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Use of the Plant Defense Protein Osmotin To Identify *Fusarium oxysporum* Genes That Control Cell Wall Properties▽‡

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***Fusarium oxysporum* is the causative agent of fungal wilt disease in a variety of crops. The capacity of a fungal pathogen such as *F. oxysporum* f. sp. *nicotianae* to establish infection on its tobacco (*Nicotiana tabacum*) host depends in part on its capacity to evade the toxicity of tobacco defense proteins, such as osmotin. *Fusarium* genes that control resistance to osmotin would therefore reflect coevolutionary pressures and include genes that control mutual recognition, avoidance, and detoxification. We identified *FOR* (*Fusarium Osmotin Resistance*) genes on the basis of their ability to confer osmotin resistance to an osmotin-sensitive strain of *Saccharomyces cerevisiae*. *FOR1* encodes a putative cell wall glycoprotein. *FOR2* encodes the structural gene for glutamine:fructose-6-phosphate amidotransferase, the first and rate-limiting step in the biosynthesis of hexosamine and cell wall chitin. *FOR3* encodes *a* homolog of *SSD1*, which controls cell wall composition, longevity, and virulence in *S. cerevisiae*. A *for3* null mutation increased osmotin sensitivity of conidia and hyphae of *F. oxysporum* f. sp. *nicotianae* and also reduced cell wall β-1,3-glucan content. Together our findings show that conserved fungal genes that determine cell wall properties play a crucial role in regulating fungal susceptibility to the plant defense protein osmotin.**

Studies of plant-pathogen interactions strongly suggest that under the pressure to survive, plants and pathogens continuously react to one another's defense arsenal and evolve to overcome these defenses (13). Plants recognize pathogen-associated molecular patterns, such as fungal cell wall fragments composed of chitin, glucans, oligosaccharides, or glycoprotein peptides (32). It has been established that pathogens evolved effector proteins to avoid plant surveillance mechanisms that recognize pathogen-associated molecular patterns and this in turn led to the evolution of plant surveillance mechanisms that recognize pathogen-specific effector proteins. All pathogen recognition mechanisms induce intracellular signaling that culminates in the synthesis of factors, such as antimicrobial plant proteins, that help in limiting the severity of infection (74). The antimicrobial proteins are therefore among the ultimate effectors of plant defense. There is evidence of recognition between plant antimicrobial proteins and pathogen-specific molecules (74). Therefore, pathogen mechanisms of resistance to the antimicrobial proteins and the antimicrobial proteins themselves must have coevolved. Consequently, we postulated that a screen for fungal genes that alter the sensitivity of a phytopathogen to an antifungal protein of the host plant (that is, a cognate plant defense effector) would lead to identification of genes involved in controlling pathogenicity, in controlling access of the antifungal protein to its target fungal molecules (such as genes controlling cell surface composition), and in controlling detoxification mechanisms.

The plant antifungal protein selected to test this hypothesis was osmotin. Osmotin is an antifungal protein that is overexpressed in and secreted by salt-adapted cultured tobacco (*Nicotiana tabacum*) cells (63). It is a member of a family of ubiquitous plant proteins, referred to as plant pathogenesis-related proteins of family 5 (PR-5), that are implicated in defense against fungi (74). Osmotin gene and protein expression is induced by biotic stresses, and overexpression of osmotin delays development of disease symptoms in transgenic plants (41, 42, 43, 84). The genetic bases of the susceptibility and resistance of *Saccharomyces cerevisiae* to osmotin have been explored in our laboratory (49, 50). The results show that specific interactions of osmotin with the plasma membrane are responsible for cell death signaling. However, because the cell wall governs access of osmotin to the plasma membrane, differences in cell wall composition largely account for the differential osmotin sensitivity of various *S. cerevisiae* strains, and specific cell wall components play a significant role in modulating osmotin toxicity (30, 31, 49, 50, 81, 82). These studies in the model nonpathogenic fungus, *S. cerevisiae*, support our hypothesis that a screen for genes that alter the sensitivity of a phytopathogenic fungus to an antifungal defense effector protein of the host plant will uncover genes involved in controlling access of the antifungal protein to its target fungal molecules.

Osmotin, like other plant defense antifungal proteins, has specific but broad-spectrum antifungal activity (74). One of the most osmotin-sensitive phytopathogenic fungi is *Fusarium*

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oxysporum. *F. oxysporum* is an ascomycete fungus, like *S. cerevisiae*, and has been touted as an appropriate multihost model for studying fungal virulence (53). It is a soilborne plant pathogen of economic significance, because it causes vascular wilt disease on a large variety of crop plants and produces toxic food contaminants (17, 58). In humans it also causes skin, nail, and eye disease that can become serious or life-threatening illnesses in immunocompromised patients (52, 69). *F. oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *nicotianae*, and *F. oxysporum* f. sp. *meloni*, like *S. cerevisiae*, are quite sensitive to osmotin (1, 51; M. L. Narasimhan, unpublished data). Furthermore, it was recently shown that overexpression in *F. oxysporum* f. sp. *nicotianae* of an *S. cerevisiae* cell wall glycoprotein that increases the osmotin resistance of *S. cerevisiae* also increases the osmotin resistance of the plant pathogen and its virulence on tobacco, the osmotin-producing host plant (51). This suggested that osmotin resistance mechanisms may be conserved between *S. cerevisiae* and *F. oxysporum* and that *S. cerevisiae* could be used as a tool to uncover *F. oxysporum* genes that control osmotin sensitivity or resistance.

In the current study, we expressed an *F. oxysporum* f. sp. *nicotianae* cDNA library in the osmotin-sensitive *S. cerevisiae* strain BWG1-7a and selected genes for their ability to increase osmotin tolerance. We report here the identification and characterization of three *FOR* (*Fusarium* Osmotin Resistance) genes that affect the cell wall in *S. cerevisiae*. The product of *FOR1* has homology with a putative cell surface glycoprotein; *FOR2* encodes glutamine:fructose-6-phosphate amidotransferase (GFAT), an enzyme that catalyzes the first step in the biosynthetic pathway leading to amino sugar-containing macromolecules, such as glycoproteins and chitin (64); and *FOR3* has high homology with *S. cerevisiae SSD1*, a gene that controls cell wall composition and virulence (31, 78). *FOR2* and *FOR3* are the functional equivalents of the corresponding *S. cerevisiae* genes. Our parallel analysis using two model fungi verifies the notion that cell wall proteins play a critical role in determining the sensitivity/resistance of fungi to osmotin. In addition, these results implicate that the tobacco defense protein, osmotin, can serve as an effective/useful tool in identifying genes that control cell wall composition not only in a model fungus, such as *S. cerevisiae*, but also in phytopathogenic fungi, such as *F. oxysporum*.

MATERIALS AND METHODS

Fungal strains, media, and culture conditions. Unless specified otherwise, *Saccharomyces cerevisiae* strain BWG1-7a (*MAT*a *ade1-100 his4-159 leu2-3,112 ura3-52*) was used throughout. *S. cerevisiae* strain RAY-3A (*MAT*a *his3 leu2 trp1 ura3*) and its isogenic Δ*ssd1*::*HIS3* derivative were kindly provided by Y. Uesono (University of Tokyo, Tokyo, Japan) and have been described elsewhere (73). YAT1588 (*pir1*::*LEU2 pir2*::*HIS3 pir3*::*URA3*) was the kind gift of A. Toh-e (University of Tokyo, Tokyo, Japan) and has been described (71). The Δ*gfa1*::*HIS3MX* mutation, which replaced nucleotides 1074 to 3100 of the *S. cerevisiae GFA1* (*ScGFA1*) open reading frame (ORF) with the *HIS3* marker, was constructed using the PCR-based gene-targeting system (75) in *S. cerevisiae* strain W303-1A (*MAT*a *his3 leu2 trp1 ade2 ura3*) (76). The *S. cerevisiae* strain JC1246-7A (*MAT*a *ade2 ade3 lys2-801 ura3-52 gfa1-97*) (83) was the kind gift of J. F. Cannon (University of Missouri—Columbia, Columbia, MO). Standard procedures were followed for the growth and genetic manipulation of yeast (2, 60). Yeast cells were grown in either synthetic minimal medium (SD) (0.67% yeast nitrogen base without amino acids, 2% glucose, pH 6.5) with appropriate nutritional supplements or in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 28 to 30°C. Null *gfa1* mutants are auxotrophic for D-glucosamine, and the *gfa1* mutant was grown in media supplemented with D-glucosamine as described previously (79).

