Fusarium verticillioides *GAP1*, a gene encoding a putative glycolipid-anchored surface protein, participates in conidiation and cell wall structure but not virulence

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Fusarium verticillioides is an important pathogen of maize that causes ear rot and produces the mycotoxins known as fumonisins. To date, knowledge of pathogenicity and the regulation of fumonisin biosynthesis in *F. verticillioides* is limited. Here, the molecular characterization of *GAP1*, a gene encoding a putative 540 aa protein that belongs to a glycolipid-anchored surface (GAS) protein family, is presented. *F. verticillioides* *GAP1* was identified as an expressed sequence tag (EST) upregulated in a culture condition conducive to conidiation and fumonisin B₁ (FB₁) production. *GAP1* null mutants GAM126 ($\Delta gap1::HYG$) and GAG8 ($\Delta gap1::GEN$) exhibited restricted growth, with more aerial hyphae than their wild-type progenitor on solid media. No defect in mycelial mass or filamentous growth was observed when the GAM126 and GAG8 strains were grown in liquid media under shaking conditions. When grown in suspended conditions, GAM126 and GAG8 strains produced significantly fewer conidia and produced comparatively densely branched hyphae. Concanavalin A staining indicated that the *GAP1* deletion altered the cell wall carbohydrate composition/deposition process. Deletion of *GAP1* did not affect the production level of FB₁ or *F. verticillioides* virulence on maize seedlings and stalks. Complementation of GAM126 with the wild-type *GAP1* gene restored growth, conidiation and cell wall abnormality phenotypes. The results suggest that *GAP1* is associated with growth, development and conidiation in *F. verticillioides*, but not with pathogenicity or regulation of FB₁.

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INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland) causes rots of maize (*Zea mays*) stalks and ears (Munkvold & Desjardins, 1997). Stalk rot of maize is widespread in dry, warm climates (White, 1999), and can cause losses in several different ways, such as stalk breakage, lodging, interruption of the normal grain-filling process, and sometimes premature death of the plant (Dodd, 1980; Michaelson, 1957; White, 1999). On maize ears, the fungus can produce

polyketide-derived carcinogenic mycotoxins called fumonisins (Nelson *et al.*, 1993; Marasas, 2001). Since the discovery of fumonisins, several efforts have been made to understand the regulation of fumonisin biosynthesis in the *F. verticillioides*–maize system (reviewed by Sagaram *et al.*, 2006). Briefly, fumonisins are synthesized by a cluster of 22 *FUM* genes, of which 15 are co-regulated (Proctor *et al.*, 1999, 2003). Recently, a putative regulatory gene within the *FUM* cluster, *FUM21*, has been identified and characterized (Brown *et al.*, 2007). However, *FUM21* gene deletion is not sufficient to completely block fumonisin biosynthesis, suggesting that transcriptional regulation of *FUM* genes is also affected by regulatory genes outside the cluster. Fumonisin biosynthesis is also impacted by several environmental factors, including available nitrogen, ambient pH and maize kernel constitution environment (reviewed by Sagaram *et al.*, 2006).

The first regulatory gene identified by the restriction enzyme-mediated integration (REMI) strategy was *FCC1*, which encodes a C-type cyclin (Shim & Woloshuk, 2001). Mutation in *FCC1* leads to a severe reduction in

Abbreviations: CFW, Calcofluor white; ConA, concanavalin A; CR, Congo red; EST, expressed sequence tag; FB₁, fumonisin B₁; GAS protein, glycolipid-anchored surface protein; GPI, glycosylphosphatidylinositol; SSH, suppression subtractive hybridization.

The GenBank/EMBL/DDBJ accession no. for the 350 bp *GAP1* gene sequence from *F. verticillioides* is DQ458798.

Figures showing the *F. verticillioides* seedling rot assay on hybrid sweetcorn, and Northern analysis of *GAP2* and *GAP3* expression in *F. verticillioides*, are available as supplementary data with the online version of this paper.

conidiation and fumonisin production. Nitrogen source and pH strongly influence the conidiation and expression of the fumonisin biosynthetic gene (*FUM1*) in a $\Delta fcc1$ strain, indicating a regulatory role for *FCC1* in fumonisin biosynthesis and fungal development (Shim & Woloshuk, 2001). Subsequent genomic approaches, such as construction of cDNA subtraction libraries using a wild-type strain and $\Delta fcc1$ strains (Shim & Woloshuk, 2001), and microarray analysis (Pirttilä *et al.*, 2004), have identified additional regulatory genes for fumonisin biosynthesis. As a result, several genes, e.g. *FCK1*, a C-type cyclin-dependent kinase, *PAC1*, a pH regulatory gene, *ZFR1*, a Zn(II)2Cys6 gene, and *AMY1*, a gene encoding a putative α -amylase (reviewed by Sagaram *et al.*, 2006), have been identified and characterized.

During the screening of *F. verticillioidea* EST index (TIGR) and suppression subtractive hybridization (SSH) cDNA libraries (Shim & Woloshuk, 2001), a 350 bp EST was identified in the wild-type SSH library that encodes a protein sequence with high homology to glycosylphosphatidylinositol (GPI)-anchored β -1,3-glucanotransferases that belong to a class of GPI-anchored glycoproteins. We designated this gene *GAP1* (GPI-anchored protein 1). GPI-anchored cell wall proteins (GPI-CWPs) are among the most common proteins in eukaryotic cell walls (De Groot *et al.*, 2005) that are covalently linked to β -1,6-glucan, which can be further linked to β -1,3-glucan or chitin, resulting in a strong anchorage of these proteins to cell walls (Kaptein *et al.*, 1996, 1997; Kollar *et al.*, 1997). The β -1,3-glucanotransferases are involved in the elongation of β -1,3-glucan side chains of the cell wall (Mouyna *et al.*, 2000). These enzymes split the β -1,3-glucan molecule and transfer the newly produced reducing end to the non-reducing end of another β -1,3-glucan molecule (Mouyna *et al.*, 2000). GPI-anchored β -1,3-glucanotransferases have been shown to play a critical role in fungal cell wall biogenesis (Mouyna *et al.*, 2000), morphogenesis and virulence in opportunistic fungal pathogens of humans (Muhlschlegel & Fonzi, 1997; DeBernardis *et al.*, 1998; Ghannoum *et al.*, 1995; Mouyna *et al.*, 2005). For example, *GEL1* and *GEL2* are GPI-anchored β -1,3-glucanotransferase-encoding genes characterized in *Aspergillus fumigatus* (Mouyna *et al.*, 2000, 2005). Disruption of *GEL1* does not result in a phenotype, while disruption of *GEL2*, and disruption of both *GEL1* and *GEL2*, result in altered cell wall composition, slower growth, unusual conidiogenesis and reduced invasive aspergillosis in a murine model (Mouyna *et al.*, 2005). Significantly, a β -1,3-glucanotransferase-encoding gene, *GAS1*, is also involved in virulence in the plant-pathogenic fungus *Fusarium oxysporum* (Caracuel *et al.*, 2005), suggesting that *GAP1* may be associated with fungal virulence in *F. verticillioidea*. Caracuel *et al.* (2005) have also reported that *F. oxysporum* *GAS1* expression is pH-independent, in contrast to *Candida albicans* *PHR1* and *PHR2* (Muhlschlegel & Fonzi, 1997).

