross of *a1 b1* $gpa3_{Q206L}$ with the incompatible strain *a1 b2* $gpa3_{Q206L.}$

Gpa3 is required for pathogenic development of solopathogenic haploid strains

To find out whether the function of Gpa3 is restricted to the transmission of the pheromone signal, we have introduced the Δ*gpa3* mutation into two different solopathogenic haploid strains CL13(*a1 bW2 bE1*) and SG200 (*a1 mfa2 bW2 bE1*) by gene replacement. CL13 is a haploid *a1* strain in which the resident *b* locus encodes *bW2* and *bE1*; SG200 is derived from CL13 and carries in addition a *mfa2* gene inserted in the *a1* locus (Bölker *et al.*, 1995). Because of the active configuration of *b* genes, both strains are solopathogenic, i.e. they do not need a mating partner to induce symptoms *in planta*. Tumour induction by CL13 further illustrates that an activated pheromone signalling pathway is not essential for pathogenicity (Bölker *et al.*, 1995), as had also been demonstrated previously with diploid strains (Banuett and Herskowitz, 1989). In contrast to CL13, SG200 shows filamentous growth on charcoal plates because the pheromone signalling pathway is activated by the additional pheromone gene (Bölker *et al.*, 1995). SG200Δgpa3 and CL13Δgpa3 were morphologically indistinguishable from haploid strains carrying the Δ*gpa3* mutation, i.e. cells were elongated (data not shown) and colonies were covered with very short filaments (Figure 4A). Much to our surprise, however, SG200Δgpa3 and CL13Δgpa3 strains were both non-pathogenic (Table I). To analyse whether mutations in other genes involved in the pheromone response pathway also affect pathogenicity, we inactivated the gene for the pheromone receptor (*pra1*) in CL13, by exchanging the *pra1* gene for the *pra1::Tn5H#3* allele which eliminates receptor function (Bölker *et al.*, 1992). When assayed *in planta*, CL13pra1::Tn5H#3 was unaffected in pathogenicity (Table I).

The requirement for pathogenic development of another component of the pheromone signalling cascade, Prf1,

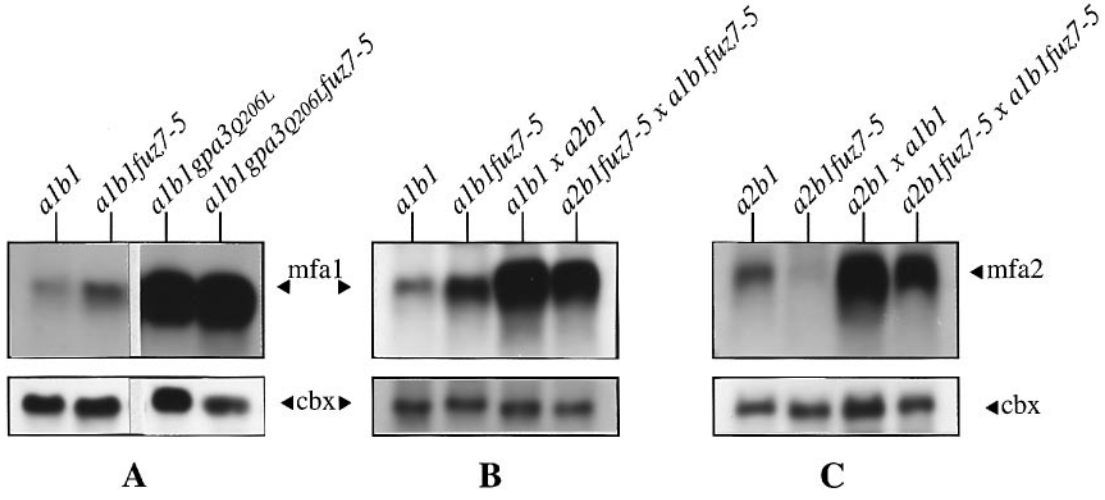

Fig. 7. Transcription of the pheromone-inducible *mfa* genes in $gpa3_{Q206L}$ and *fuz7-5* mutant strains. Strains and strain combinations listed were grown on CM-charcoal plates for 48 h. Total RNA was prepared and subjected to Northern analysis using *mfa1* (**A** and **B**) or *mfa2* (**C**) as probes. The *cbx* gene served as an internal control for loading.

