***Fusarium oxysporum gas1* Encodes a Putative β -1,3-Glucanosyltransferase Required for Virulence on Tomato Plants**

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Glycosylphosphatidylinositol-anchored (β)-1,3-glucanosyltransferases play active roles in fungal cell wall biosynthesis and morphogenesis and have been implicated in virulence on mammals. The role of β -1,3-glucanosyltransferases in pathogenesis to plants has not been explored so far. Here, we report the cloning and mutational analysis of the *gas1* gene encoding a putative β -1,3-glucanosyltransferase from the vascular wilt fungus *Fusarium oxysporum*. In contrast to *Candida albicans*, expression of *gas1* in *F. oxysporum* was independent of ambient pH and of the pH response transcription factor PacC. Gene knockout mutants lacking a functional *gas1* allele grew in a way similar to the wild-type strain in submerged culture but exhibited restricted colony growth on solid substrates. The restricted growth phenotype was relieved by the osmotic stabilizer sorbitol, indicating that it may be related to structural alterations in the cell wall. Consistent with this hypothesis, Δ *gas1* mutants exhibited enhanced resistance to cell wall-degrading enzymes and increased transcript levels of *chsV* and *rho1*, encoding a class V chitin synthase and a small monomeric G protein, respectively. The Δ *gas1* mutants showed dramatically reduced virulence on tomato, both in a root infection assay and in a fruit tissue-invasion model, thus providing the first evidence for an essential role of fungal β -1,3-glucanosyltransferases during plant infection.

Additional keyword: glucan.

Pathogenic fungi have evolved mechanisms to successfully invade eukaryotic hosts, overcome their defenses, and colonize their tissue, thereby causing disease. One of the key factors underlying fungal pathogenesis is the capacity to grow as hyphae (Madhani and Fink 1998), a process that requires the polarized biogenesis of the cell wall at the apical tip (Harris and Momany 2004). The fungal cell wall is a complex and dynamic structure composed mostly of carbohydrates, which determines hyphal shape and resistance to adverse environmental conditions (Klis et al. 2002). The major structural component of the fungal wall is β -1,3-glucan, which serves as an anchor for other polysaccharides, such as chitin and galactomannan (Fontaine et al. 1997). The β -1,3-glucan is synthesized by a plasma membrane-bound glucan synthase, is

extruded as a linear chain into the periplasmic space (Shematek and Cabib 1980), and is subjected to a number of downstream processing steps that lead to extensive branching and cross-linking to other cell wall components (Klis et al. 2002). Among the key enzymes involved in downstream processing are the β -1,3-glucanosyltransferases, a family of glycosylphosphatidylinositol (GPI)-anchored glycoproteins exemplified by Gas1p of *Saccharomyces cerevisiae* (Popolo et al. 1993). The β -1,3-glucanosyltransferases internally split the β -1,3-glucan molecule and transfer the newly generated reducing end to the nonreducing end of another β -1,3-glucan molecule, thus resulting in the elongation of the β -1,3-glucan side chains (Mouyna et al. 2000a). Yeast mutants lacking Gas1p exhibit reduced growth and gross structural alterations in the cell wall, including decreased glucan and increased chitin contents (Popolo et al. 1993). *GAS1* orthologs have been identified in a number of filamentous fungi, including the human pathogens *Candida albicans* and *Aspergillus fumigatus*, and these orthologs were shown to functionally complement the yeast Δ *gas1* mutant (Mouyna et al. 2000a).

The role of β -1,3-glucanosyltransferases in fungal virulence has been explored in the human pathogen *C. albicans*. Deletion of the *GAS1* ortholog *PHR1*, which is specifically expressed at alkaline pH, causes defects in growth and morphogenesis that are evident at neutral to alkaline pH but not at an acidic pH. Conversely, the null mutants in a second gene, *PHR2*, which is preferentially expressed at an acidic ambient pH, exhibit analogous defects at acid pH values (Muhlschlegel and Fonzi 1997). Intriguingly, the *PHR1* null mutants are avirulent in a mouse model of systemic infection at a neutral ambient pH, whereas *PHR2* deletion mutants have impaired virulence in a vaginal infection model at acidic pH levels (De Bernardis et al. 1998; Ghannoum et al. 1995). Taken together, these results indicate that β -1,3-glucanosyltransferases play a key role during fungal infection of mammalian hosts.