The fact that the *GAP1* EST was isolated in the *F. verticillioidea* wild-type SSH cDNA library (Shim &

Woloshuk, 2001) prompted us initially to hypothesize that *GAP1* is primarily associated with fumonisin biosynthesis. However, we also argued that *GAP1* could be primarily associated with fungal development and conidiation (Mouyna *et al.*, 2000; Shim & Woloshuk, 2001), which in turn may affect fungal virulence (Caracuel *et al.*, 2005). Thus, we formulated a hypothesis that *GAP1* regulates fungal development and virulence in *F. verticillioidea*. To test this hypothesis, we generated *GAP1* deletion mutants of *F. verticillioidea* and investigated growth, conidiation and cell wall biogenesis. In addition, we analysed fungal virulence on maize and fumonisin production. In this study, we demonstrate that *GAP1* is required for proper growth and conidiation on solid synthetic media and conidiation in liquid media. Our study revealed that *GAP1* influences cell wall structure by organizing the carbohydrate deposition in the cell walls when the fungus is growing on solid synthetic media. We also show that *GAP1* is not involved in *F. verticillioidea* virulence on maize, and does not positively regulate fumonisin biosynthesis.

METHODS

Fungal strains, media and growth conditions. The wild-type *F. verticillioidea* strain 7600 (M3125; Fungal Genetics Stock Center) and mutant strains generated for this study were stored in 30% (v/v) glycerol at -80°C . For genomic DNA extraction, the fungus was grown in YEPD medium (3 g yeast extract l^{-1} , 10 g peptone l^{-1} , and 20 g dextrose l^{-1}) or potato dextrose broth (PDB) (BD). For production of initial inoculum [for RNA isolation, growth measurements, spore counts, virulence assays and fumonisin B₁ (FB₁) analysis] the fungal strains were grown on V8 juice agar plates (200 ml V8 juice l^{-1} , 3 g CaCO_3 l^{-1} , 20 g agar l^{-1}) for 7–10 days. The spores were harvested in sterile water, passed through cheesecloth and quantified using a haemocytometer. For RNA isolation, 10^6 spores were inoculated into BSA liquid (BSAL) medium (pH 3.5, 6.0 and 8.5) (1 g $\text{NH}_4\text{H}_2\text{PO}_4$ l^{-1} , 40 g sucrose l^{-1} , 3 g KH_2PO_4 l^{-1} , 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ l^{-1} , 5 g NaCl l^{-1}) or 10^7 spores were inoculated into defined liquid (DL) medium (initial pH 3.0 and 8.0) (1 g $\text{NH}_4\text{H}_2\text{PO}_4$ l^{-1} , 40 g sucrose l^{-1} , 3 g KH_2PO_4 l^{-1} , 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ l^{-1} , 5 g NaCl l^{-1}) and allowed to grow for 7 days at room temperature ($22\text{--}23^{\circ}\text{C}$) at 125 r.p.m.

Nucleic acid manipulation. Bacterial plasmid DNA was isolated with the Wizard miniprep DNA purification system (Promega). Fungal genomic DNA was extracted by methods described previously (Shim & Woloshuk, 2001). Total RNA was extracted with an RNeasy Plant Mini kit (Qiagen) or Trizol reagent (Invitrogen) as per the manufacturers' protocols. Southern and Northern analyses were performed following standard procedures (Sambrook & Russell, 2001). For Southern analysis, genomic DNA (10 μg) was digested with *EcoRI* and subjected to electrophoresis on a 1% agarose gel. The DNA was transferred onto a nylon membrane and probed with a ^{32}P -labelled DNA fragment amplified from *F. verticillioidea* genomic DNA with primers FvGAS-F4t and FvGAS-R3 (Table 1). For Northern analysis, total RNA was subjected to electrophoresis on a 1.2% denaturing agarose gel, transferred onto a nylon membrane and hybridized with ^{32}P -labelled gene-specific probes. The probes used in all hybridization experiments were ^{32}P -labelled with a Prime-It Random Primer Labeling kit (Stratagene).

PCR. All PCR reactions were performed in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems). The primers used in this

Table 1. Primers used in this study

Name	Sequence (5'–3')
FvGAS-F5	TTCGAGTTGTCGTCGTCTGAACCA
FvGAS-R2t	<u>TCACTGGCCGTCGTTTACAATAAAGCAGTCTAGCTACGGGACCA*</u>
FvGAS-F2	AACAACGGCATAACCAGAAACGCC
FvGAS-F4t	<u>CATGGTCATAGCTGTTTCCTGATGCCATCTTGCGCGATTGCTATG†</u>
FvGAS-R3	TTGAGCGTGTCTAATGGAGCTGGT
FvGAS-R4	TTCCAAGTCAGGTTCGCAACAACG
FvGAS-R5	ACCAAAGTTCTCGTCATCCTCGCT
FvGAS-F7t	<u>CATGGTCATAGCTGTTTCCTGTTTCGAGTTGTCGTCGTCTGAACCA†</u>
M13 F	TTGTAAAACGACGCCAGTGA
M13-R	CAGGAAACAGCTATGACCATG
FvGaseRho-D1	AAACAGCCTTATCATGCAACGGGC
FvGaseRho-U1	AGAGCTTGATTTGGCGCTTGTTGG
FvGAP2-F1	GATCCCGATGTTTGCAAGCGTGAT
FvGAP2-R1	ATCATCATCATCAGAGGCAGAGGC
FvGAP3-F1	ACACAGCCTACCTTAATCGCACCT
FvGAP3-R1	TGTGTCTGCATCGTTCTTACCGGA

*The underlined sequence represents the complementary sequence of the M13-F primer used in double-joint PCR.