had already been demonstrated. The pathogenicity defect of *prf1* mutants could be suppressed by overexpressing an active *b* gene complex (Hartmann *et al.*, 1996). Therefore, we transformed SG200Δgpa3 mutant strains with pb^{con} in which expression of *bE1* and *bW2* is driven by constitutive promoters (Hartmann *et al.*, 1996). Interestingly, pathogenic development could not be reconstituted (Table I), while filamentous growth was restored in these transformants (not shown). This shows that induction of pathogenic development by the active bE–bW heterodimer requires an intact *gpa3* gene.

The MAP kinase kinase Fuz7 does not act downstream of Gpa3

In *U.maydis*, a gene, *fuz7*, coding for a MAPKK has been identified. Δfuz7 mutants have a strong mating defect and are attenuated in pathogenicity (Banuett and Herskowitz, 1994). This may imply that the MAPKK Fuz7 acts downstream of Gpa3 in the pheromone signalling pathway. Therefore, we were interested to determine the epistatic relationship of these genes. To this end, *fuz7* disruption mutants (*fuz7-5*) were generated (see Materials and methods) and analysed for the expression patterns of the pheromone inducible *mfa1* and *mfa2* genes. With respect to the basal level of pheromone gene transcription, we note a small increase of *mfa1* mRNA in *a1 b1 fuz7-5* compared with the *a1 b1* wild-type strain (Figure 7B, lanes 1 and 2) while the level of *mfa2* mRNA appears ~5-fold lower in *a2 b1 fuz7-5* relative to the *a2 b1* wild-type strain (Figure 7C, lanes 1 and 2). At present, we cannot explain the opposing effects of the *fuz7* mutation on the basal expression levels of *mfa1* and *mfa2*.

With respect to pheromone stimulation, however, a mixture of *fuz7-5* mutant strains that are different in *a* but identical in *b* show a comparable increase in pheromone gene expression as observed in the mixture of corresponding wild-type strains (Figure 7B and C, lanes 3 and 4). This suggests that the described mating defect of *fuz7* mutants (Banuett and Herskowitz, 1994) is not caused by a block in pheromone induction (see Discussion). To further substantiate this conclusion, we have generated a $gpa3_{Q206L}$ *fuz7-5* double mutant in strain FB1 (*a1 b1*) (see Materials and methods). If Fuz7 acts downstream of Gpa3, we expected that in the double mutant the constitutively active Gα subunit should no longer be able to stimulate the expression of *mfa1*. Northern analysis demonstrated that the *a1 b1* $gpa3_{Q206L}$ *fuz7-5* double mutant strain displayed the same increased level of *mfa1* expression as the *a1 b1* $gpa3_{Q206L}$ mutant strain (Figure 7A). We thus conclude that Fuz7 and Gpa3 are unlikely to be components of the same signalling pathway.

Discussion

In this communication we describe the identification of four different genes encoding Gα subunits in *U.maydis* and show that one of these Gα proteins, Gpa3, plays a critical role in transmission of the pheromone signal. In addition, Gpa3 has an essential function during pathogenic development.

Multiple Gα subunits in U.maydis

The finding of four genes encoding distinct Gα subunits in the Basidiomycete *U.maydis* contrasts with the general situation in fungi, where no more than two genes in a single organism have been identified so far (Borkovich, 1996). In addition, the availability of the complete sequence of the *S.cerevisiae* genome gives no hints to the presence of additional Gα subunits in this species.

In fungal systems except the yeasts, knowledge about the biological function of the Gα subunits identified is limited because most of these genes were identified by sequence homology. Notable exceptions are the *cpg1* gene of *C.parasitica* that plays a role in regulating fungal virulence (Choi *et al.*, 1995) and the *gna1* gene of *N.crassa*, which participates in multiple sexual and asexual differentiation steps (Ivey *et al.*, 1996). The most thoroughly analysed Gα genes are those of the yeasts *S.cerevisiae* and *S.pombe*. In both organisms, one of the respective Gα subunits is involved in pheromone signalling, whereas the other is implicated in nutritional sensing (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987; Nakafuku *et al.*, 1988; Obara *et al.*, 1991; Isshiki *et al.*, 1992).