The importance of β -1,3-glucanosyltransferases in pathogenesis to plants is currently unknown. To address this question, we have examined the role of *gas1* encoding a putative β -1,3-glucanosyltransferase of the vascular wilt fungus *Fusarium oxysporum*, a ubiquitous soilborne pathogen that attacks over a hundred different plant species (Di Pietro et al. 2003). The cell wall of *F. oxysporum* contains an inner layer of chitin and glucans that is covered by an external layer of glycoproteins (Schoffemeer et al. 1999). We find that *F. oxysporum* mutants lacking a functional *gas1* copy exhibit a characteristic restricted-growth phenotype on solid substrates, indicative of structural alterations in the cell wall. The mutants also show increased resistance of cell wall-degrading enzymes and transcriptional activation of genes

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Nucleotide sequence data for the *Fusarium oxysporum gas1* gene is available in the GenBank database under accession number AY884608.

involved in cell wall biogenesis. Finally, the $\Delta gas1$ strains are dramatically reduced in virulence on tomato plants, providing the first evidence for an essential role of β -1,3-glucanosyltransferase in fungal pathogenesis on plants.

RESULTS

Cloning of the *F. oxysporum gas1* gene encoding a putative β -1,3-glucanosyltransferase.

The *gas1* gene encoding a putative β -1,3-glucanosyltransferase of *F. oxysporum* f. sp. *lycopersici* was cloned from a lambda EMBL3 genomic library, using as probe a 0.5-kb polymerase chain reaction (PCR) fragment amplified from genomic DNA with primers derived from an expressed sequence tag (EST) clone of *Fusarium venenatum* (discussed below). Sequencing of a hybridizing λ genomic clone identified an open reading frame (ORF) of 1,617 bp that encoded a putative 539-amino acid protein with significant similarity to fungal GPI-anchored β -1,3-glucanosyltransferases. Alignment of the *F. oxysporum gas1* gene with the sequences in the databases indicated the presence of a putative intron of 49 bp, whose position was subsequently confirmed by cloning and sequencing of the complete *gas1* cDNA (data not shown). Southern analysis of total genomic DNA treated with different restriction enzymes suggested that *gas1* is a single-copy gene (data not shown). However, the presence of additional bands hybridizing at lower intensity indicated that *F. oxysporum* contains at least two more genes that are structurally related to *gas1*, similar to *A. fumigatus*, which contains at least three structurally related genes, *GEL1*, *GEL2*, and *GEL3* (Mouyna et al. 2000b). In agreement with this result, a BLASTP search of the complete genome database of the closely related species *Fusarium graminearum* detected three significant protein alignments, one showing very high identity with *F. oxysporum* Gas1 (BLASTP value 0.0) and two with lower degrees of identity (values 6e-63 and 5e-49). Among the five Gas paralogs reported in yeast, Gas1p showed the highest degree identity with the *F. oxysporum* Gas1 (41.3%).

To explore the capacity of *F. oxysporum gas1* to complement the previously reported phenotypic defects of the $\Delta gas1$ mutant of *S. cerevisiae* (Popolo et al. 1993, 1997), the coding region of *F. oxysporum gas1* was placed under the control of the yeast *GAL1* promoter and was introduced into a yeast strain deleted for the *gas1* gene. Five randomly chosen Ura⁺ transformants were grown in minimal medium containing galactose and were examined for phenotypic parameters related to the $\Delta gas1$ phenotype, such as cell clumping in stationary

phase (65% of $\Delta gas1$ mutant cells versus 10% of wild-type cells were present in aggregates of more than two cells). Cell clumping of the Ura⁺ transformants expressing the *F. oxysporum gas1* gene was very similar to that of the $\Delta gas1$ mutant (between 60 and 70%). The characteristic round shape and larger size of the $\Delta gas1$ cells was also observed in the Ura⁺ transformants (results not shown). Moreover, a significant increase of fluorescence upon Calcofluor staining of the $\Delta gas1$ mutant as compared with the wild type (Popolo et al. 1997) was also detected in the Ura⁺ transformants, and sensitivity to Calcofluor White was indistinguishable between the $\Delta gas1$ mutant and the Ura⁺ transformants (results not shown). We concluded that expression of the complete cDNA of the *F. oxysporum gas1* gene under the control of the *GAL1* promoter failed to rescue the phenotypic defects of the yeast $\Delta gas1$ mutant.

Expression of *F. oxysporum gas1* is not regulated by ambient pH or by the pH-response transcription factor PacC.