†The underlined sequence represents the complementary sequence of the M13-R primer used in double-joint and single-joint PCR.

study are listed in Table 1. PCR amplification of DNA was performed in 25 or 50 µl total volumes with *Taq* DNA polymerase (Promega) or Expand Long Polymerase (Roche). The PCR conditions were 2 min of pre-denaturation at 94 °C followed by 30 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 55–57 °C, and 1–2 min of extension at 72 °C for *Taq* DNA polymerase and 68 °C for Expand Long Polymerase.

Deletion and complementation vector construction. Two deletion constructs were generated by the double-joint PCR strategy (Yu *et al.*, 2004) to replace the *GAP1* ORF in *F. verticillioides* strain 7600. First, 1150 bp 5' and 1050 bp 3' flanking regions of the *GAP1* gene were amplified from *F. verticillioides* genomic DNA using *Taq* DNA polymerase. The primers FvGAS-F2 and FvGAS-R2t were used to amplify the 5' flanking region, and primers FvGAS-F4t and FvGAS-R4 were used to amplify the 3' flanking region. Simultaneously, the hygromycin phosphotransferase (*HYG*)-encoding gene and the geneticin (G418)-resistance gene (*GEN*) were amplified from plasmid vectors pBP15 and pBS-G, respectively, using the primers M13-F and M13-R. The drug-resistance markers were amplified using Expand Long Polymerase, which has proof-reading activity. Subsequently, the three amplicons were mixed in a single tube in a 1:3:1 (5' fragment:marker:3' fragment) molar ratio and joined by PCR without using any primers. Finally, nested primers FvGAS-F5 and FvGAS-R4 were used to amplify the 3.9 kb amplicon carrying the *HPH* or *GEN* marker fused to the *GAP1* flanking regions. These fused products, named GAS1DV2-H and GAS1DV2-G, which harbour *HYG* and *GEN*, respectively, were used as *GAP1* disruption constructs.

The *GAP1* deletion mutant strain GAM126 ($\Delta gap1::HYG$) was complemented with a wild-type *GAP1* gene that was fused to *GEN*. *GEN* was amplified with primers M13-F and M13-R from pBS-G, and *GAP1* [including 1530 bp 5' untranslated region (UTR) and 1200 bp 3' UTR] was amplified from genomic DNA with FvGAS-F7t and FvGAS-R3 using Expand Long Polymerase. *GEN* and *GAP1* were fused by a single-joint PCR strategy to generate the complementation

construct (Shim *et al.*, 2006; Yu *et al.*, 2004). The joined-PCR product GAS1CV1 was amplified with the primers M13-F and FvGAS-R4 using Expand Long Polymerase and used for transformation to complement GAM126.

Fungal transformation. Fungal protoplasts were prepared and transformed as described previously (Shim & Woloshuk, 2001), except that mureinase (2 mg ml⁻¹) was replaced by Driselase (5 mg ml⁻¹) (Sigma), and 1 g wet mycelium was digested in 20 ml enzyme solution [10 mM NaH₂PO₄ (pH 5.8), 20 mM CaCl₂, β -glucuronidase (1343 U ml⁻¹; Sigma), Driselase (5 mg ml⁻¹; Sigma), 1.2 M KCl]. Transformants were selected on regeneration medium (343 g sucrose l⁻¹, 0.2 g yeast extract l⁻¹, 10 g agar l⁻¹) containing 150 µg ml⁻¹ hygromycin B (Calbiochem) or G-418 (Cellgro).

Mycelial staining, microscopy and cell wall assays. For mycelial staining, 10⁵ spores were inoculated on potato dextrose agar (PDA) and allowed to grow. After 2 days, the mycelia were scraped and collected in a 1.5 ml tube. Concanavalin A (ConA) type VI conjugated to FITC (Sigma, catalogue no. C7642) was used at a final concentration of 100 µg ml⁻¹ using previously published conditions (Shaw & Hoch, 1999). ConA in a 100 µl volume was added to fungal mycelia and incubated for 45 min. Later, samples were washed twice with 100 µl water by successive centrifugation and resuspension. Finally, the samples were resuspended in 100 µl water before observation under the microscope. Microscopic imaging used an Olympus BX51 microscope (Olympus America) fitted with Uplanapo objectives and an Olympus DP70 cooled charge-coupled device (CCD) digital camera. Further details of this imaging system have recently been described (Upadhyay & Shaw, 2006). Images were acquired using DP70-BSW software (version 01.01) and prepared for publication with Adobe Photoshop. For FITC fluorescence visualization, an Olympus U-MNIBA2 (Olympus) filter cube was used with excitation wavelengths from 470 to 480 nm, emission wavelengths from 510 to 550 nm, and a dichroic mirror at 505 nm FITC. Susceptibility assays with Congo red (CR) (Matheson Coleman & Bell) and Calcofluor white (CFW) (fluorescent brightener F3453,

Sigma) were conducted as described in Ram & Klis (2006), with few modifications. Ten drops 5 M KOH was added to dissolve CFW. The assays were conducted on PDA with initial pH adjusted to 8.0.