In *U.maydis*, we have not been able to ascribe functions to three of the *gpa* genes identified. This could indicate functional redundancy or involvement in signalling pathways that neither play an essential role during growth in complete medium nor during growth *in planta*. Interestingly, one of these Gα subunits, Gpa4, showed significantly less similarity to all known Gα proteins and differs from the consensus at several conserved positions. In particular, the motif involved in GTP binding, GQAGAGKT, deviates from the consensus in the third amino acid position (GXGXXGKS/T), and the highly conserved glutamine in the GTPase domain DXXGQ has been exchanged for a serine residue (see Figure 1) (Kaziro *et al.*, 1991; Hamm and Gilchrist, 1996). These substitutions in Gpa4 might affect the affinity for GDP/GTP or the activity of the intrinsic GTPase.

Gpa3 and transmission of the pheromone signal

It has been proposed that binding of the pheromone to the receptor in wild-type *U.maydis* cells leads to transcriptional activation of the pheromone and receptor genes (Hartmann *et al.*, 1996; Urban *et al.*, 1996a). In this study, the expression level of both pheromone genes, *mfa1* and *mfa2*, was used to monitor the pheromone response in *U.maydis*. Pheromone stimulation was triggered by co-cultivation of two haploid strains that were different at *a* and carried identical or non-identical *b* alleles, respectively. In these experiments, expression levels of pheromone-inducible genes were significantly higher when strains carrying identical *b* alleles were mixed compared with mixtures where both strains carried different *b* alleles. This most likely reflects a negative effect of the active bE–bW heterodimer on *mfa* gene expression that has been observed before in a diploid strain (Urban *et al.*, 1996a). Strains deleted for *gpa3* were unresponsive to pheromone, whereas a haploid *a1 b1* strain carrying the constitutive active $gpa3_{Q206L}$ allele showed a dramatic increase in *mfa1* expression in the absence of pheromone stimulation. In addition, two haploid strains identical in *a* but different in *b* could form an infectious dikaryon if they both carried the $gpa3_{Q206L}$ allele. Pheromone-independent mating through constitutive activation of the respective signalling cascade demon-

strates that, in *U.maydis*, the GTP-bound form of Gpa3 plays the active role in transmission of the pheromone signal. This is reminiscent of the situation in *S.pombe*, where it is also the Gα subunit that actively transmits the pheromone signal (Obara *et al.*, 1991). In *S.cerevisiae*, on the other hand, it is the βγ heterodimer of the G protein that activates the downstream effector (Whiteway *et al.*, 1989). Since Δ*gpa3* mutant strains show additional phenotypes, e.g. morphological alterations that cannot easily be explained by loss of pheromone signalling, it is feasible that the free βγ heterodimer can activate other signalling pathways. In accordance, neither receptor mutant strains nor *prf1* mutants display any morphological changes.

The Δ*gpa3* mutants also show a decrease in the basal expression levels of both *mfa* genes. To explain this effect, we consider the possibility that the pheromone receptor activates the G protein at low levels in the absence of the cognate pheromone. Alternatively, the free βγ heterodimer in Δ*gpa3* mutants could exert an inhibitory effect, or the Gα subunit could be activated by other receptors, since environmental signals also have been shown to affect the basal level of *mfa* gene expression (Spellig *et al.*, 1994).

Another question remaining is why compatible combinations of strains carrying the constitutively active $Gpa3_{Q206L}$ show a significantly more vigorous mating reaction than incompatible combinations (Figure 5, compare panels B2 and C2). This could imply that a certain fraction of $Gpa3_{Q206L}$ is in the inactive form and can still be activated through the pheromone receptor. Alternatively, one could propose that binding of pheromone to the receptor induces an additional G protein-independent signalling process during mating, as has been proposed for *S.cerevisiae* (Jackson *et al.*, 1991).