Expression of the β -1,3-glucanosyltransferase genes *PHR1* and *PHR2* from *C. albicans* is regulated by ambient pH via the pH-response transcription factor RIM101 (Davis et al. 2000;

Fig. 1. Expression of *Fusarium oxysporum gas1* is not regulated by ambient pH or by the pH-response transcription factor PacC. Total RNA was extracted from mycelia of the wild-type strain, the *pacC*^{-/-} loss-of-function mutant, and the *pacC*⁺ dominant activating mutant, was grown in synthetic medium buffered at different pH values, was fractionated in an agarose gel, was blotted onto a nylon membrane, and was hybridized to the *gas1* probe. As a loading control, RNA was stained directly on the filter with 0.02% methylene blue (rRNA).

Fig. 2. Targeted replacement of the *Fusarium oxysporum gas1* gene. **A**, Physical map of the *gas1* locus and construction of the gene replacement vector pDgas1. The relative positions of the primers used for amplification of the linear DNA fragment employed for gene replacement and of the probe used for Southern and Northern analyses are indicated. E = *EcoRV*; K = *KpnI*. **B**, Analysis of transformants by Southern blot. Genomic DNA from the wild-type strain and of three independent transformants was digested with *EcoRV*, was separated in a 0.7% agarose gel, was blotted onto a nylon membrane, and was hybridized with the *gas1* probe indicated in A. Molecular sizes of the hybridizing fragments are indicated on the left.

Muhlschlegel and Fonzi 1997). To examine whether the expression of the *F. oxysporum gas1* gene is also dependent on ambient pH, we determined the transcript levels in mycelia of the wild-type strain grown at either pH 4 or 8. In addition, we analyzed *gas1* transcript levels in *pacC⁺* and *pacC⁻* mutants carrying, respectively, a loss-of-function or a gain-of-function allele of the pH-response transcription factor PacC (Caracul et al. 2003). As shown in Figure 1, *gas1* transcript levels were similar in mycelia grown at acidic or alkaline ambient pH and did not vary significantly between the wild-type strain and the different *pacC* mutants. We concluded that expression of *gas1* in *F. oxysporum* is not regulated by ambient pH or by the pH-response transcription factor PacC.

Generation of $\Delta gas1$ loss-of-function mutants by targeted gene replacement.

To investigate the function of *gas1* in *F. oxysporum*, we generated loss-of-function mutants carrying a disrupted copy of the gene, using a one-step gene replacement. A hygromycin-resistance cassette was inserted into a *KpnI* site at nucleotide position 293 of the *gas1* coding region (Fig. 2A), resulting in disruption of the ORF. A linear PCR fragment of this construct was introduced into *F. oxysporum* 4287, and a number of hygromycin-resistant transformants were analyzed by PCR with different combinations of gene-specific primers. Five independent transformants produced PCR amplification products that were indicative of a homologous integration-mediated gene replacement (data not shown). Southern blot analysis confirmed the replacement in these transformants of a 3-kb *EcoRV* fragment corresponding to the wild-type *gas1* allele by a 6-kb fragment (Figure 2B shows two of the transformants, $\Delta gas1-1$ and $\Delta gas1-2$). In contrast, a hygromycin-resistant

transformant, *gas1ND1*, still contained the wild-type 3-kb fragment together with an additional hybridizing fragment of higher molecular weight, indicative of ectopic insertion of the replacement vector. We concluded that transformants $\Delta gas1-1$ through $\Delta gas1-5$ carry a disrupted copy of *gas1*, whereas *gas1ND1* contains an intact copy of the gene. The five independent $\Delta gas1$ strains showed very similar phenotypes in all the subsequent experiments.

Mutation of *gas1* affects hyphal development on solid substrates.

To examine the role of *gas1* in hyphal development of *F. oxysporum*, we compared the growth phenotype of the $\Delta gas1$ mutants with that of the wild-type strain 4287. No significant differences in hyphal morphology and growth rate were detected in mycelia cultured in liquid minimal medium with or without 1.2 M sorbitol (Fig. 3A). In contrast, the $\Delta gas1$ strains exhibited a significantly restricted colony morphology on solid media (Fig. 3B). Stereomicroscopic examination of the $\Delta gas1$ colonies revealed burying of the colony margin into the agar (results not shown). The restricted colony phenotype was partially relieved by addition of the osmotic stabilizer sorbitol (1.2 M) to the medium (Fig. 3B). In additional assays, we tested growth of the $\Delta gas1$ strains in the presence of a detergent (0.1% sodium dodecyl sulfate) or agents that disrupt the cell wall (50 μ g of Calcofluor per milliliter or 50 μ g of Congo red per milliliter) or the cell membrane (50 μ g of α -tomatine per milliliter), as well as under conditions of oxidative (3 mM H_2O_2) or temperature stress (37°C). No additional growth phenotypes of the $\Delta gas1$ mutants were detected under these conditions, beyond the restricted colony growth mentioned above (results not shown). We concluded that mutation of the *gas1* gene specifically affects hyphal development on solid surfaces.