Stalk rot and seedling rot assay. Stalk rot assays were performed on 8-week-old plants of the B73 corn line. Internodal regions of the stalk were punctured (2 mm deep) with a sterile needle and 10^4 spores were inoculated into the punctured hole. Plants were incubated in a growth chamber at 25 °C and 40 % humidity. Stalks were split open longitudinally after 21 days and the disease symptoms were photographed. For seedling rot assays, the B73 corn line and Golden Cross Bantam hybrid sweetcorn (Ed Hume Seeds) were surface-sterilized with 100 % bleach for 15 min, washed twice with sterile water, and soaked in sterile water for 1 h. The seeds were placed in between two layers of moist filter paper in a sterile glass saucer (30 × 15 cm) and allowed to germinate. After 7 days, the top filter paper was removed and the ungerminated seeds were discarded. Seeds were inoculated at the embryo region with 10^4 spores in a 5 µl volume. Eight seeds were inoculated for each fungal strain. The glass saucer was covered with Saran wrap and incubated at room temperature (22–23 °C) for 8 days. The experiments were repeated twice with similar results.

FB₁ analysis. FB₁ analysis was performed as described previously, with some modifications (Shim & Woloshuk, 1999). Fungal strains were grown on cracked corn (B73 line; 1 g dry weight) medium for 15 and 25 days at room temperature (22–23 °C). FB₁ was extracted with acetonitrile: water (1:1, v/v) for 48 h. The crude extracts were passed over equilibrated PrepSep SPE C18 columns (Fisher Scientific) to eliminate unwanted metabolites coming from corn and fungus. The FB₁ concentration of samples was analysed by HPLC using the Shimadzu LC-20AT HPLC system (Shimadzu Scientific Instruments) equipped with an analytical Zorbax ODS column (4.6 × 150 mm) (Agilent Technologies) and a Shimadzu SPD-20A Prominence UV/Vis detector (335 nm). The HPLC system was operated following the protocol described by Shim & Woloshuk (1999). FB₁ was quantified by comparing HPLC peak areas with those of FB₁ standards (Sigma). Four biological replicates were included in the experiment and the experiment was repeated twice.

***F. verticillioides* sexual cross experiments** Sexual crosses were performed as described in Shim *et al.* (2006), with modifications. Fresh carrots (400 g) were washed, diced and boiled for 20 min in 400 ml distilled water. The carrots were macerated and the mixture was passed through cheesecloth. Agar (20 g) was added to the filtrate and the final volume was brought to 1 l with distilled water. The carrot agar medium was autoclaved for 20 min and poured into 100 mm diameter Petri plates. Both mating types were grown on V8 agar prior to mating. *F. verticillioides* 7600, GAM126, GAG8 and GASC1 strains (all with genotype *MAT1-1*) were crossed with *F. verticillioides* strain 7598 (genotype *MAT1-2*) (Fungal Genetics Stock Center) on carrot agar plates. The growth conditions were maintained at 25 °C, with a 14 h light and 10 h dark cycle.

RESULTS

Molecular characterization of GAP1

We isolated a 350 bp EST sequence (wt_0_G15) from the *F. verticillioides* SSH cDNA library (Shim & Woloshuk, 2001) that shared high similarity to *Saccharomyces cerevisiae* GAS protein ($E=1 \times 10^{-33}$) and *C. albicans* pH responsive protein ($E=3 \times 10^{-33}$). The complete GAP1 sequence was obtained from a search of the *F. verticillioides*

7600 strain genome (Broad Institute of Harvard and MIT; <http://www.broad.mit.edu>). GPI-anchored surface proteins are known to be differentially expressed with changes in ambient pH (Saporito-Irwin *et al.*, 1995; Muhlschlegel & Fonzi, 1997). In *F. verticillioides*, GAP1 expression was repressed at pH 8.5 and induced proportionately at acidic pH (Fig. 1). The GAP1 gene is 1669 bp long with a single putative intron (49 bp) and encodes a putative protein of 540 aa. The nucleotide sequence and putative translated protein sequence were submitted to GenBank (accession no. DQ458798). A database search for conserved domains in the Conserved Domain Database (CDD; National Center for Biotechnology Information) revealed the presence of a characteristic glycolipid-anchored surface (GAS) protein (GAS1) domain at the N-terminal region of the protein (Marchler-Bauer *et al.*, 2005). Amino acid comparison using BLASTP analysis indicated that Gap1 shares high homology with predicted protein sequences of other filamentous fungi and yeast (Altschul *et al.*, 1997). Sequence accession numbers of homologues in other fungi with percentage identity to putative Gap1 are: *F. oxysporum* AAX78216 (94 %); *Fusarium graminearum* EAA69544 (81 %); *Magnaporthe grisea* XP_362679 (53 %); *Neurospora crassa* CAD70754 (51 %); *Aspergillus nidulans* XP_661334 (50 %) and *A. fumigatus* EAL84955 (49 %).

Generation of GAP1 deletion mutant

To test our central hypothesis, we generated *F. verticillioides* GAP1 deletion mutant strains, GAM126 ($\Delta gap1::HYG$) and GAG8 ($\Delta gap1::GEN$), using a double homologous recombination strategy. After protoplast transformation, hygromycin-resistant colonies were selected and screened for gene deletion by PCR (data not shown). The homologous recombination event was verified by Southern analysis

Fig. 1. Northern analysis of GAP1 expression. Total RNA (15 µg) was subjected to electrophoresis on a 1.2 % denaturing agarose gel, transferred onto a nylon membrane and hybridized with a ³²P-labelled probe. A 1 kb GAP1 genomic DNA fragment amplified with primers FvGAS-F1 and FvGAS-R5 (Table 1) was used as probe. rRNA stained with ethidium bromide is shown as an equal loading control.

(Fig. 2d). The wild-type strain produced a 5 kb band, whereas the deletion mutant GAM126 produced a 3 kb band, indicating that the *GAP1* gene was replaced with the disruption construct GAS1DV2-H (Fig. 2b, d). To further validate the GAM126 phenotype, we generated GAG8, a *GAP1* knockout strain harbouring a different selectable marker. The deletion of *GAP1* in GAG8 was also confirmed by Southern analysis (not shown). Northern analysis showed that the *GAP1* expression was completely abolished in both GAM126 and GAG8 strains, whereas the wild-type strain produced a 2.7 kb transcript (Fig. 3).