The wild-type strain of *F. oxysporum* f. sp. *nicotianae* was obtained from G. Chilosi (Universitá della Tuscia, Viterbo, Italy). Conditions and media used for cultivating the fungus have been described (51). Potato dextrose broth (PDB) and potato dextrose agar (PDA) used for cultivating the fungus were purchased from Sigma (St. Louis, MO). The gene replacement vector p*for3*::HYG was used to create a *F. oxysporum* Δ*for3* mutant. It contained *FOR3* genomic DNA (−125 bp to +3941 bp) with a hygromycin resistance cassette inserted between +697 bp and +3205 bp, replacing approximately 2.4 kb of intervening *FOR3* sequence. To construct p*for3*::HYG, a 0.7-kb fragment containing the 3′ part of the *FOR3* ORF (+3205 bp to +3941 bp) was amplified from *F. oxysporum* genomic DNA by PCR using the primer pair 5′-ATCGAGTCTTGCCGACGAT-3′/5′-TGAG ATCCGTCTTCAGGATC-3′ and inserted into the EcoRV site of the pSTblue-1 vector (Novagen, Madison, WI). After sequencing, to establish the fidelity of the PCR and the direction of the insert, a 1.4-kb SalI fragment of plasmid pCB1003 (Fungal Genetics Stock Center) containing a hygromycin resistance gene cassette was inserted into the SalI site on this plasmid, yielding a construct that contained the hygromycin gene cassette fused to the 5′ end of the *FOR3* fragment (+3205 bp to +3941 bp). Finally, a 0.8-kb fragment of *FOR3* (−125 bp to +697 bp) was amplified by PCR from *F. oxysporum* genomic DNA using the primer pair 5′-GCTCTAGACAGCAGCAGCAGTCTTCTCAA-3′/5′-CCGCTCGAGGGC GTTGGTTCATTGCTT-3′ and inserted as an XbaI/XhoI fragment into the corresponding restriction enzyme sites in the previous construct to yield p*for3*::HYG. To disrupt *FOR3*, p*for3*::HYG was linearized by digestion with PstI and XbaI. Linearized p*for3*::HYG DNA was used to transform protoplasts of *F. oxysporum* f. sp. *nicotianae* as described previously (51), except that protoplasts were incubated at 37°C for 15 min before addition of DNA. *F. oxysporum* is somewhat recalcitrant to targeted gene disruption due to a high frequency of ectopic integration of the vector (16). However, a dramatic increase in the frequency of gene replacement has been reported under conditions which induce expression and chromatin remodeling of the target gene locus (25, 68). Since heat shock treatment induces *SSD1* expression in yeast, it was considered likely that *FOR3* would also be induced by heat shock. Therefore, *F. oxysporum* protoplasts were subjected to heat shock before the addition of transforming DNA, and this was indeed found to increase the frequency of homologous integration events at the *FOR3* locus. Hyg^r transformants were purified by single-spore isolation, and disruption of *FOR3* was confirmed by PCR using the primer pairs 5′ ATCGTG ATCAATCACCTTCGC-3′/5′-CAAGTTATCGTGCACCAAGCA-3, 5′-ATCG TGATCAATCACCTTCGC-3′/5′-TGTATTGACCGATTCCTTGCG-3′, and 5′ ATCGTGATCAATCACCTTCGC-3′/5′-TGAGATCCGTCTTCAGGATC-3′. The PCR conditions used were 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 4 min 30 s, with a 5-s extension at every cycle, and a final elongation step at 72°C for 10 min.

Antifungal tests. Osmotin was purified from salt-adapted tobacco cell suspension cultures (*Nicotiana tabacum* L. var. Wisconsin 38) to apparent homogeneity as described previously (63). The IC_{50} (amount of osmotin that reduces growth by 50%) of different osmotin batches was determined as described previously using *S. cerevisiae* strain BWG1-7a as a standard (82) and ranged between 6 and 8 μg/ml. For quantitative measurement of growth inhibition by osmotin, overnight cultures *S. cerevisiae* in YPD or selective media were diluted to an A_{600} value between 0.01 and 0.05 in the same medium, and the diluted culture (320 μl) was mixed with various concentrations of osmotin in sterile water (80 μl). Cultures were then incubated at 28 to 30°C with shaking for 20 to 22 h, and growth was measured as the absorbance at 600 nm after appropriate dilution. For measurement of growth inhibition of *S. cerevisiae* spheroplasts by osmotin, cells were harvested in the exponential growth phase. They were suspended in 1 M sorbitol, and cell walls were digested by treatment with lyticase (Sigma, St. Louis, MO). Spheroplasts were collected by centrifugation, washed with YPD containing 1 M sorbitol, and embedded in osmotin-supplemented YPD agar containing 1 M sorbitol. Viable counts were determined after incubation at 28 to 30°C for 2 to 3 days.

To determine the osmotin sensitivity of *F. oxysporum*, conidia were harvested from cultures grown on PDA plates in 2× PDB. The conidial suspension was filtered through two layers of cheesecloth to remove mycelial fragments, and conidial numbers were determined using a hemacytometer. The effect of osmotin on hyphal elongation was tested exactly as described earlier (51). The test of osmotin's effect on conidial germination was performed essentially in the same manner. Equal volumes (100 μl) of conidial suspension (1×10^6 conidia/ml) and sterile osmotin solution (or water) were mixed in wells of a 24-multiwell tissue culture plate, aliquots (10 μl) were withdrawn from each well at the end of the 16 h of the incubation period, and the concentration of germinated conidia was

determined in each aliquot using a hemacytometer. Each well was sampled three times for counting, and three replicates were used for each treatment.

DNA and RNA methods. Procedures for isolation and fractionation of *F. oxysporum* genomic DNA and total RNA for Southern and Northern analyses have been described previously (51). For Southern analyses, nitrocellulose membrane blots of the gel were hybridized overnight with ^{32}P-labeled probe at 55°C in Church buffer (7% SDS–1 mM EDTA–0.5 M potassium phosphate buffer, pH 7.2). The membrane was subjected to successive washes in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 15 min, 1× SSC–0.1% SDS for 10 min, and 0.5× SSC–0.1% SDS for 10 min at room temperature (to detect *FOR2* and *FOR3*) or 65°C (to detect *FOR1*) and then exposed to X-ray film at −80°C for 18 to 48 h. Probes were prepared by random primer labeling (Ready-to-go labeling system; Amersham Biosciences, Piscataway, NJ). The entire 1.2-kb EcoRI/XhoI cDNA insert of p*FOR1*, a 1-kb BglII/NcoI fragment, p*FOR2* (base pairs 225 to 1505 of the *FOR2* ORF), and a 1-kb SphI/SacI fragment of p*FOR3* (base pairs 1030 to 2111 of the *FOR3* ORF) were used as probes except for the Δ*for3* mutation. An 822-bp fragment of *FOR3* (−125 bp to +697 bp) was used for probing Southern blots for confirming the Δ*for3* mutation.