Since a heterotrimeric G protein is involved in pheromone signalling, it is anticipated that a MAP kinase cascade transmits the pheromone signal into the nucleus (Herskowitz, 1995). In *U.maydis*, a proposed component of such a MAPK module, Fuz7, had been isolated (Banuett and Herskowitz, 1994). By demonstrating that transcriptional activation of pheromone-inducible genes is not affected in *fuz7* mutants, we can exclude an epistatic relationship of the two genes. This result forces us to propose two distinct stages during the pheromone response. One is an early response, involves *gpa3*, and results in the activation of the pheromone-inducible genes without morphological alterations. By using the green fluorescent protein fused to the *mfa1* gene promoter, such a stage has recently been documented (Spellig *et al.*, 1996). In the second stage, the pheromone signal is converted to a morphological transition and this transition requires a second signalling cascade, including *fuz7*. In this context we find it interesting that *fuz7* mutants do not only display defects in conjugation tube formation, but also show defects in filamentous growth, maintenance of the filamentous stage and promycelium formation (Banuett and Herskowitz, 1994). Although the cellular structures involved appear distinct, a participation of Fuz7 in all these morphological transitions could point to a general pathway for morphogenesis and it should be an exciting task to identify other components of this apparently unprecedented signalling pathway.

Gpa3 and pathogenic development

In *U.maydis*, there is ample documentation that pheromone signalling is not a prerequisite for tumour induction. Diploid strains homozygous at *a* and heterozygous at *b*, as well as haploid strains carrying a single *a* allele and a composite *b* locus consisting of one *bE2* and one *bW1* gene, were all shown to be solopathogenic (Holliday, 1961; Puhalla, 1968; Banuett and Herskowitz, 1989; Bölker *et al.*, 1995). In addition, we have demonstrated in this communication that the pheromone receptor gene can also be deleted without adverse effects on pathogenic development. Therefore, it was a rather unanticipated finding that the deletion of *gpa3* prevents pathogenic development. Interestingly, it has recently been shown for the chestnut blight fungus *C.parasitica* that fungal virulence is attenuated by virus-mediated down-regulation of a Gα subunit. In this system, growing evidence suggests that virulence is affected by alteration of G protein-regulated cAMP levels (Choi *et al.*, 1995; Chen *et al.*, 1996).

To explain the loss of pathogenicity in *U.maydis gpa3* mutants, one could formally propose that the free βγ heterodimer in the *gpa3* mutant might inhibit an essential step during pathogenic development. A more attractive hypothesis that could at the same time explain the complex phenotype of *gpa3* mutants is to assume that Gpa3 is involved in multiple signalling pathways and could coordinate additional inputs from the host plant or the environment. Integrity of such additional signalling pathway(s) may be a prerequisite for pathogenicity. These signalling pathways might use the same or a different MAPK module but must differ in the transcription factors that are activated. This conclusion stems from the observation that loss of pathogenicity in *prf1* mutants can be fully restored by overexpressing the *b* genes (Hartmann *et al.*, 1996) while overexpression of *b* does not suppress the pathogenicity defect of Δ*gpa3* mutants. Such an alternative signalling pathway might be required for induction of pathogenicity genes either together with the bE–bW heterodimer or independently of *b*. Since overexpression of the *b* genes in Δ*gpa3* mutants induces filamentous growth but not pathogenicity, the transition to filamentous growth may not rely on the proposed additional signal input which appears essential for pathogenicity. If this proposal holds up, pathogenic development of *U.maydis* may not only require the genetic switch exerted by the *b* locus but, in addition, the ability to respond to signals from its host plant.

Materials and methods

Strains and growth conditions

For cloning purposes, the *Escherichia coli* K-12 derivative DH5α (Bethesda Research Laboratories) was used.

U.maydis strains FB1 (*a1 b1*), FB2 (*a2 b2*), FB6b (*a1 b2*), FB6a (*a2 b1*), CL13 (*a1 bW2 bE1*), SG200 (*a1 mfa2 bW2 bE1*), FBD12-17 (*a2 a2 b1 b2*) and FBD11-7 (*a1 a1 b1 b2*) have been described previously (Banuett and Herskowitz, 1989; Bölker *et al.*, 1995). Strains were grown in YEPS at 28°C (Tsukuda *et al.*, 1988). The mating reaction was monitored by co-spotting strains on solid potato dextrose medium (PDA, Difco) containing 1% activated charcoal. Plates were sealed with parafilm and incubated at room temperature for 2–4 days. Plant infections were performed as described previously (Gillissen *et al.*, 1992). The transformation of *U.maydis* followed the protocol of Schulz *et al.* (1990).