Mutation of *GAP1* results in cell wall alterations

Mutational analyses of GPI-anchored proteins in other fungi have resulted in a variety of cell wall development

Fig. 2. (a) Schematic representation of a 5.5 kb *F. verticillioides* *GAP1* locus. The dashed line and double arrowhead represent the genomic regions used as probes for Northern and Southern blots, respectively. E, *EcoRI* restriction site. (b) Schematic representation of targeted replacement of *GAP1* with the hygromycin phosphotransferase gene (*HYG*) via a double homologous integration event. (c) The complementation construct (GAS1CV1) used to complement GAM126 with a *GAP1* wild-type copy. GAS1CV1 was generated by fusing the geneticin-resistance gene (*GEN*) to the *GAP1* gene by single-joint PCR. (d) Southern analysis of transformants. Molecular sizes are indicated on the left. Lanes: 1, wild-type (WT); 2, $\Delta gap1::HYG$ (GAM126); 3, ectopic integration; 4, complemented strain (GASC1). The wild-type strain produced a 5 kb band. Homologous recombination of *GAP1* with *HYG* resulted in production of a 3 kb band due to the insertion of an *EcoRI* site. The complemented strain produced a mutant band (3 kb) and a 4 kb band due to ectopic insertion of the complementation construct.

Fig. 3. Northern analysis of gene expression in the wild-type, deletion mutants and complemented strains. Total RNA (15 μ g) was subjected to electrophoresis on a 1.2% denaturing agarose gel, transferred onto a nylon membrane and hybridized with appropriate 32 P-labelled genomic DNA fragments. rRNA stained with ethidium bromide is shown as an equal loading control. Target genes are indicated on the left. Fungal strains: 1, wild-type; 2, GAM126; 3, GAG8; 4, GASC1; 5, PAC2A (*PAC1* deletion strain).

defects (Chabane *et al.*, 2006; Davydenko *et al.*, 2005; Kottom *et al.*, 2001; Mouyna *et al.*, 2005), and therefore one of the key biological functions of *GAP1* that we were interested in was its involvement in cell wall biogenesis. We noted a brighter CFW staining pattern for the *GAP1* deletion strains GAM126 and GAG8 compared to that for the wild-type and the complemented strain GASC1 (data not shown). To better visualize this difference, we assayed the staining pattern of all four strains incubated in ConA conjugated to the fluorophore FITC. ConA is a lectin that selectively binds to glucan and mannan moieties (Shaw & Hoch, 1999). The GAM126 and GAG8 deletion strains each exhibited a significantly altered and brighter staining pattern than that of the wild-type and GASC1 when observed with identical staining protocols and camera settings (Fig. 4). Deletion strains each exhibited uniformly brightly stained cell walls punctuated by regularly spaced discrete binding foci. The wild-type and the GASC1 strain both lacked significant ConA binding.

Since the cell walls of the deletion strains showed an unusual patterning, we decided to study the expression pattern of *RHO1*, a gene encoding a small monomeric G protein that is a key regulator of cell wall biogenesis (Bickle *et al.*, 1998; Madaule *et al.*, 1987). *RHO1* of *F. verticillioides* was identified by using the nucleotide sequence of *rho1* of *F. oxysporum* (AY884607) as a query in BLASTN analysis of the *F. verticillioides* genome sequence (<http://www.broad.mit.edu>). The putative translated *RHO1* of *F. verticillioides* is 98% identical to *RHO1* of *F. oxysporum*. A 500 bp DNA fragment that spans the *RHO1* gene was amplified from *F. verticillioides* genomic DNA using primers FvRhoGase-D1 and FvRhoGase-U1, and used as a probe for Northern

Fig. 4. Staining of fungal cell walls with ConA. Fungal strains: (a) wild-type; (b) GAM126; (c) GAG8; (d) GASC1. Bar, 10 μ m. It should be noted that GAM126 and GAG8 strains exhibited a significantly brighter staining pattern separated by regularly spaced discrete binding foci (arrowheads) compared to that of the wild-type and GASC1. Identical staining protocols, exposure time (1/55 s) and camera settings were used.

analysis. Since *GAP1* itself is pH-regulated, we decided to test whether the *GAP1*-mediated regulation of *RHO1* is affected by different pHs. Since *PACC*-group transcriptional regulators control pH-responsive genes such as *PHR1* and *PHR2* in *C. albicans* (Muhlschlegel & Fonzi, 1997; Porta *et al.*, 1999; Saporito-Irwin *et al.*, 1995), we included an *F. verticillioides* *PAC1*-deletion strain to test if *PAC1* has a direct effect on *GAP1* and/or *RHO1*. Surprisingly, *RHO1* transcript levels in GAM126 and GAG8 mutants were similar to those of the wild-type under both pH conditions tested (Fig. 3). Our results also

indicated that *GAP1* expression is induced under alkaline conditions in a *PAC1*-deletion mutant, whereas *RHO1* expression is unaffected (Fig. 3). These results suggest that the deletion of *GAP1* in *F. verticillioides* might alter the composition of the cell wall through a mechanism that does not involve *RHO1*, unlike in *F. oxysporum* (Caracul *et al.*, 2005). Alternatively, it can be hypothesized that the involvement of Rho1 in cell wall biogenesis in *F. verticillioides* is post-transcriptional.

We also examined the effect of cell wall stressors such as CR and CFW on the susceptibility of GAM126 and GAG8 (Ram & Klis, 2006). Surprisingly, the deletion strains were resistant to these compounds compared to their wild-type progenitor. The wild-type strain produced significantly less radial growth on plates containing CR (25 and 50 μ g ml⁻¹) in comparison to a negative control (Fig. 5). In contrast, there was no noticeable difference in the growth of GAM126 and GAG8 in the presence of CR (Fig. 5). The resistant phenotype was not obvious in the first few days after inoculation, but was clearly expressed when observed 5 and 7 days after inoculation (not shown). The complemented strain GASC1 showed a phenotype similar to that of the wild-type. Similar results were observed with CFW at 25 μ g ml⁻¹ (not shown). At CFW concentrations of 50 μ g ml⁻¹ or higher, all the strains were highly susceptible, and as a result no growth was observed (not shown).