The *F. oxysporum* cDNA library for yeast expression was constructed in p416GPD, a single-copy plasmid vector for constitutive high-level expression of the inserted gene from the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. *F. oxysporum* mRNA was isolated from total RNA using the Quick prep micro mRNA purification kit (Amersham Biosciences, Piscataway, NJ) and mRNA separator kit (Clonetech, Mountain View, CA).

cDNA synthesis was accomplished using a cDNA synthesis kit (Stratagene, La Jolla, CA). The DNA fragments were ligated between the EcoRI and XhoI restriction enzyme sites of the p416GPD vector and transformed into *Escherichia coli* strain XL10-Gold (Stratagene, La Jolla, CA). Approximately 10^7 *E. coli* transformants were obtained, with an average insert size of 1 kb. Plasmid DNA was extracted from pools of these *E. coli* transformants and used to transform cells of *S. cerevisiae* strain BWG1-7a using the lithium acetate method of Elble (21). Primary transformants were selected on minimal medium lacking uracil, the auxotrophy selection marker of p416GPD. Approximately 3×10^5 URA$^+$ primary transformants were collected as 6 different pools and stored at −80°C in selective minimal medium supplemented with 15% glycerol.

The *F. oxysporum* cDNA library in *S. cerevisiae* strain BWG1-7a was screened by plating approximately 10^5 cells from each pool of yeast transformants on an YPD agar plate containing osmotin (30 μg/ml). The osmotin resistance phenotype of the colonies that appeared after incubation at 28 to 30°C for 2 to 3 days was confirmed by quantitative measurement of growth inhibition by osmotin. Plasmid DNA was extracted from each osmotin-resistant yeast transformant as described previously (56) and amplified by passage through *E. coli.* Each plasmid DNA was then used to transform *S. cerevisiae* strain BWG1-7a, and the osmotin resistance phenotype of the transformant was retested by quantitative measurement of growth inhibition by osmotin. Inserts of plasmids that consistently increased osmotin resistance of *S. cerevisiae* strain BWG1-7a were sequenced using the vector primers GPD2-5′ (5′-CTTAGTTTCGACGGATTC-3′) and Tcyc-3′ (5′-TTCGGTTAGAGCGGATGTGG-3′).

DNA was sequenced at the Recombinant DNA/Protein Resource Facility (Iowa State University, Ames, IA). DNA and protein homology searches were performed using BLAST in the Saccharomyces Genome Database (http://www.yeastgenome.org), Fusarium Group Database (http://www.broad.mit.edu), and GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). Sequence alignments were performed by using the CLUSTAL W software program (70) at the Pole Bioinformatique Lyonnais Network Protein Sequence Analysis database at http://npsa-pbil.ibcp.fr/. Signal sequence prediction was made using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (5). N-glycosylation sites and PHD transmembrane helix predictions were made using a PROSCAN search and the PHD secondary structure prediction method (57), respectively, at the Pole Bioinformatique Lyonnais Network Protein Sequence Analysis database at http://npsa-pbil.ibcp.fr/. Domain searches were performed using the Pfam23.0 database search (http://pfam.sanger.ac.uk/) (22). Glycosylphosphatidylinositol (GPI) anchor prediction was made using the big-PI Fungal predictor GPI Fungal Prediction Server, version 3.1 (http://mendel.imp.ac.at/) (20).

Immunoelectron microscopy. Conidia were harvested in PDB from *F. oxysporum* cultures grown on PDA plates and filtered through two layers of cheesecloth to remove mycelial fragments. They were fixed and embedded as described by Mulholland et al. (48). Immunoreactions were carried out on thin sections essentially as described previously (51) with monoclonal antibodies to β-(1,3)-glucan (Biosupplies Australia, Parkville, Australia; 1:500 dilution). To establish specificity of the immunoreaction, sections were also incubated with monoclonal antibodies to β-(1,3)-glucan that had been previously adsorbed with laminarin (100 μg/ml; Sigma, St. Louis, MO) for 1 h at 4°C, as described previously (6). Rabbit anti-mouse IgG conjugated to 10-nm gold particles (1:50 dilution) was used as a secondary antibody. A Philips CM-10 Biotwin TEM (FEI Company, Hillsboro, OR) instrument was used for electron microscopy.

RESULTS

Isolation of cDNA clones. An *F. oxysporum* f. sp. *nicotianae* cDNA library was constructed in the low-copy-number yeast shuttle vector p416GPD, which allows constitutive high-level expression from yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. The cDNA library was transformed into *S. cerevisiae* strain BWG1-7a, an unusually osmotin-sensitive strain that has been successfully used to characterize the genetic basis of osmotin tolerance/sensitivity in yeast (31, 49, 81, 82). From a screen of about 3×10^5 primary transformants on medium containing 30 μg/ml osmotin, 52 osmotin-resistant colonies were selected. Plasmids from 15 of these 52 osmotin-resistant colonies were confirmed to confer an osmotin-resistant phenotype upon retransformation into *S. cerevisiae* strain BWG1-7a. These plasmids represented 7 unique inserts, all of which had significant homology to predicted cDNAs encoded in the genome of *F. oxysporum* f. sp. *lycopersici* (www.broad.mit.edu). Transformation with three plasmids, designated p*FOR1*, p*FOR2*, and p*FOR3*, was reproducibly associated with significant increases in the IC_{50} of osmotin (Fig. 1A, B, and C, respectively), and the inserted sequences appeared to be full-length cDNAs. Southern analysis of *F. oxysporum* f. sp. *nicotianae* genomic DNA, using labeled fragments of p*FOR1*, p*FOR2*, or p*FOR3* inserts as a probe, showed that the corresponding *FOR1*, *FOR2*, and *FOR3* genes occurred as single-copy genes in the *F. oxysporum* f. sp. *nicotianae* genome (Fig. 1D). *FOR1*, *FOR2*, and *FOR3* were therefore selected for further study. Results obtained with the other genes will be presented elsewhere.

Characterization of *FOR1*. The p*FOR1* insert (1.5 kb) included an open reading frame of 468 nt with significant homology to hypothetical proteins encoded in the genomes of *F. oxysporum* f. sp. *lycopersici* (FOXG_05281.2; 99% identity), *Fusarium graminearum* (FGSG_10125.3; 86% identity), and *Fusarium verticillioides* (FVEG_02849; 94% identity) (see Fig. S1 in the supplemental material). FOR1 has 43% identity to *S. cerevisiae* SED1 (ScSED1) (YDR077w) and 24% identity to *S. cerevisiae* SPI1 (ScSPI1) (YER150w). However, a 43-amino-acid stretch in FOR1 is highly conserved in ScSED1 (70% identity) and ScSPI1 (60% identity) (see underline in Fig. S1). This sequence occurs as an interrupted repeat sequence in ScSED1. It is also found in hypothetical proteins of several filamentous fungi, such as *Neurospora crassa* (XM_95593.1; 80% identity), *Coccidioides immitis* (XM_001247153; 69% identity), and *Chaetomium globosum* (XM_001219801; 69% identity).

ScSED1(338 amino acids) is a serine- and threonine-rich, GPI-anchored, highly N- and O-glycosylated protein that accumulates as the major protein in cell walls of stationary-phase *S. cerevisiae* cells (8, 20, 61). Consequently, the phenotype of Δ*sed1* mutants is increased sensitivity to the cell wall hydrolyzing enzyme, zymolyase. *SED1* is induced by stress and presumably has a role in strengthening the cell wall under stress conditions (28). ScSPI1 (149 amino acids) is also a serine- and threonine-rich, GPI-anchored, stress-induced cell wall glycoprotein (29). FOR1 is predicted to have a signal sequence