Mutation of *gas1* increases cell wall integrity and transcription of genes involved in cell wall biogenesis.

The effect of the osmostabilizer sorbitol in alleviating the growth defects of the $\Delta gas1$ mutants on solid media prompted

Fig. 3. Mutation of *gas1* affects hyphal growth on solid but not on liquid growth media. Growth of the wild-type strain and the $\Delta gas1$ mutant on liquid (upper panels) or solid synthetic medium with or without 1.2 M sorbitol (lower panels). Photographs from submerged cultures were recorded using a Leica microscope with a Leica DC 300F digital camera (200 $\times$ magnification).

Fig. 4. Mutations in *gas1* and *chsV* affect sensitivity of *Fusarium oxysporum* to cell wall-degrading enzymes. Germlings of the wild-type strain (diamonds), the $\Delta gas1$ strain (squares), and the $\Delta chsV$ mutant (triangles) were incubated with protoplasting enzyme mix at 30°C in osmotically stabilized buffer, and the number of protoplasts released was determined microscopically. The decrease observed in the $\Delta chsV$ mutant at 2 h is caused by protoplast death. Each datapoint represents the mean of three replicates. Bars indicate standard deviations.

us to examine the relative integrity of the cell walls of these mutants. To this end, germinated microconidia of the wild-type strain or the mutant $\Delta gas1-1$ were exposed to a mixture of cell wall-degrading enzymes, and the release of protoplasts was monitored over time. For comparative purposes, a mutant lacking the class V chitin synthase *ChsV* (Madrid et al. 2003) was included in the experiment. As shown in Figure 4, the $\Delta gas1$ strain was significantly more resistant to the activity of the protoplasting enzymes than was the wild-type strain. In contrast, the $\Delta chsV$ mutant was more sensitive than the wild type. These results are indicative of an increase in the structural integrity of the cell wall in the $\Delta gas1$ mutants.

Next, we examined whether the enhanced cell wall integrity of the $\Delta gas1$ mutants could be explained by changes in the expression levels of cell wall-biogenesis genes. Northern blot analysis detected higher transcript levels of the *chsV* gene in the $\Delta gas1$ mutant as compared with that in the wild-type strain (Fig. 5A). In contrast, no significant differences in *gas1* transcript accumulation were detected between the wild-type strain and the $\Delta chsV$ mutant.

To further explore the hypothesis that a mechanism for maintenance of cell wall integrity may be activated in the $\Delta gas1$ mutants, we examined the expression of the *F. oxysporum rho1* gene. Rho is a small monomeric G protein that functions as an essential signaling component of the fungal cell wall-integrity pathway (Bickle et al. 1998; Madaule et al. 1987). A 1.2-kb DNA fragment containing part of the *F. oxysporum rho1* gene was amplified from genomic DNA of strain 4287, using PCR with primers derived from conserved regions of fungal *rho* genes (discussed below). The obtained fragment was used as probe to screen a λ EMBL3 genomic library of *F. oxysporum* isolate 4287. Sequencing of a 4.1-kb *HindIII* frag-

ment obtained from a hybridizing genomic clone identified an ORF of 585 nucleotides interrupted by 4 introns. The GenBank accession number of *F. oxysporum rho1* is AY884607. The deduced polypeptide shared 91, 80, and 76% identity, respectively, with *A. nidulans* RhoA (Guest et al. 2004), *Cryptococcus neoformans* Rho1 (Chang and Penoyer 2000), and *S. cerevisiae* Rho1 (Madaule et al. 1987). Northern blot analysis of the *F. oxysporum rho1* gene detected significantly higher transcript levels in the $\Delta gas1$ mutant as compared with the wild-type and the $\Delta chsV$ strains (Fig. 5B). These results indicate that both a structural (*chsV*) and a regulatory (*rho1*) component of the cell wall-integrity response are transcriptionally upregulated in the $\Delta gas1$ mutant.