Deletion of *GAP1* affects growth and microconidia production

Deletion of *GAP1* affected growth on synthetic solid media and in suspension in liquid medium (Table 2, Fig. 6). When grown on agar medium, the growth of both the GAM126 strain and the GAG8 strain was restricted when compared to that of the wild-type. The deletion mutants produced a compact mass of mycelium with restricted edges that tended to grow into the agar medium, while their wild-type progenitor produced thin and spreading

Fig. 5. Effect of CR on *F. verticillioides* wild-type and mutant strains. Spore suspension (2 μ l) containing 2×10^4 (upper rows) and 2×10^3 (lower rows) spores was spot-inoculated on PDA plates with or without CR, and the plates were incubated in the dark at 25 °C. The plates shown here were photographed 5 days after inoculation. Fungal strains: 1, wild-type; 2, GAM126; 3, GAG8; 4, GASC1.

Table 2. Comparison of growth and microconidial production between the wild-type and *GAP1* deletion strains

Data were analysed using analysis of variance (ANOVA), and significantly different means were separated by least significant difference (LSD). Significantly different means for microconidial number (in the same column) are represented with a, b and c ($P<0.05$). All experiments were repeated twice with similar results.

Strain*	Growth†			Microconidia‡		
	0.5× PDA (mm)	V8 agar (mm)	0.5× PDB (mg)	0.5× PDA§, 10 ⁵	V8 agar§, 10 ⁶	0.5× PDB, 10 ⁴
Wild-type	52±1	66±1	58±1	131±18 a	70±7 a	112±7 a
GAM126	11±1	20±1	58±1	33±2 b	12±1 c	53±6 b
GAG8	11±1	19±1	60±1	32±6 b	14±1 c	47±3 b
GASC1	48±1	60±0	58±1	146±14 a	57±12 b	109±5 a

*Fungal strains were spot-inoculated with 10⁴ spores (agar plate) or 10⁶ spores (in 100 ml stagnant broth) and allowed to grow for 7 days at room temperature (22 °C).

†Growth was measured as diameter (mm) for agar plates and dry mass (mg) for broth. Values represent the mean ± SD of three biological replicates.

‡Mean ± SD of number of conidia per millilitre.

§Agar plates were flooded with 10 ml 0.1 % Triton, passed through eight layers of cheesecloth (VWR) to eliminate mycelium, and microconidia were counted using a haemocytometer.

borders on the surface of the medium (Fig. 6a). Microscopic examination of spore germination revealed that deletion strains produced short and highly branched hyphae, whereas the wild-type strain produced long and less-branched hyphae that extended on the surface of agar

(Fig. 6b). However, we observed no defect in deletion strains with regard to mycelial mass production (Table 2) or filamentous growth (Fig. 6c) when grown in liquid media under shaking conditions. In YEPD suspension, when compared to their wild-type progenitor, GAM126

Fig. 6. Effect of *GAP1* mutation on growth and hyphal development. (a) Colony edges of fungal strains grown on 0.5× PDA. (b) Spores (10³) in a 10 µl volume were spot-inoculated on 0.5× PDA. Pictures of individual colonies were taken 4 days after inoculation. (c) Growth of *GAP1* deletion mutants and complemented strains in 0.5× PDB in comparison with that of the wild-type strain. In (a) and (b) it should be noted that the deletion strain produced short and hyper-branching hyphae that resulted in compact and restricted growth on solid media. (a, b) ×20 magnification; (c) ×10 magnification. Bar, 50 µm.

and GAG8 produced thin and densely branched hyphae that tended to remain submerged (not shown). In PDB suspension, GAM126 and GAG8 produced densely branched hyphae, but hyphal diameter was not noticeably different from that of the wild-type (not shown).

GAP1 deletion strains produced significantly ($P>0.05$) fewer microconidia than the wild-type when grown on $0.5 \times$ PDA and V8 agar (Table 2). We speculated that the production of fewer conidia on agar medium might be due to the restricted growth of deletion strains. Hence, we tested the microconidia production in a liquid medium (PDB). Under this condition, GAM126 and GAG8 produced approximately half the microconidia produced by their wild-type progenitor (Table 2).

Deletion of *GAP1* did not influence pathogenicity and FB_1 production

Previous reports showing that GAS proteins in filamentous fungi are involved in virulence (Caracuel *et al.*, 2005; Martinez-Lopez *et al.*, 2004; Mouyna *et al.*, 2005) prompted us to examine the involvement of *GAP1* in *F. verticillioides* virulence. In the maize stalk rot assay, we tested the ability of deletion strains to infect and invade stalk tissue. As observed 21 days after infection, the deletion strains successfully colonized and rotted the tissue in a similar manner to the wild-type (Fig. 7a). In the maize seedling assay, the deletion strains were as efficient as the wild-type in infecting and colonizing seedlings (Fig. 7b). After 8 days, all the strains colonized the kernels completely, and invaded the radicle and plumule regions of the seedlings. Similar results were observed in both the maize lines tested (Fig. 7b, Supplementary Fig. S1). Based on these results, we concluded that *GAP1* is not involved in virulence in *F. verticillioides*.

To determine the effect of the *GAP1* mutation on FB_1 biosynthesis, we compared the FB_1 production ability of *GAP1* deletion strains with that of the wild-type strain. Observations made at 25 days post-inoculation revealed that GAM126 and GAG8 strains grew similarly, but TLC analysis showed higher FB_1 levels than those of the wild-type strain (not shown). However, further HPLC analysis indicated that only the GAG8 strain produced significantly ($P>0.05$) higher levels of FB_1 (47.63 ± 2.87 p.p.m.) than its wild-type progenitor (29.69 ± 6.87 p.p.m.). The FB_1 levels produced by GAM126 (37.38 ± 4.79 p.p.m.) and GASC1 (26.25 ± 6.36 p.p.m.) were not significantly different from those the wild-type strain. Contradictory to our hypothesis, the data clearly indicate that *GAP1* is not a positive regulator of FB_1 biosynthesis in *F. verticillioides*.