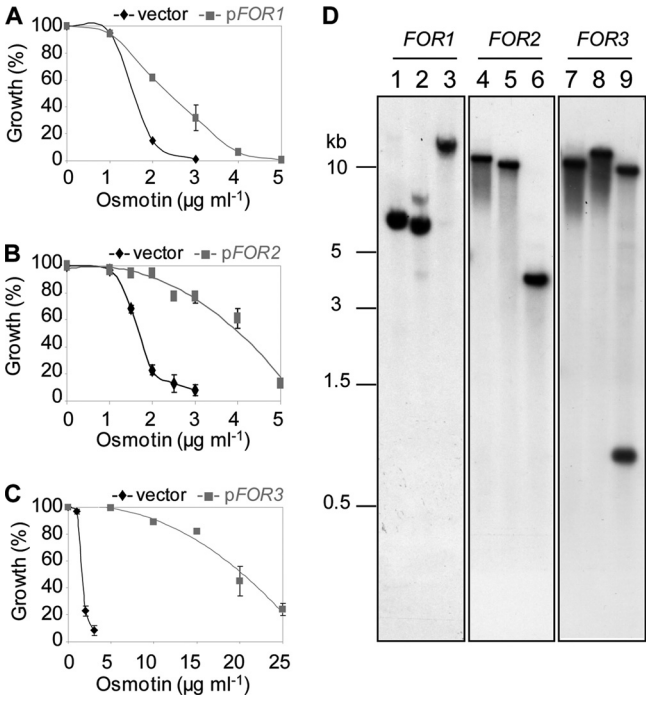

FIG. 1. *FOR1*, *FOR2*, and *FOR3* increase osmotin resistance of *S. cerevisiae* cells and are single-copy genes in *F. oxysporum* f. sp. *nicotianae*. (A, B, and C) Shown are osmotin sensitivities of cells of *S. cerevisiae* strain BWG1-7a transformed with p416GPD (vector) or the cDNA clone p*FOR1* (A), p*FOR2* (B), or p*FOR3* (C), which were compared by estimating growth in the presence of various concentrations of osmotin. Growth of cells was measured in liquid culture in selective minimal medium supplemented with the indicated osmotin concentrations. Data were normalized to viable counts of samples without osmotin. Data are the averages ± standard errors (SE) of results from at least three experiments with duplicate samples. (D) Shown are blots of restriction enzyme-digested genomic DNA (15 μg) probed with ^{32}P-labeled *FOR1* (lanes 1, 2, and 3), *FOR2* (lanes 4, 5, and 6), and *FOR3* (lanes 7, 8, and 9). The restriction enzymes used were EcoRI (lanes 1, 4, and 7), PstI (lanes 2, 5, and 8), and XbaI (lanes 3, 6, and 9).

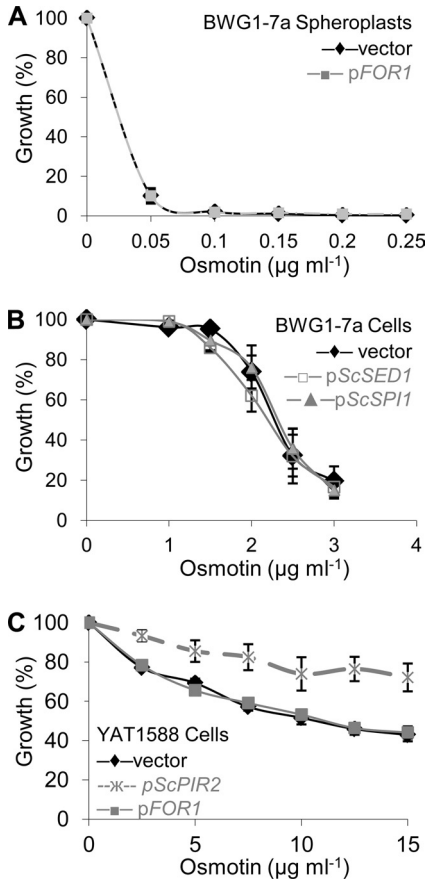

FIG. 2. The role of *FOR1* in osmotin resistance. (A) Overexpression of *FOR1* does not increase osmotin resistance of yeast spheroplasts. Shown are osmotin sensitivities of spheroplasts of *S. cerevisiae* strain BWG1-7a transformed with p416GPD (vector) or *FOR1* in the same vector (p*FOR1*). Growth of spheroplasts was measured by embedding 500 spheroplasts in YPD agar supplemented with 1 M sorbitol and the indicated concentration of osmotin and determining the number of viable colonies after 3 days of incubation at 30°C. Data were normalized to viable counts of samples without osmotin. Data are the averages ± standard deviations of results from at least two experiments with duplicate samples. (B) Overexpression of *ScSED1* and *ScSPI1* does not increase osmotin resistance of yeast cells. Cells of *S. cerevisiae* strain BWG1-7a transformed with p416GPD (vector), as well as p416GPD containing *ScSED1* (p*ScSED1*) or *ScSPI1* (p*ScSPI1*) inserts, were grown in liquid culture in selective minimal medium supplemented with the indicated osmotin concentrations. Growth was measured as the absorbance at 600 nm and normalized to the absorbance of control cultures grown without osmotin. Data are the averages ± SE of results from at least two experiments with three samples. (C) The function of *FOR1* is distinct from that of the *ScPIR* genes. Cells of *S. cerevisiae* strain YAT1588 (Δ*pir1* Δ*pir2* Δ*pir3*) transformed with p416GPD (vector), p*FOR1*, or *ScPIR2* in the same vector (p*ScPIR2*) were grown in liquid culture in selective minimal medium supplemented with the indicated osmotin concentrations. Growth was measured as the absorbance at 600 nm and normalized to the absorbance of control cultures grown without osmotin. Data are the averages ± SE of results from at least two experiments with three samples.

and a Ser/Thr-rich 43-amino-acid stretch that is conserved in ScSED1 or ScSPI1 (see Fig. S1 in the supplemental material). Bioinformatic analyses predict that FOR1 also has N-glycosylation sites, a GPI anchor addition signal, and a 17-amino-acid C-terminal hydrophobic stretch (see Fig. S1). Thus, FOR1 is very likely to be a cell wall glycoprotein.

The bioinformatic analyses suggesting that FOR1 encodes a cell wall protein were supported by the observation that osmotin resistance conferred to *S. cerevisiae* by *FOR1* requires the cell wall (Fig. 2A). Enzymatic removal of the cell wall of *S. cerevisiae* transformed with p*FOR1* or the corresponding p416GPD vector yielded spheroplasts that were equally sensitive to osmotin. Since FOR1 is predicted to be a cell wall protein and shares a highly conserved domain with ScSED1 and ScSPI1 (see Fig. S1 in the supplemental material), we tested the effect of *ScSED1* or *ScSPI1* overexpression on the osmotin resistance phenotype of yeast cells. Unlike *FOR1* overexpression, *ScSED1* or *ScSPI1* overexpression did not increase osmotin resistance (Fig. 2B). So far, the only fungal cell wall proteins known to increase osmotin resistance in *S. cerevisiae* are the PIR proteins (82). PIR proteins of *S. cerevisiae* and *Candida albicans* are a family of covalently linked O-glycosylated cell wall proteins that have a role in strengthening the cell wall under stress (35, 36). *S. cerevisiae* PIR (ScPIR) proteins were identified as osmotin resistance determinants in a screen similar to the one used to identify FOR1 (82). Although the

amino acid sequences of the PIR proteins and FOR1 do not share significant homology and a characteristic repeated sequence (TA AAVSQIGDGQIQATTKT) present in PIR proteins is absent in FOR1 (see Fig. S1), it is possible that they function interchangeably in the cell wall, because PIR proteins are anchored to the cell wall (19) and bioinformatic analyses predict that FOR1 is GPI anchored (see Fig. S1). To test whether PIR proteins and FOR1 function interchangeably, we transformed *FOR1* into an *S. cerevisiae* Δ*pir1* Δ*pir2* Δ*pir3* strain. It is known that the PIR family proteins are functionally redundant for conferring osmotin resistance in *S. cerevisiae*, so that simultaneous deletion of at least three family members is required to increase sensitivity to osmotin and, conversely, overexpression of any *ScPIR* gene increases osmotin resistance of an *S. cerevisiae* Δ*pir1* Δ*pir2* Δ*pir3* strain (82) (Fig. 2C). However, overexpression of *FOR1* did not increase osmotin resistance of the *S. cerevisiae* Δ*pir1* Δ*pir2* Δ*pir3* strain (Fig. 2C). Thus, FOR1 cannot substitute for PIR proteins to confer osmotin resistance. However, it is worth noting that while overexpression of *FOR1* does not confer resistance to a Δ*pir1* Δ*pir2* Δ*pir3* mutant (Fig. 2C), it increases osmotin resistance of *S. cerevisiae* strain BWG1-7a (Fig. 1A). *S. cerevisiae* strain BWG1-7a has the *ssd1-d* allele but contains a significant amount of PIR proteins in its cell wall, unlike the Δ*pir1* Δ*pir2* Δ*pir3* mutant (82, 31). Therefore, it follows that FOR1 requires cell wall-localized PIR proteins to function in osmotin resistance, and this lends further support to the notion that FOR1 is cell wall localized.