Fig. 5. The $\Delta gas1$ mutants show increased expression of genes involved in cell wall biogenesis. Total RNA was extracted from mycelia of the indicated strains, was grown on synthetic medium with or without 1.2 M sorbitol, and was fractionated in agarose gels, blotted onto nylon membranes, and hybridized to the probes indicated to the right. As a loading control, RNA was stained directly on the filter with 0.02% methylene blue (rRNA). **A**, Accumulation of *chsV* and *gas1* transcripts in the wild-type strain and the $\Delta gas1$ and $\Delta chsV$ mutants. **B**, Accumulation of *rho1* transcript in the wild-type strain and the $\Delta gas1$ and $\Delta chsV$ mutants.

Fig. 6. *Gas1* is required for virulence of *Fusarium oxysporum* on tomato. **A**, Incidence of *Fusarium* wilt after root inoculation of tomato plants (cultivar Vemur). Severity of disease symptoms was recorded at the indicated times after inoculation, using an index ranging from 1 (healthy plant) to 5 (dead plant). Symbols refer to plants inoculated with the following strains: wild type (closed circles), $\Delta gas1-1$ (open diamonds), $\Delta gas1-2$ (closed squares), *gas1-ND1* (closed triangles), and the uninoculated control (open circles). Error bars indicate standard deviations calculated from 10 plants for each treatment. **B**, Invasive growth on tomato fruits. Inoculation was performed by injecting 5×10^5 microconidia of the indicated strains. The photograph was taken after incubation for four days at 28°C and 100% relative humidity.

***F. oxysporum gas1* is required for infection of tomato plants.**

Two different assay systems were used to determine the effect of the $\Delta gas1$ mutation on the virulence of *F. oxysporum* on tomato. First, root-infection assays were performed by immersing the roots of tomato plants in a microconidial suspension of the strains, and the severity of wilt symptoms was scored at different times after inoculation (Fig. 6A). Disease symptoms in plants inoculated with the wild-type strain 4287 increased steadily throughout the experiment. Initially, leaf tips turned yellow and curved, then the stalk weakened and decayed, and most of the plants were dead 20 days after inoculation. Plants inoculated with the ectopic transformant *gas1ND1* showed a highly similar pattern of disease development. In stark contrast, the plants inoculated either with the $\Delta gas1-1$ or the $\Delta gas1-2$ mutant showed a very low incidence of disease. We also assayed the capacity of the $\Delta gas1$ strains to grow invasively on tomato fruit tissue, by injecting a microconidial suspension directly into the fruits. Both the wild-type strain and the ectopic transformant efficiently colonized and macerated the tomato fruit tissue and produced abundant aerial mycelium on the surface (Fig. 6B). In contrast, all five $\Delta gas1$ mutants were almost completely impaired in invasive growth. We thus concluded that the β -1,3-glucanosyltransferase Gas1 is required for efficient infection of tomato plants and for invasive growth of *F. oxysporum* on the host tissue.

DISCUSSION

In the present study, we have cloned and characterized the *gas1* gene from the vascular wilt fungus *F. oxysporum*. The deduced Gas1 polypeptide contains the three characteristic domains reported for fungal β -1,3-glucanosyltransferases (glycoside hydrolase family GH72 [the CAZy database]), an N-terminal domain with two catalytic Glu residues (Glu₁₅₇ and Glu₂₅₈), the presence of six highly conserved interspersed cysteine residues, and a hydrophobic C-terminus characteristic of GPI-anchored proteins (Mouyna et al. 2000a and b; Popolo and Vai 1999). The presence of these conserved domains as well as the analogies between the phenotypes of the *F. oxysporum* and *S. cerevisiae* $\Delta gas1$ mutants suggest that *F. oxysporum gas1* encodes a functional β -1,3-glucanosyltransferase. The failure of the *F. oxysporum gas1* gene to rescue the phenotypic defects of the yeast $\Delta gas1$ mutant may be due to structural and functional divergences between the Gas1 proteins of these two organisms, such as the high number of serine residues in the C-terminal region of *S. cerevisiae* Gas1p that are absent from the *F. oxysporum* protein. Alternatively, the failed complementation may also be due to other factors, such as insufficient expression levels provided by the GAL1 promoter, poor protein yields, or inadequate processing of the *F. oxysporum* Gas1 protein in *S. cerevisiae*.