Gene complementation

To reconfirm that restricted growth and the reduced conidiation phenotype in GAM126 and GAG8 strains is due to the deletion of *GAP1*, we complemented GAM126 with a wild-type copy of *GAP1*. Geneticin-resistant transformants were analysed for a functional *GAP1* gene by PCR (data not shown) and Southern analysis (Fig. 2c, d). The complemented strain GASC1 contains a 4 kb fragment, indicative of complementation construct insertion at an ectopic location, in addition to a 3 kb fragment resulting from GAS1DV2-H. While we observed that the restoration of *GAP1* expression in GASC1 was not to that of the wild-type (lane 4, Fig. 3a and b), all the phenotypes observed in GAM126 and GAG8 strains, such as conidiation, growth and cell wall phenotypes, were completely restored to wild-type levels (Figs 4, 5 and 6), providing evidence that *GAP1* deletion was the direct cause of GAM126 and GAG8 mutant phenotypes.

Fig. 7. *F. verticillioides* stalk rot and seedling rot assay. (a) Eight-week-old maize stalks were inoculated with 10^4 spores of fungal strains at the internodal region and incubated in a growth chamber for 21 days at 25 °C. Subsequently, the maize stalks were split longitudinally to assay the severity of stalk rot. Four independent biological repetitions were performed, and two biological repetitions were selected and are shown in the figure. Maize stalk inoculated with sterile water is shown as the negative (Neg) control. (b) Germinated corn seedlings were inoculated with 10^4 spores of fungal strains at the embryo region. Eight seedlings were infected with each strain. Seedlings were periodically observed and were photographed 8 days after infection. A close-up picture of a single seedling for each strain, showing the fungal colonization, is shown.

Effect of *GAP1* deletion on *F. verticillioides* sexual development

We demonstrated that the *GAP1* deletion had no impact on sexual development in *F. verticillioides*. Wild-type, GAM126, GAG8 and GASC1 strains were crossed to the opposite mating type wild-type strain 7598, and after 10 days' incubation at 25 °C with a 14 h light and 10 h dark cycle we observed the development of perithecia as black spots embedded in the mycelium (not shown). Later, we observed viable ascospore formation in all the crosses performed (not shown).

DISCUSSION

We regard *FCC1* as a key regulatory gene that impacts two important biological features, fumonisin biosynthesis and conidiation, in *F. verticillioides* (Shim & Woloshuk, 2001). The *F. verticillioides* *GAP1* gene was isolated from the wild-type SSH cDNA library, suggesting that *GAP1* is downstream of *FCC1* in the signalling pathway that regulates fumonisin biosynthesis or conidiation, or both. *In silico* analysis showed that *GAP1* encodes a putative protein that shares high structural similarity to a family of GPI-anchored glycoproteins that are known to be involved in cell development in filamentous fungi (Chabane *et al.*, 2006; Mouyna *et al.*, 2000, 2005). Therefore, we hypothesized that *GAP1* is primarily associated with conidiation and fungal development. While earlier studies have shown that osmotic stabilization with 1.2 M sorbitol partially remedies the restricted colony phenotype in a *gas1* mutant (Caracuel *et al.*, 2005), the restricted colony growth phenotypes in GAM126 and GAG8 were not remediated upon addition of osmotic stabilizers, including 1.2 M KCl and 1.2 M sorbitol (not shown).

When mycelia of wild-type, GAM126 and GAG8 were stained with CFW, we noticed that the cell walls of the GAM126 and GAG8 strains were more brightly stained than those of the wild-type, indicating either that the mutant strains produced more chitin and β -glucans in their cell walls, or that these moieties were more readily stained by CFW in the mutant (not shown). When stained with ConA-FITC, GAM126 and GAG8 exhibited uniform binding of the lectin to cell walls, punctuated by discrete sites of greater binding (Fig. 4). In contrast, neither the wild-type nor GASC1 revealed any significant ConA binding sites. ConA is routinely used to assay the presence of glucan and mannan moieties in fungal cell walls (Shaw & Hoch, 1999), and is generally believed to bind to terminal α -mannan and α -glucan (Smith & Goldstein, 1967). One might have hypothesized that the *GAP1* mutants would exhibit fewer ConA binding sites than the wild-type, since the *GAP1* protein is thought to function in the linking of β -linked glucan groups. Our result is not surprising, however, since the alteration of the cell wall by lack of *GAP1* function may lead to a more amorphous cell wall that is less cross-linked, therefore leading to exposure of more ConA

binding sites. While it is difficult to unambiguously explain this ConA staining pattern, it is clear from our results that the *GAP1* mutant cell wall is significantly altered from that of the wild-type. In this study, we also observed that GAM126 and GAG8 strains were resistant to the cell wall-stressing agents CR and CFW. This result contrasts with those obtained with *S. cerevisiae* *gas1* mutants, which are sensitive to CFW (Popolo *et al.*, 1993). Interestingly, resistance to CR and CFW has also been observed in *S. cerevisiae* mutants that are defective in chitin synthesis (Imai *et al.*, 2005; Roncero *et al.*, 1988). In general, the cell wall mutants that are disrupted in the synthesis of β -1,3 or β -1,6 glucans, GPI-anchored glycoprotein biosynthesis or mannosylation of mannoproteins have elevated chitin levels and are hypersensitive to CR and CFW (Imai *et al.*, 2005; Ram *et al.*, 1994). However, in *F. verticillioides*, we hypothesize that deletion of *GAP1* results in reduced chitin production that renders the mutants resistant to CR and CFW.

The expression of pH-responsive genes in filamentous fungi is regulated by key transcriptional regulators such as *pacC* in *A. nidulans* (Tilburn *et al.*, 1995) and *PRR1* in *C. albicans* (Porta *et al.*, 1999). For example, the expression of *PHR1* and *PHR2*, the β -1,3-glucanotransferase-encoding genes in *C. albicans*, is regulated by changes in the external pH via *PRR1* (Muhlschlegel & Fonzi, 1997; Porta *et al.*, 1999; Saporito-Irwin *et al.*, 1995). In contrast, *GEL1* and *GEL2*, the two characterized β -1,3-glucanotransferase-encoding genes in *A. fumigatus*, are constitutively expressed over a range of growth conditions (Mouyna *et al.*, 2000). Interestingly, the expression of *GAS1* in *F. oxysporum* is not controlled by ambient pH, or by the responsive regulator PacC (Caracuel *et al.*, 2005). In contrast to the results of *F. oxysporum* studies, the expression of *GAP1* in *F. verticillioides* is induced under acidic conditions (Fig. 1) and seems to be dependent upon the pH-responsive transcription factor *PAC1* (Fig. 3). Our Northern analysis revealed that *GAP1* expression is upregulated under alkaline conditions in a *PAC1* deletion mutant compared to that of the wild-type (Fig. 3b, lane 5). We propose that under alkaline conditions, induction of *PAC1* suppresses the expression of *GAP1* and vice versa. However, further studies are needed to confirm the regulation of *GAP1* by *PAC1*. Since ambient pH plays a critical role in the expression of *GAP1*, and *GAP1* plays an important role in growth, we decided to check if the pH variation affects the growth of *GAP1* deletion strains. Unlike the *C. albicans* deletion strains (Muhlschlegel & Fonzi, 1997; Saporito-Irwin *et al.*, 1995), differences in pH (4.5 and 7.5) did not affect the growth of the GAM126 and GAG8 strains in either solid or liquid media (not shown).