Characterization of *FOR2*. The sequence of the *FOR2* gene revealed an open reading frame of 2,100 nucleotides (nt), encoding a protein of 699 amino acids with homology to *GFA* (see Fig. S2 in the supplemental material), the gene encoding glutamine:fructose-6-phosphate amidotransferase (GFAT). GFAT has been identified from bacterial, fungal, plant, and mammalian sources (3, 18, 46, 77). It catalyzes the formation of glucosamine-6-phosphate, the first step in the biosynthetic pathway leading to amino sugar-containing macromolecules, such as glycoproteins and chitin (64). A single locus with significant sequence identity to *FOR2* exists in the genomes of *F. oxysporum* f. sp. *lycopersici* (FOXG_00190.2; 100% identity), *F. graminearum* (FGSG_01199.3; 94% identity), and *F. verticillioides* (FVEG_01326.3; 97% identity) (data not shown). FOR2 has significant but lower homology with GFAT of *S. cerevisiae* (60% identity), *C. albicans* (60% identity), *Escherichia coli* (36% identity), and *Homo sapiens* (52% identity) (see Fig. S2; also data not shown). However, GFAT-specific domains were particularly conserved in FOR2 (data not shown; also see Fig. S2). FOR2 has a glutamine amidotransferase class 2 domain (amino acids 1 to 199) and two sugar isomerase (SIS) domains, structures that are contained in all GFAT enzymes (see Fig. S2). SIS domains are found in many phosphosugar isomerases and phosphosugar binding proteins (4). These sequence comparisons/features strongly suggest that *FOR2* is the structural gene for the *F. oxysporum* glutamine: fructose-6-phosphate amidotransferase.

A null mutation in *ScGFA1*, the gene encoding ScGFAT, increases osmotin sensitivity of the osmotin-tolerant *S. cerevisiae* strain W303-1A (Fig. 3A, inset). Transformation of the osmotin-sensitive W303-1A Δ*gfa1* mutant with p*FOR2*, which expresses *FOR2* from the constitutive GPD promoter in the p416GPD vector, restores osmotin resistance comparable to that of the wild-type *S. cerevisiae* strain W303-1A (Fig. 3A).

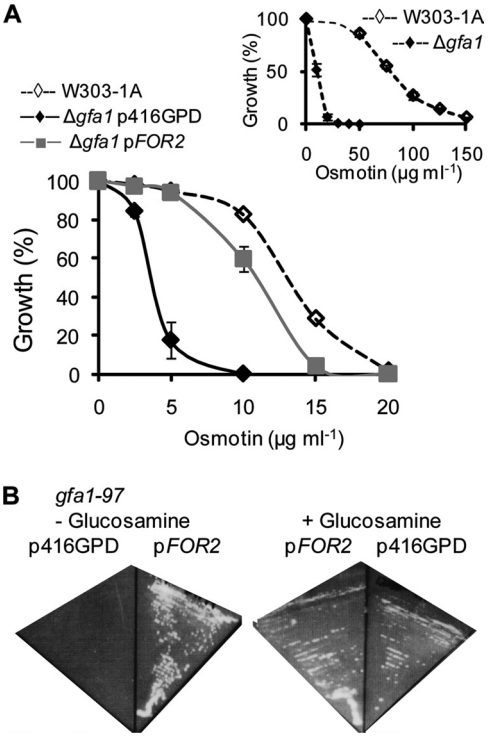

FIG. 3. *FOR2* is the functional homolog of *ScGFA1*, the gene for GFAT. (A) Cells of *S. cerevisiae* strain W303-1A and an isogenic Δ*gfa1*::*HIS3MX* strain transformed with vector (Δ*gfa1*p416GPD) or *FOR2* in the same vector (Δ*gfa1*p*FOR2*) were grown in liquid culture in selective minimal medium supplemented with the indicated osmotin concentrations. Growth was measured as the absorbance at 600 nm and normalized to the absorbance of control cultures grown without osmotin. Data are the averages ± SE of results from two experiments with three independent transformants for each strain. Inset: relative osmotin sensitivities of *S. cerevisiae* strain W303-1A and the isogenic Δ*gfa1*::*HIS3MX* strain (Δ*gfa1*) in YPD medium in a similar assay are shown. Data are the averages ± SE of results from two independent experiments. (B) Shown are plates of *S. cerevisiae* strain JC1246-7A (*gfa1-97*) transformed with p416GPD or p*FOR2*, grown for 3 days at 37°C in selective minimal medium without (−) or with (+) glucosamine (1 mg/ml) supplement.

Having established that *FOR2* can replace the function of *ScGFA1* in osmotin resistance, we next examined whether *FOR2* functions in glucosamine biosynthesis. The *S. cerevisiae* strain JC1246-7A (*gfa1-97*) has a point mutation in *GFA1* that confers a temperature-sensitive glucosamine-auxotrophic phenotype (83). Thus, transformants of this strain that carry the vector p416GPD are glucosamine auxotrophs at 37°C (Fig. 3B). However, transformants carrying p*FOR2* revert to glucosamine prototrophy at 37°C, indicating that FOR2 functions in glucosamine biosynthesis, as expected from its sequence homology with GFAT from various sources. Collectively, these data establish that *FOR2* is a structural gene for glutamine: fructose-6-phosphate amidotransferase.

Characterization of *FOR3*. The p*FOR3* insert includes an open reading frame of 3,954 nt, encoding a protein of 1,318 amino acids that has significant sequence similarity to SSD1 of various fungi (see Fig. S3 in the supplemental material). Hypothetical proteins with high similarity to FOR3 are encoded by single loci in the genomes of *F. oxysporum* f. sp. *lycopersici* (FOXG_01798.2;

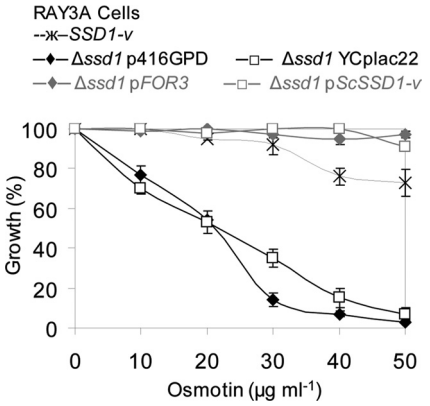

FIG. 4. *FOR3* is the functional homolog of *ScSSD1*. Shown is the relative growth of cells of isogenic *S. cerevisiae* strains RAY3A (*SSD1-v*) and RAY3AΔ*ssd1*::*HIS3MX* transformed with the vector p416GPD (Δ*ssd1*p416GPD), *FOR3* in p416GPD (Δ*ssd1*p*FOR3*), vector YCplac22 (Δ*ssd1*YCplac22), or *ScSSD1-v* in YCplac22 (Δ*ssd1*p*ScSSD1*-v) in minimal selective medium supplemented with the indicated osmotin concentrations. Values are the averages ± SE of results from two independent experiments and are normalized to the A_{600} of control cultures without osmotin.

90% identity), *F. verticillioides* (FVEG_08172.3; 89% identity), and *F. graminearum* (FGSG_07009.3; 87% identity) (see Fig. S3; also data not shown). FOR3 has significant but lower identity with SSD1 of other fungi: *Colletotrichum lagenarium* (76% identity), *Magnaporthe grisea* (78% identity), *C. albicans* (44% identity), and *S. cerevisiae* (40% identity) (see Fig. S3; also data not shown). Furthermore, the C-terminal RNase II homology domain that is essential for ScSSD1 function is conserved in FOR3 (see Fig. S3) (73, 80).