The *gas1* gene appears to be constitutively expressed during hyphal growth of *F. oxysporum*. We found no significant changes in transcript levels between mycelia grown at alkaline or acidic pH levels or between the wild-type strain and loss-of-function or gain-of-function mutants in the transcription factor PacC. Thus, in contrast to *C. albicans* (Davis et al. 2000; Muhlschlegel and Fonzi 1997) and similar to *S. cerevisiae* and *A. fumigatus* (Mouyna et al. 2000a; Popolo et al. 1993) expression of *gas1* in *F. oxysporum* is independent of the ambient pH.

The role of Gas1 in hyphal growth and cell wall–integrity control.

To study *gas1* function, we generated *F. oxysporum* strains lacking a functional *gas1* allele. Interestingly, hyphal growth of these mutants was indistinguishable from that of the wild-

type strain in submerged culture but was severely restricted on solid surfaces. This striking difference suggests that *gas1* carries out a specific function during hyphal growth on solid surfaces that is not required during submerged culture. Whether this specific role is related to the physical contact with the substrate or with the air and whether it involves merely a structural function or a possible role in sensing and transducing signals from the underlying surface remains to be determined. A solid surface–related role in hyphal growth has previously been reported for the protein kinase A isoform Tpk1p of *C. albicans* (Bockmuhl et al. 2001). Tpk1 is specifically required for hyphae formation on solid but not on liquid-inducing media, suggesting that contacts to solid support structures by as-yet-unknown pathways trigger Tpk1p activation. The processes relevant in this regard during the infection process in mammalian hosts are as yet unknown but could include contacts to host cells and structures such as the extracellular matrix (Bockmuhl et al. 2001).

The restricted colony growth of the *F. oxysporum* $\Delta gas1$ mutants was partially relieved by the osmotic stabilizer sorbitol, suggesting a possible link between the $\Delta gas1$ phenotype and the presence of structural alterations in the cell wall. Consistent with this hypothesis, the $\Delta gas1$ mutants exhibited increased resistance to cell wall–degrading enzymes, an observation reminiscent of the results reported in *S. cerevisiae*. Yeast *gas1* mutants exhibit morphogenetic defects that are suggestive of changes in the cell wall matrix (Popolo et al. 1993) and are relieved in the presence of osmotic stabilizers (Turchini et al. 2000). Moreover, yeast *gas1* mutants are more resistant to Zymolyase than are the wild type during the exponential growth phase (Popolo et al. 1993) and have a higher chitin content (Popolo et al. 1997; Ram et al. 1998). The increase in cell wall chitin is brought about by a surge in chitin synthase activity, in particular by Chs3p, which is essential for viability of the *gas1* mutants (Carotti et al. 2002; Valdivieso et al. 2000). A recent genome-wide expression analysis in yeast identified the *chs3* gene to be transcriptionally upregulated as part of the compensatory response of the *gas1* mutant (Lagorce et al. 2003). Interestingly, we observed that the *F. oxysporum* $\Delta gas1$ mutant has significantly increased transcript levels of the *chsV* gene, encoding a class V chitin synthase that was discovered as a virulence determinant during a random insertional screen (Madrid et al. 2003). Class V chitin synthases are specific for filamentous fungi and, thus, are absent in *S. cerevisiae*; however, the closest yeast relative of ChsV is Chs3p, which belongs to class IV (Roncero 2002). Thus, both in *S. cerevisiae* and in *F. oxysporum*, deletion of *gas1* leads to activation of a mechanism that enhances cell wall integrity and induces transcription of chitin synthase genes.

The mechanism through which this cell wall–integrity response is activated in *S. cerevisiae* has been studied in detail. Yeast cells have an exquisite cell wall monitoring system that responds to chemical and mechanical insults through a signal transduction pathway mediated by protein kinase C (PKC1) and the mitogen-activated protein (MAP) kinase Mpk1/Slit2 (Errede and Levin 1993). This signaling pathway ensures proper maintenance of cell wall integrity under conditions of stress and is conserved in filamentous fungi (Xu et al. 1998). One of the key upstream components of the cell-integrity pathway is the small GTPase Rho1p (Madaule et al. 1987), which is activated in response to cell wall alterations, including those caused by loss of *gas1* (Bickle et al. 1998). Our observation that expression of the *rho1* gene is increased in the $\Delta gas1$ mutants suggests that a similar Rho1-dependent signaling mechanism is activated in *F. oxysporum* upon deletion of the *gas1* gene.