Plasmids

For subcloning and sequencing, the plasmids pTZ18R, pTZ19R, pSL1180 (Pharmacia), pUC118 and pUC18 were used. pHLN (Schulz *et al.*, 1990) and pTZHyg (Schauwecker *et al.*, 1995) contain the hygromycin resistance cassette in which the bacterial *hph* gene is under control of the *U.maydis* hsp70 promoter (Tsukuda *et al*., 1988). For overexpression of the *bE1* and *bW2* genes, the plasmid pb^{con} (Hartmann *et al.*, 1996) was used. pUMa1::Tn5H#3 carries a Tn5H insertion in the *pra1* gene (Urban *et al.*, 1996b).

Cloning of gpa genes and construction of plasmids for gene replacement

gpa1. The degenerated primers oMP20 and Ta29 (Strathmann *et al.*, 1990) were used for amplification with 100 ng FB6b DNA as template in a volume of 100 µl. Reactions contained 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 20 pmol primers and 1.5 U Taq polymerase (Boehringer). Amplification was achieved in 35 cycles of 1 min at 94°C, 30 s at 42°C, and 30 s at 72°C. PCR products were cloned after digestion with *Bam*HI into pTZ19R for sequencing. The amplified *gpa1* fragment was used to screen a genomic λEMBL3 library (Schulz *et al.*, 1990). *gpa1* was cloned as 2.8 kb *Pst*I fragment in pUC18 and the resulting plasmid was designated pGPA1. pΔGPA1 was generated from pGPA1 by replacing a 100 bp *Nco*I fragment from the coding region of *gpa1* with the hygromycin resistance cassette isolated as a *Pvu*II fragment from pHLN.

gpa2. The open reading frame of the *S.cerevisiae* GPA2 was amplified by PCR with 1 µg yeast DNA (wild-type strain D273-10B; ATCC 25657) and the oligonucleotides Ysc-gpa2/1 (5′-CGGGATCCAATAT-CATGGGTCTCTGCG-3′) and Ysc-gpa2/2 (5′-CGGGATCCGCATT-CATTGTAACACTCCAG-3′) as primers. Standard reaction conditions were used. The PCR product was isolated and cloned as a 1.4 kb *Bam*HI fragment in pTZ19R. This fragment was used to hybridize a *U.maydis* cosmid library (Bölker *et al.*, 1995) under low-stringency conditions according to Church and Gilbert (1984) at 60°C. A 1.3 kb *Sph*I–*Pst*I fragment containing the ORF of the *gpa2* gene was cloned into pUC118 resulting in plasmid pGPA2. pGPA2–Hyg was derived from pGPA2 by inserting the hygromycin resistance cassette from pTZHyg into the internal *Bam*HI site of the *gpa2* gene.

gpa3. Degenerate primers GAGES (5′-CGGGATCCGGNGCNGGNG-ARWSNGGNAA-3′) and YFXDY (5′-CGGGATCCTARTCNGRRA-ARTA-3′) with R = A/G, N = A/C/T/G, W = A/T and S = G/C were used for amplification of *U.maydis* DNA from strain 521 (*a1 b1*, from the collection of R.Holliday) using the same reaction conditions as for *gpa1*. 35 cycles of 1 min at 94°C, 1 min at 47°C, and 2 min at 72°C plus one additional cycle of 10 min at 72°C were performed. PCR products were cloned as *Bam*HI fragments into pTZ19R for sequencing. The 238 bp *gpa3* fragment was used to screen both a cDNA library (Schauwecker *et al.*, 1995) and a genomic λEMBL3 library (Schulz *et al.*, 1990). Two incomplete cDNA clones of *gpa3* were isolated and to generate pGPA3, the genomic copy of *gpa3* was cloned as a 2.5 kb *Mlu*I fragment into a pSL1180 derivative, in which the *Bam*HI restriction site was eliminated by deletion of the *Bam*HI–*Bgl*II region of the polylinker. pΔGPA3 was generated from pGPA3 by replacing the 238 bp *Bam*HI fragment from the coding region of *gpa3* with the hygromycin resistance cassette derived from pTZHyg.