ScSSD1 is the major determinant of osmotin resistance in *S. cerevisiae*, with the natural polymorphism of SSD1 in strains of *S. cerevisiae* accounting for most of their differential sensitivities to osmotin (31). The *SSD1-v* allele, which encodes a 160-kDa protein, can suppress mutations in genes that regulate cellular integrity, cell cycle progression, growth at high temperatures, differentiation, and life span, whereas the *SSD1-d* allele, which encodes an 83-kDa C-terminally truncated version of the protein, does not have these suppressor functions (33, 34, 39, 45, 65, 72). The osmotin-sensitive phenotype of *S. cerevisiae* strain BWG1-7a, which has the *SSD1-d* allele, can be suppressed by the *SSD1-v* allele (31). It is interesting to note that *FOR3*, which is the structural equivalent of the *SSD1-v* allele in *S. cerevisiae*, produces the greatest increase in osmotin resistance of *S. cerevisiae* strain BWG1-7a of the three *FOR* genes isolated (see Fig. S3 in the supplemental material) (Fig. 1C). The functional equivalence of *FOR3* and *ScSSD1* was further confirmed by comparing the osmotin sensitivities of an *S. cerevisiae* Δ*ssd1* strain (Ray3A Δ*ssd1*) transformed with plasmids expressing or not expressing either *FOR3* or *ScSSD1-v* (Fig. 4). The Δ*ssd1* transformants expressing *FOR3* or *ScSSD1-v* were resistant to osmotin, just like the wild-type strain Ray3A (*SSD1-v*), while the vector transformants remained sensitive to osmotin, as expected (31). These results demonstrate the functional equivalence of *FOR3* and *ScSSD1* in *S. cerevisiae*.

***FOR3* controls cell wall composition and osmotin sensitivity in *F. oxysporum*.** To investigate the function of *FOR3* in *F. oxysporum*, a gene disruption vector, p*for3*::HYG, was constructed in which 2,466 bp of the *FOR3* coding region was replaced by the hygromycin resistance cassette from pCB1003 (Fungal Genetics Stock Center) (Fig. 5A). Protoplasts of *F. oxysporum* f. sp. *nicotianae* were transformed with linearized p*for3*::HYG, and hygromycin-resistant colonies were screened by PCR for the presence of the insertion in the *FOR3* gene. Among single-spore isolates of several independent PCR-positive transformants, one transformant (Δ*for3*) exhibited a banding pattern on Southern blots that was consistent with a homologous recombination event and replacement of the *FOR3* gene (Fig. 5B). Northern analysis confirmed the absence of the *FOR3* transcript in the mutant (Fig. 5C). The Δ*for3* strain was therefore used for further study.

To ascertain the effect of the Δ*for3* mutation on osmotin sensitivity, conidia from the Δ*for3* and wild-type strains of *F.*

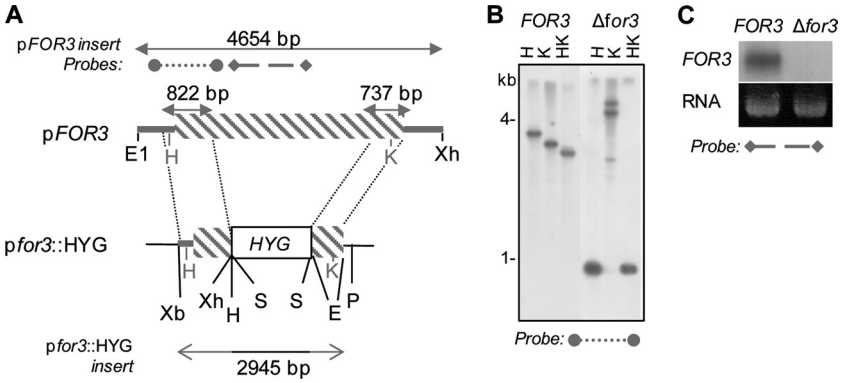

FIG. 5. Disruption of the *FOR3* gene in *F. oxysporum* f. sp. *nicotianae*. (A) Schematic view of the *FOR3* gene disruption strategy. The DNA inserts of p*FOR3* and p*for3*::HYG are shown. The *FOR3* coding region is indicated by the gray striped box and noncoding regions by the gray line. The vector sequences are indicated by the black line. Restriction sites on the cloning vector are indicated in black, and those in *FOR3* are indicated in gray. The sizes of cloned DNA fragments (double-headed arrows), as well as the probes used for Southern (dotted line) or Northern (dashed line) blot analyses, are indicated. Restriction site abbreviations are as follows: E, EcoRV; E1, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SalI; Xb, XbaI; Xh, XhoI. (B) Southern blot of restriction-digested genomic DNA of the wild-type (*FOR3*) and Δ*for3* mutant strains. Restriction site abbreviations are as in panel A. (C) Northern blot of total RNA of the wild-type (*FOR3*) and Δ*for3* mutant strains with a *FOR3* probe (*FOR3*) and the corresponding ethidium bromide signal (RNA) shown.

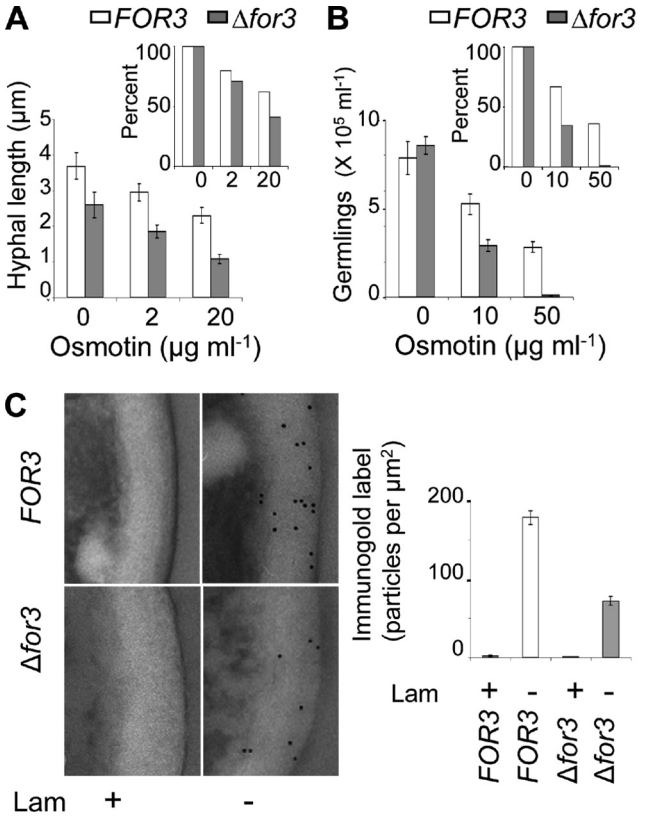

FIG. 6. Disruption of the *FOR3* gene increases osmotin sensitivity and alters cell wall composition. (A) Inhibition of hyphal elongation by osmotin. Hyphal lengths of germlings of the wild type (*FOR3*) and Δ*for3* mutant strain (Δ*for3*) after 16 h of incubation with the indicated osmotin concentration are shown. The inset shows the same data normalized to the average hyphal length in the absence of osmotin. Each value is the mean of three different observations ± SE. (B) Inhibition of conidial germination by osmotin. Conidia (10^6/ml) were incubated with the indicated osmotin concentrations, and the concentration of germinated conidia of the wild type (*FOR3*) or the Δ*for3* mutant strain (Δ*for3*) after 16 h of incubation is shown. The inset shows the same data normalized to the average concentration of germlings in the absence of osmotin. Values are the means of three different observations ± SE. (C) Immunocytochemical measurement of cell wall β-1,3-glucan content. Shown are ultrathin sections of conidia of the wild-type (*FOR3*) and Δ*for3* mutant strains that were incubated with β-1,3-glucan antibody preadsorbed (+) or not (−) with laminarin (Lam). Secondary antibodies were conjugated to 10-nm gold particles, which appear as small black dots at sites of positive reaction. The graph shows the average number of gold particles on the cell walls in these samples ± SE (n = 10 cells).