The role of Gas1 in fungal pathogenicity on plants.

The *F. oxysporum* Δ *gas1* mutants showed dramatically reduced virulence on tomato plants, both in a root-infection assay and in a fruit-invasion model, suggesting that Gas1 is essential for efficient colonization of the host tissue. To our knowledge, this is the first evidence for an essential role for a fungal β -1,3-glucanosyltransferase during plant infection, although such a role has been previously reported in virulence on mammals (De Bernardis et al. 1998; Ghannoum et al. 1995). The exact function of *gas1* in plant infection remains to be determined. One straightforward hypothesis is that Gas1 is required for proper hyphal morphogenesis on a solid surface, which includes the host plant. Because hyphae are essential structures for fungal pathogens, allowing them to actively invade the host tissue and obtain nutrients from host cells, a defect in hyphal morphogenesis would lead to an inability to cause disease. Alternatively, being a cell-surface protein, Gas1 may play a more subtle role in the exchange of chemical or physical signals, or both, between fungus and host. Deletion of *gas1* may perturb proper recognition of the host surface by the pathogen. Thus, the contact with a hard surface was shown to trigger the pathogenic response in conidia of *Colletotrichum gloeosporioides*, possibly via a Ca^{2+} -calmodulin signaling cascade (Kim et al. 1998). On the other hand, loss of *gas1* may alter the pattern of recognition of the fungus by the plant. Deletion of *GAS1* in yeast was shown to result in the release of a β -1,3-glucan- β -1,6-glucan-protein complex into the surrounding medium (Ram et al. 1998). Because fungal β -1,3-glucan- β -1,6-glucan-protein complexes are among the most active known elicitors of the plant defense response (Hahn 1996), inappropriate release of cell-wall elicitor molecules from the *F. oxysporum* Δ *gas1* mutants may lead to early recognition by the host plant, followed by a defense response that effectively blocks pathogenic development.

MATERIALS AND METHODS

Strains and growth media.

F. oxysporum f. sp. *lycopersici* 4287 (race 2) was originally obtained from J. Tello, Universidad de Almería, Spain, and was stored at -80°C with glycerol as a microconidial suspension (Di Pietro and Roncero 1998). The pathotype of the isolate on tomato was routinely confirmed by plant-infection assays. The generation and characterization of the Δ *chsV* mutant lacking a class V chitin synthase was described previously (Madrid et al. 2003). For extraction of genomic DNA and for microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit) or synthetic medium (SM) containing 1% sucrose as the carbon source, as described previously (Di Pietro and Roncero 1998). For analysis of gene expression, freshly obtained microconidia were germinated for 14 h in PDB, germlings were washed twice in sterile water, and then, were transferred for 2 h to SM with or without 1.2 M sorbitol or to SM buffered with 100 mM Na_2HPO_4 at pH 4 or 100 mM NaH_2PO_4 and 100 mM NaCl at pH 8. For phenotypic analysis of colony growth, drops containing 2×10^5 freshly obtained microconidia were transferred onto plates of SM containing 1.5% agar, 1% (wt/vol) sucrose as carbon source, and 0.1% NaNO_3 as nitrogen source. Where indicated, sorbitol was added to the media at a concentration of 1.2 M. Congo red, Calcofluor White, α -tomatine, or H_2O_2 (all from Sigma, St. Louis) were dissolved in water, sterile-filtered, and added to the medium at the indicated concentrations after autoclaving. For determination of sensitivity to cell wall-degrading enzymes, germlings were incubated with protoplasting enzyme (Lallzyme; Lallemand S.A., St. Simon, France) at 30°C as described previously (Di Pietro and Roncero 1998), and protoplast release over time was determined microscopically. Microscopic obser-

vations were carried out in a Leica DMR microscope using the Nomarsky technique. Photographs were recorded with a Leica DC 300F digital camera.

Nucleic acid manipulations and cloning of the *gas1* and *rho1* genes.

Total RNA and genomic DNA were extracted from *F. oxysporum* mycelium as previously described (Chomczynski and Sacchi 1987; Raeder and Broda 1985). Southern and Northern analyses and probe labeling were carried out as described (Di Pietro and Roncero 1998), using the nonisotopic digoxigenin labeling kit (Roche Diagnostics SL, Mannheim, Germany). Other routine nucleic acid manipulations were performed according to standard protocols (Sambrook and Russel 2001).