The mutant allele $gpa3_{Q206L}$ in which the glutamine at position 206 is exchanged for leucine was generated by PCR using the oligonucleotides GPA3-470 (5′-GCTCGATCACGCCATGGCG-3′) and GPA3-Q206L (5′-ACGTTCCGAACGGAGTCCTCCTACG-3′) as primers for site-directed mutagenesis and 100 ng FB1 DNA as template. In a second round of PCR, 500 ng of the 250 bp PCR product and GPA3-860 (5′-caaatagaacgagcgactcgg-3′) were used as primers to amplify a 390 bp fragment that harbours the mutation. The following protocol was used for amplification: one cycle of 5 min at 94°C; five cycles of 1 min at 94°C, 4 min at 50°C and 1 min at 74°C; 25 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 74°C, plus one cycle of 5 min at 74°C. The internal 238 bp *Bam*HI fragment was cloned into pTZ19R, sequenced and used to replace the hygromycin cassette of pΔGPA3. The hygromycin resistance cassette was reintroduced as a *Pvu*II fragment into the *Eco*RV site of the polylinker and the resulting plasmid was designated $pGPA3_{Q206L}$.

gpa4. The gene *gpa4* was isolated in a screen for differentially expressed genes (T.Spellig and R.Kahmann, in preparation). The ORF was cloned as a 3 kb genomic *Bam*HI fragment of a cosmid clone (Bölker *et al.*, 1995) into pTZ19R to generate pGPA4. From a cDNA library (Schauwecker *et al.*, 1995) a full-length cDNA clone of *gpa4* could be isolated. pΔGPA4 was derived from pGPA4 by replacing 0.6 kb of the *gpa4* coding region with a hygromycin resistance cassette. Oligonucleotides with *Not*I linkers at their 5′ ends hybridizing to positions 1122 to 1104 (ATTTGCGGCCGCCGAGAGTAGTTGTCGAGAAAG) and 1705 to 1725 (ATTTGCGGCCGCTTGAGGGAGAACCTCAAACTG) of *gpa4*, respectively, were used in a PCR reaction using Tfl-polymerase (Biozym) and pGPA4 as template, resulting in a 5.3 kb fragment that lacks 600 bp of the coding region of *gpa4*. The fragment was digested with *Not*I and religated. The hygromycin resistance cassette of pHLN was ligated to *Not*I linkers and inserted into the *Not*I site of this plasmid to generate pΔGPA4.

Strain construction

With the exception of the *a2 b1* Δ*gpa3* strain, all strains in which the resident *gpa* genes were replaced by the mutant *gpa* alleles were generated by homologous recombination. The respective plasmids were linearized with *Ssp*I (pΔGPA1), *Kpn*I (pGPA2–Hyg), *Mlu*I (pΔGPA3) and with *Bam*HI (pΔGPA4) and transformed into the haploid *U.maydis* strains FB1 and FB2. pΔGPA3 was also transformed in CL13 and SG200. Transformants were screened for loss of the wild-type copy by Southern analysis (not shown). Gene replacement occurred with a frequency of 65% for *gpa1*, 45% for *gpa2*, 11% for *gpa3* and 29% for *gpa4*.

The strain *a2 b1* Δ*gpa3* was isolated from the progeny of a cross between FB1 and FB2Δgpa3. The *a* mating type was determined by testing for secreted pheromone by co-spotting with the pheromone tester strains FBD12-17 (*a2 a2 b1 b2*) and FBD11-7 (*a1 a1 b1 b2*), respectively (Spellig *et al.*, 1994). The nature of the *b* allele was determined by RFLP analysis. To this end, a 1.1 kb fragment encompassing the variable region of the *b* locus was amplified with the primers Kla18R (5′-CTGAAAGTCGGAACTTCTC-3′) and 122-115 (5′-GCATGTG-GTACGCTGGAAGATCCT-3′). Upon cleavage with *Bgl*II, *b1* and *b2* could be distinguished. Presence of the Δ*gpa3* mutation was verified by Southern analysis.