oxysporum f. sp. *nicotianae* were incubated in growth medium for 16 h in the presence of various concentrations of osmotin. In both strains, all the conidia germinated at the lower concentrations of osmotin used, and the lengths of the emergent hyphae were affected inversely in relation to the concentration of osmotin. At higher concentrations of osmotin, only a fraction of the conidia were able to germinate. Thus, the effect of osmotin on hyphal length and ability to germinate were quantified separately (Fig. 6A and 6B). By analysis of variance (ANOVA) at the level of $\alpha = 0.05$, the P value of the hyphal lengths of the wild type and Δ*for3* mutant in the absence of osmotin was <0.05, indicating that the Δ*for3* mutant had significantly shorter hyphae even in the absence of osmotin (Fig. 6A). The average hyphal lengths after osmotin treatment were therefore normalized to the average hyphal lengths of control samples without added osmotin. Figure 6A shows that the inhibition of hyphal elongation by osmotin was greater in the Δ*for3* mutant than in the wild-type strain of *F. oxysporum* f. sp. *nicotianae* (about 1.5-fold difference at 20 μg/ml osmotin; Fig. 6A, inset). The effect of the Δ*for3* mutation on the ability of conidia to germinate (or hyphal emergence), measured as the number of germlings obtained after 16 h of incubation of conidia in growth medium, is shown in Fig. 6B. By ANOVA at the level of $\alpha = 0.05$, the P value of the number of germlings of the wild type and Δ*for3* mutant in the absence of osmotin was >0.05, indicating no significant effect of the Δ*for3* mutation on the ability of conidia to germinate. Osmotin significantly inhibited the ability of conidia to germinate in both wild-type and Δ*for3* strains, but the inhibitory effect was much more pronounced in the Δ*for3* mutant. In order to visualize the results more clearly, the average number of germlings observed in osmotin-treated samples was normalized to the average number of germlings observed in the sample without osmotin and expressed as a percentage (Fig. 6B, inset). The results clearly demonstrated that the inhibition of conidial germination (or hyphal emergence) by osmotin was greater in the Δ*for3* mutant than in the wild-type strain (about a 33-fold difference at 50 μg/ml osmotin; Fig. 6B, inset). These data show that a null mutation in *FOR3* results in increased sensitivity to osmotin in *F. oxysporum.*

In *S. cerevisiae*, *SSD1* controls cell wall composition (31, 78). To determine whether *FOR3*, the homolog of *SSD1* in *F. oxysporum*, regulates cell wall composition, conidia of the wild-type and Δ*for3* strains of *F. oxysporum* f. sp. *nicotianae* were subjected to immunocytochemical analysis in ultrathin sections with an antibody that specifically recognizes linear β-1,3-glucan. β-1,3-Glucan occurs specifically in fungal cell walls, and accordingly immunogold labeling was observed only in the cell wall of both strains (Fig. 6C). Preadsorption of the antibody with laminarin, a linear β-1,3-glucan, completely abolished labeling, confirming that the immunoreaction was specifically detecting linear β-1,3-glucan. Quantitative measurement of immunogold labeling as the average densities of the gold particles per μm^2 showed that the cell wall content of linear β-1,3-glucan is reduced in the Δ*for3* mutant to 40% of that of the wild-type strain (Fig. 6C). Taken together, these data demonstrate that *FOR3* controls osmotin sensitivity as well as cell wall composition in *F. oxysporum* and is both the structural and functional equivalent of *S. cerevisiae SSD1*.

DISCUSSION

We have identified *FOR1*, *FOR2*, and *FOR3* as three *F. oxysporum* genes that increase osmotin resistance in a heterologous fungal species. Interestingly, all three genes affect the cell wall of *S. cerevisiae*. The cell walls of *S. cerevisiae* and other ascomycetous fungi are similar and contain mainly β-1,3-glucans, glycoproteins, and chitin, along with smaller amounts of α-1,3-glucans or β-1,6-glucans (15, 38, 59). It is therefore not surprising that some osmotin resistance determinants are conserved between ascomycetous fungi.

FOR3. Several lines of evidence suggest that *FOR3* is the structural and functional homolog of *ScSSD1* (Fig. 4, 5, and 6). First, FOR3 has significant similarity with SSD1 of other fungi (see Fig. S3 in the supplemental material). Second, overexpression of *FOR3* confers osmotin resistance to the *S. cerevisiae* BWG1-7a strain, which carries a truncated *ssd1-d* allele (Fig. 1). Third, *FOR3* complements the osmotin sensitivity phenotype of an *S. cerevisiae* Δ*ssd1* mutant (Fig. 4). Like *FOR3*, *SSD1* orthologs from other fungi show considerable sequence similarity with the full-length *S. cerevisiae SSD1-v* allele and can complement phenotypes of *S. cerevisiae* strains carrying the truncated *ssd1-d* allele or null Δ*ssd1* mutations. *C. albicans SSD1* can suppress synthetic lethality of *swi4 ssd1-d* mutations in *S. cerevisiae* that are also suppressed by the *ScSSD1-v* allele (12). *SSD1* of the cucumber anthracnose fungus *Colletotrichum lagenarium* can suppress the caffeine sensitivity phenotype of an *S. cerevisiae* Δ*ssd1* mutant (67). Fourth, null mutation of *FOR3* reduced the β-1,3-glucan content of the cell wall and increased osmotin sensitivity of *F. oxysporum* f. sp. *nicotianae.* Likewise, null mutation of *ScSSD1*, the major osmotin resistance determinant of *S. cerevisiae*, results in a slight deficiency of β-glucans accompanied by an increase in mannan and chitin in both laboratory and feral strains (31, 78). It is not surprising that *FOR3* was isolated as the major osmotin resistance determinant in our screen, since the *S. cerevisiae* strain BWG1-7a that was transformed with the *F. oxysporum* cDNA library has the defective truncated *ssd1-d* allele (31). This finding is consistent with our previous work indicating SSD1 as the major osmotin resistance determinant in *S. cerevisiae* (31).

FOR2. *FOR2* encodes a functional glutamine:fructose-6-phosphate amidotransferase (GFAT) since it was able to complement both the osmotin sensitivity and glucosamine auxotrophy phenotypes of *S. cerevisiae gfa1* mutants (Fig. 3; see also Fig. S2 in the supplemental material). GFAT is the highly conserved first and rate-limiting enzyme in the highly conserved biosynthetic pathway for UDP-*N*-acetylglucosamine, which is the donor of *N*-acetylglucosamine monomer units incorporated into glycoproteins and chitin (64). In *Aspergillus nidulans*, an increase in cell wall chitin content is associated with increased osmotin resistance (14). In *S. cerevisiae*, it has been shown that the availability of donor monomer units for chitin biosynthesis is normally limiting (10). The expression level of *GFA1* is therefore a major determinant of cell wall chitin content (40). Therefore, it is plausible that increased cell wall chitin content contributes to the osmotin resistance associated with overexpression of *FOR2* in *S. cerevisiae.* In *S. cerevisiae*, *F. oxysporum*, *A. niger*, and *Penicillium chrysogenum*, *GFAT* is induced by cell wall stressors such as calcofluor white (7, 24, 40, 55). The induced expression of *GFAT* is accompanied by an increase in cell wall chitin content, suggesting that GFAT content could be a major factor limiting chitin content of the cell walls of many ascomycete fungi (55). Therefore, it is very likely that *FOR2* will be an osmotin resistance determinant in *F. oxysporum*, just as *GFA1* controls osmotin resistance in *S. cerevisiae.*