For cloning of the *gas1* gene, genomic DNA of *F. oxysporum* 4287 was used for PCR amplification on a Perkin Elmer Gene-Amp System 2400 (Norwalk, CT, U.S.A.), using the primers Gas1-1 (5'-GACTCCGACCTCTGCGACT-3') and Gas1-2 (5'-CTTTTACAGAAGGACCATACCG-3'). The primer sequences were derived from an EST clone of *F. venenatum* that showed homology to the *PHR1* gene of *C. albicans*. For cloning of the *rho1* gene, a PCR fragment was amplified from genomic DNA of *F. oxysporum*, using the primers Rho1-1 (5'-GAAAGCTCG TCATTGTGCGGC-3') and Rho1-2 (5'-AACACTTCACGGAC ACCCTC-3'), which were deduced from conserved regions of fungal *rho* genes. PCR reactions were routinely performed with the Long Template PCR system (Roche Diagnostics SL). The amplified fragments were cloned into the pGEM-T vector (Promega) and, after confirming the identity by sequencing, the fragments were used as probes to screen a λ EMBL3 genomic library of *F. oxysporum* f. sp. *lycopersici* isolate 4287 (Di Pietro and Roncero 1998). Library screening, subcloning, and other routine procedures were performed, following standard protocols (Sambrook and Russel 2001). Sequencing of both DNA strands of the obtained clones was performed at the Servicio de Secuenciación Automática de DNA, SCAI (University of Córdoba, Spain), using the Dyedexy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, U.S.A.) on an ABI Prism 377 genetic analyzer apparatus (Applied Biosystems). DNA and protein sequence databases were searched, using the BLAST algorithm (Altschul et al. 1990) at the National Center for Biotechnology Information (Bethesda, MD, U.S.A.).

Yeast complementation experiments.

For yeast complementation tests, a *S. cerevisiae* strain deleted for the *gas1* gene (Euroscarf accession number Y00897) and the corresponding wild-type strain BY4741 (*MATa*; *his3 Δ 1*; *leu2 Δ 0*; *met15 Δ 0*; *ura3 Δ 0*) were used. The complete *F. oxysporum* *gas1* cDNA was obtained by reverse transcription-PCR with gene-specific primers and was cloned as an *EcoRI/SalI* fragment downstream of the *GAL1* promoter in the plasmid p426*GAL1* (Mumberg et al. 1994). The accuracy of the obtained construct was confirmed by sequencing, and the plasmid was transformed into the Δ *gas1* strain, using a standard protocol (Gietz et al. 1992). The phenotype of the resulting *Ura*⁺ transformants was assayed microscopically, as previously described (Popolo et al. 1993, 1997), and was compared with Δ *gas1* and the wild-type strain. For determination of cell clumping, the number of cell aggregates (more than two cells) versus individual or budding cells (one or two) was determined by counting 100 cells and cell aggregates five times. Calcofluor staining was performed as described (Popolo et al. 1997).

Construction of plasmid vectors and fungal transformation.

Gene replacement vector pD*gas1* was constructed by inserting a *KpnI* fragment containing the hygromycin B-resistance

gene under control of the *A. nidulans gpdA* promoter (Punt and van den Hondel 1992) into the *KpnI* site at position 244 of the *gas1* ORF. A linear fragment containing the interrupted *gas1* allele was generated by amplifying the entire construct with primers Gas1-1 and Gas1-2 (discussed above) and was used to transform protoplasts of *F. oxysporum* 4287 to hygromycin resistance, following a protocol described previously (Di Pietro and Roncero 1998). Transformants were selected, purified by monoconidial isolation, and were stored as microconidial suspensions at -80°C .

Virulence assays.

Root-inoculation assays were performed as described (Di Pietro and Roncero 1998). Briefly, two-week-old tomato seedlings (cultivar Vemar) were inoculated with *F. oxysporum* strains by immersing the roots in a microconidial suspension, were planted in vermiculite, and were maintained in a growth chamber. At different times after inoculation, severity of disease symptoms was recorded using an index ranging from 1 (healthy plant) to 5 (dead plant). Ten plants were used for each treatment. Assays for invasive growth on tomato fruits (cultivar Daniela) were carried out as described (Di Pietro et al. 2001). Virulence experiments were performed three times with similar results.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The CAZy (Carbohydrate-Active enZymes) database:
afmb.cnrs-mrs.fr/CAZY