To generate strains with a chromosomal $gpa3_{Q206L}$ mutation, $pGPA3_{Q206L}$ was transformed as a circular plasmid into FB1. A transformant in which $pGPA3_{Q206L}$ was inserted into the chromosomal *gpa3* gene by a single crossing over event was identified by Southern analysis using the 2.5 kb *Mlu*I fragment encompassing *gpa3* as probe. In this strain, plasmid excision was stimulated by UV-induced mitotic recombination (Holliday, 1961). Hygromycin-sensitive strains were isolated and probed for the presence of the $gpa3_{Q206L}$ allele. To this end, a 390 bp region from position 470 to 860 of *gpa3* was amplified with the primers GPA3-470 and GPA3-860 and screened for the presence of an additional *Hin*fI site which is indicative for the $gpa3_{Q206L}$ allele. This allowed the identification of $FB1gpa3_{Q206L}$. The *U.maydis* strains $ER22gpa3_{Q206L}$ (*a2* $gpa3_{Q206L}$ *b2*) and $ER21gpa3_{Q206L}$ (*a1* $gpa3_{Q206L}$ *b2*) were isolated from the progeny of a cross between $FB1gpa3_{Q206L}$ (*a1* $gpa3_{Q206L}$ *b1*) and FB2 (*a2 b2*). The presence of the $gpa3_{Q206L}$ allele was verified as above.

The *pra1*-deficient strain CL13pra1::Tn5H was generated by transforming pUMa1::Tn5H#3 linearized with *Mlu*I into the strain CL13 and screening for gene replacement by Southern analysis.

To generate a gene disruption in the *fuz7*, gene the plasmid pFuz7::Tn5H#248 was constructed. A *U.maydis* cosmid library was screened with an internal fragment of the *fuz7* gene, which was amplified by PCR with oligonucleotides F471 (5′-AGAGCTTCAGATCCT-GCACG-3′) and F472 (5′-TGACACTGTACTGATCTCCC-3′). From a hybridizing cosmid clone, a 8 kb *Pvu*II genomic fragment encompassing the ORF of *fuz7* was cloned into pSP72 and mutagenized with the Tn5H transposon according to the procedure of Urban *et al.* (1996b). The resulting plasmid pFuz7::Tn5H#248 was sequenced and carries a Tn5H mutation at nucleotide position 775 of the *fuz7* gene (Banuett and Herskowitz, 1994). pFuz7::Tn5H#248 was linearized with *Sfi*I and transformed into the *U.maydis* strains FB1, FB6a and $FB1gpa3_{Q206L}$ to create the mutant strains FB1fuz7-5, FB6afuz7-5 and $FB1fuz7-5gpa3_{Q206L}$. Replacement of the resident *fuz7* gene by the *fuz7::Tn5H* allele was verified by Southern analysis.

DNA and RNA procedures

U.maydis DNA was isolated according to the protocol of Hoffman and Winston (1987). Radioactive labelling of the DNA was performed with the megaprime DNA labelling kit (Amersham). DNA sequencing was carried out with the T7 DNA sequencing kit (Pharmacia). RNA was isolated from strains grown on charcoal-containing CM plates (Holliday, 1974) for 48 h and Northern blot analyses were performed as described previously (Schauwecker *et al.*, 1995). All other molecular techniques

followed standard procedures (Sambrook *et al.*, 1989). As probes for Northern blot analyses, a 680 bp *Eco*RV fragment from pUMa1 (Urban *et al.*, 1996a) served as probe for *mfa1*, a 386 bp *Pst*I–*Spe*I fragment of pUMa2 for **mfa**2 (Urban *et al.*, 1996a), and a 1.7 kb *Eco*RI–*Eco*RV fragment from pCBX122, encompassing the constitutively expressed *cbx* gene, was used as control (Keon *et al.*, 1991).

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