***GFA1* and *SSD1* are conserved master controllers of cell wall strength and osmotin resistance.** The cell walls of *S. cerevisiae* and other ascomycetous fungi are similarly organized (15, 37). The inner layer of the cell wall of *S. cerevisiae* contains chitin and alkali-insoluble cross-linked glucan and thereby makes a major contribution to the mechanical strength of the cell wall (9, 27, 37, 38, 54). SSD1 and GFA1 control the structure and composition of this layer. SSD1 controls the content of alkali-insoluble cross-linked glucan, and GFA1 controls chitin content (30, 40). In addition, SSD1 controls deposition of PIR glycoproteins that are known to be cross-linked to cell wall β-1,3-glucan fibrils of the inner wall (31, 36). Cell wall and electron microscopy analyses show that Δ*ssd1* mutants of *S. cerevisiae* have weak walls and altered composition, which are suggestive of cross-linking defects (31, 54). Null *SSD1* mutants of *S. cerevisiae* also exhibit increased sensitivity to cell wall perturbing agents (66). Deletion of orthologs of *SSD1* in other fungi is also shown to weaken the cell wall. For instance, deletion of the *SSD1* ortholog in the human pathogenic yeast *Cryptococcus neoformans* results in increased sensitivity to cell wall-degrading enzymes (26). Null *ssd1* mutants of *C. lagenarium* and *Magnaporthe grisea* have a lower rate of hyphal growth and increased sensitivity to caffeine, which is an indicator of cell wall weakening (67). In fact, we also observed that hyphal growth was retarded in the Δ*for3* mutant of *F. oxysporum* compared to the wild-type strain even in the absence of osmotin (Fig. 6A). Deletion of the gene encoding GFAT in *S. cerevisiae* and *A. niger* results in glucosamine insufficiency and is therefore lethal (55, 83). A temperature-sensitive mutant of *S. cerevisiae GFA1* fails to grow normally when glucosamine is limiting unless osmotic stabilizers are added to the medium, indicating that GFA1 is a major contributor to cell wall strength (83). Conversely, increasing gene dosage of *SSD1* and *GFA1* compensates for cell wall weakening in *S. cerevisiae* (33, 34, 40, 44) and also confers resistance to osmotin (82). All these data provide further evidence that cell wall weakening is closely associated with increased sensitivity to osmotin. Genes such as *GFA1* and *SSD1*, which are master controllers of cell wall strength, are therefore conserved fungal osmotin resistance determinants.

***FOR1* and the divergent relationship between cell wall glycoprotein structure and function.** Cell wall glycoproteins of ascomycetous fungi form a layer surrounding the core β-glucan and chitin-containing layer (37). Due to the bulk of the carbohydrate moieties involved in N glycosylation and the presence of disulfide bridges, the cell wall glycoproteins determine permeability of the cell wall to macromolecules (38). Cell wall glycoproteins also have a limited role in maintaining and strengthening the cell wall (15). We have shown that heterologous expression of *ScPIR2* in *F. oxysporum* targets the protein to the *F. oxysporum* cell wall and increases osmotin resistance, suggesting that cell wall targeting mechanisms are conserved between these fungi (51). However, FOR1 presents an example of a divergent structure-function relationship of glycoprotein between fungi. Although FOR1 is predicted to be a cell wall protein similar to ScSED1/ScSPI1 based on sequence analysis (see Fig. S1 in the supplemental material) and is unable to confer osmotin resistance to *S. cerevisiae* if cell walls are removed (Fig. 2A), neither ScSED1 nor ScSPI1 was able to confer osmotin resistance to the osmotin-sensitive yeast strain BWG1-7a as FOR1 was (Fig. 2B). Additionally, while FOR1 cooperates with ScPIR proteins, it cannot replace their function in increasing osmotin resistance of *S. cerevisiae* (Fig. 2C). We can only speculate about the mechanism by which FOR1 increases osmotin resistance in *S. cerevisiae*. It does not contain

the signature DGQJQ sequence (J is any hydrophobic amino acid) that is essential for cross-linking of PIR glycoproteins of *S. cerevisiae* to β-1,3-glucan and thereby strengthening the cell wall (11, 36, 37, 47). However, FOR1 has a predicted GPI anchor sequence for covalent linkage to cell wall β-1,6-glucan (see Fig. S1) (37). Glycoproteins that are anchored by the remnant of a GPI anchor to cell wall β-glucan are known to contribute to cell wall strengthening (28, 61, 62). FOR1 could also be bound to other cell wall proteins by disulfide bonds or noncovalent linkages, mechanisms that are known to anchor proteins in the *S. cerevisiae* cell wall (37). The existence of putative N-glycosylation sites, a Ser/Thr-rich domain containing multiple cysteine residues, and a C-terminal domain with weak homology to collagen domains (see Fig. S1; collagen domains are annotated as Pfam PF01391 and can form triple helices and multimers of these helices) suggests that FOR1 could exist in the cell wall as high-molecular-weight bulky oligomers of an N- and O-glycosylated monomer. The expression level of PIR proteins correlates inversely with cell wall permeability in *S. cerevisiae* (37). Therefore, on the basis of the data in Fig. 2C, we can consider the possibility that the mechanism by which FOR1 increases osmotin resistance in yeast consists of FOR1 aggregates reducing permeability of the cell wall to osmotin when the inner cell wall layer is already rendered somewhat impermeable by cross-linking of β-1,3-glucan with PIR proteins, a process that neither mandates nor excludes covalent linking of FOR1 to the cell wall.

Conservation of osmotin resistance determinants between fungi. Together, our results validate our hypothesis that osmotin resistance mechanisms may be conserved between *S. cerevisiae* and *F. oxysporum* to a great extent and that *S. cerevisiae* could be used as a tool to uncover *F. oxysporum* genes that control osmotin sensitivity or resistance. Most importantly, our results show that resistance of fungi to the plant defense protein osmotin is determined by the impermeability of their cell walls to this protein. Consequently, genes such as *GFA1* and *SSD1* that control deposition of multiple cell wall constituents are major, and conserved, fungal osmotin resistance determinants. Individual cell wall glycoproteins can make small contributions to osmotin resistance and are not conserved osmotin resistance determinants.

Effect of *for3* mutation on pathogenicity. Fungal cell walls are unique and are therefore the targets of antifungals of medical and agronomic importance. GFAT is an essential and ubiquitous enzyme and is therefore less attractive as an antifungal target than SSD1, which is unique to fungi. It has been shown that deletion of *ScSSD1* orthologs of the cucumber anthracnose fungus *C. lagenarium* and the rice blast fungus *M. grisea* impaired their ability to initially establish infection on their hosts (67). Compared with the wild-type strains, the Δ*ssd1* mutants of *C. lagenarium* and *M. grisea* were shown to have weaker cell walls and were also able to induce host defenses more strongly, which accounted for their reduced infectivity. Similarly, null *ssd1* mutants of *C. albicans* were found to have decreased virulence (23). The null *ssd1* mutants of *C. albicans* were more sensitive to host defense antimicrobial peptides than the isogenic wild-type strain. Since the null *for3* mutation depleted cell wall β-1,3-glucan, which could weaken the wall, and increased sensitivity to the tobacco defense protein osmotin, we expected the Δ*for3* mutant of *F. oxysporum* f. sp. *nicotianae* to also be impaired in its ability to cause disease symptoms in the host tobacco plant. However, we were unable to observe significant differences in disease symptom development between soil-grown tobacco plants inoculated by the root dip method with the wild-type strain of *F. oxysporum* or the Δ*for3* mutant (see Fig. S4 in the supplemental material). Wheeler et al. (78) observed that *SSD1* knockout mutants of feral strains of *S. cerevisiae* had altered cell surface properties and changes in cell wall composition, were more-potent elicitors of a proinflammatory response in cultured macrophages, and were also more virulent in mouse models of infection. Our results, when considered with the reports of Wheeler et al. (78) and Tanaka et al. (67), suggest that cell surface changes induced by inactivation of SSD1 affect pathogenicity in a unique manner for every pathogen-host combination. The results support the conclusion that SSD1 is not a good antifungal target.

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