In addition to investigating the developmental phenotypes of GAM126 and GAG8, we studied the impact of $\Delta gap1$ on key biological features that are linked to the *FCC1* in *F. verticillioides*. In our study we observed a decrease in production of conidia due to the deletion of *GAP1* (Table 2). Flaherty *et al.* (2003) have shown that *PAC1* is

critical to maintain proper growth and conidiation under alkaline conditions. However, the ambient pH did not have any impact on growth in the GAM126 and GAG8 strains. Hence, we speculate that the reduction in microconidia production in our deletion mutants was due to a developmental defect similar to the abnormal conidiophores observed in *A. fumigatus* Δ gel2 and Δ gel2 Δ gel1 mutants (Mouyna *et al.*, 2005), rather than molecular regulation of conidiation genes. Overall, very little information is available regarding GPI-anchored proteins in relation to conidiation. Identification and characterization of developmental-related regulatory genes in *F. verticillioides* are necessary to clarify this relationship. We were intrigued that *GAP1* deletion did not have a negative impact on fumonisin production, which was in contrast to the conidiation data. Rather, we observed an increased level of FB₁ production in the GAG8 strain. These data provide indirect evidence that the primary functional role of *GAP1* is to regulate proper hyphal development and conidiation in *F. verticillioides*. The discovery of additional genes in the *FCC1*-mediated signalling pathways will allow us to investigate the epistatic relationship between the genes and clarify the pathway–phenotype association.

In this report we also demonstrated that *GAP1* in *F. verticillioides* is not essential for invasion and colonization of maize stalks and seedlings. In a way it is interesting that the GAM126 and GAG8 strains, which are severely impaired in hyphal development on synthetic solid surfaces, can grow as effectively as the wild-type in maize stalks and on seedlings that in theory can be considered to be solid surfaces. Based on our observations it is unlikely that *GAP1* plays a signalling role in the interaction between maize and *F. verticillioides*. Our finding is significant, based on the fact that supposedly orthologous proteins (94% identical) performed dissimilar functions (Caracuel *et al.*, 2005; this study), although both fungi might have different modes of infection. However, our finding is not surprising, since the fungus might have a compensatory mechanism to overcome the loss of *GAP1*. Similar results have been observed in other filamentous fungi. For example, deletion of *GEL1* in *A. fumigatus* does not have an impact on virulence, but deletion of its homologue *GEL2* affects morphogenesis and virulence (Mouyna *et al.*, 2005). Surprisingly, *GEL1* and *GEL2* of *A. fumigatus* both complement a *GAS1* deletion strain of *S. cerevisiae*, indicating that both proteins can act as functional homologues in yeast (Mouyna *et al.*, 2005). In addition, under *in vitro* conditions, Gel2p exhibits the same enzymic activity as Gel1p, reconfirming the redundancy in function (Mouyna *et al.*, 2005). The regulation of expression of structurally similar but functionally different genes could be under the control of external signals such as pH, nutrient source, etc. On the other hand, *PHR1* and *PHR2* are GPI-anchored proteins in *C. albicans* that structurally and functionally exhibit opposing relationships between pH and morphology: *PHR1* and *PHR2* mutants are morphologically abnormal at alkaline and acidic pH, respectively (Muhlschlegel & Fonzi, 1997; Saporito-Irwin *et al.*, 1995).

The fact that deletion of *GAP1* had no effect on pathogenicity, whereas its structural homologue in *F. oxysporum* has a significant impact on virulence (Caracuel *et al.*, 2005), further indicates that the function of GPI proteins is specific to each organism.

Analyses of publicly available fungal genomes revealed that more than one GAS protein family gene member is present in filamentous fungi, and as expected two additional GPI-anchored glycoprotein-encoding genes were identified in the *F. verticillioides* genome. This might indicate that β -1,3-glucanotransferases are functionally homologous or that they perform a different function. To address this question, we first studied the expression of the other two GAP protein-encoding genes in *F. verticillioides*. The complete *GAP2* (supercontig 3; locus FVEG_02799.3) and *GAP3* (supercontig 5; locus FVEG_04666.3) sequences were obtained from a genome search of the *F. verticillioides* 7600 strain (Broad Institute of Harvard and MIT; <http://www.broad.mit.edu>). Our Northern analysis indicated that *GAP2* expression is also dependent on ambient pH (Supplementary Fig. S2). In contrast to *GAP1* expression, *GAP2* is induced under alkaline conditions (Supplementary Fig. S2). No detectable *GAP3* transcript levels were observed under the conditions tested (Supplementary Fig. S2). Our studies also revealed that *GAP2* expression is unaltered in the GAM126 and GAM8 strains under both pH (3.0 and 8.0) conditions tested (not shown). This suggests that GAS proteins (at least Gap1 and Gap2) in *F. verticillioides* are regulated by ambient pH, probably through pH transcription factor Pac1, and might perform their functions independently of one other. Further mutational studies are needed to better understand the role of *GAP2* and *GAP3* proteins in *F. verticillioides*.

Finally, it is evident that β -1,3-glucanotransferases share a common function of involvement in cell wall biogenesis. In spite of their homology, GPI proteins of fungi are regulated in different ways and perform different cellular functions, probably to meet the specific requirements of an organism.

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