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GENES CONTROLLING CYTOTOXICITY OF OSMOTIN, A PLANT ANTIFUNGAL PROTEIN

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INTRODUCTION

Phytopathogenic fungi are the predominant cause of agricultural losses resulting from infectious diseases in plants. The annual economic impact of these losses is significant and can be devastating after fungal epidemics (7). It is recognized that logical agronomic strategies to control fungi require an understanding of the defense responses elicited in plants. Therefore a great deal of attention has been given in recent years to the mechanism by which plants respond to invading pathogens (6).

The plant defense response to pathogens is summarized in Fig. 1 (6). Briefly, plant receptor proteins perceive pathogen derived signals which may include physical contact, fungal enzymes or cell wall fragments, plant cell wall fragments and direct or indirect products of *Avr* genes. This signaling results in the rapid generation of several signaling components including reactive oxygen species, jasmonates, ethylene, benzoic acid, salicylic acid and induction of defense genes including pathogenesis-related (PR) proteins or hypersensitivity (apoptosis) genes. These outputs are induced by signaling events that include ion fluxes, G proteins, kinases and phosphatases. Amplification of the initial defense response by the generated signaling components then occurs. Activation of cellular protection mechanisms to minimize consequences of oxidative stress is likely to accompany the defense response. These would include detoxification of peroxides and free radicals, and recycling of damaged proteins. The damage caused by these reactions on the host and pathogen generate in turn, a second generation of elicitors for temporal and spatial regulation of the plant defense response by amplification or repression of the original defense response or induction of cell death. Coordination of the defense responses is achieved by cross-talk between the induced signal transduction pathways. Although this general pattern of plant defense response has been recognized, the details are still the focus of intense research.

The fungal arsenal is equally formidable (7). Penetration of the host plant is achieved by the secretion of a mixture of hydrolytic enzymes

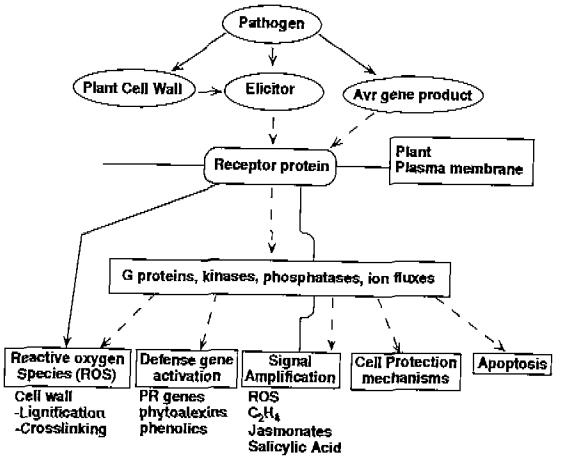

Fig. 1. Plant Defense Responses. Adapted from Hammond-Kosack & Jones (1996)

including chitinases, cellulases, pectinases and proteases. In many fungal pathogens it is also accomplished by formation of specialized penetration organs called appressoria at the tip of the germ tubes. Fungi that have not evolved a penetration mechanism, somehow locate natural openings on the plant such as stomata. Penetration is thus likely to be controlled by fungal compounds, plant surface structures and properties as well as activators or inhibitors of fungal spore germination and germ tube formation. After penetration, the usual fungal strategy is to secrete phytotoxins or plant hormone-like compounds that kill host cells or to subvert host cellular machinery for nutrient acquisition. The exact role of most phytotoxins is poorly understood. Successful colonization demands that the fungus should have developed strategies to avoid or suppress host defense, which further implies that fungi recognize plant metabolites and redirect their own cellular machinery appropriately.

Reasoning that the design of effective fungal disease control strategies (in particular, strategies designed to overcome evolving fungal resistance) would benefit from the precise mechanism by which end products of the plant defense response act against fungi, we decided to develop a genetic system to investigate the mechanism of action of osmotin, a pathogenesis-related protein of family 5 (PR-5). Osmotin is a member of the PR-5 family that was originally identified as the predominant protein that accumulated in tobacco cells as a function of osmotic adaptation (13). Subsequently, osmotin and other osmotin-like proteins were shown to have antifungal activity in vitro against a broad range of fungi, including several plant pathogens (18). Leaves of transgenic potato plants expressing tobacco osmotin exhibited partial resistance to *Phytophtora infestans* (9). The fungal growth inhibition by osmotin and zeamatin, a maize PR-5 protein, correlated with plasma membrane permeabilization and dissipation of the membrane potential (1,11), suggesting a physical interaction between PR-5 proteins and the plasma membrane of sensitive fungi, but the precise mechanism of cytotoxicity remains unknown.

Many of the PR proteins, including osmotin, exhibit clear specificity of their toxicity against fungi, indicating that there must be determinants of sensitivity and resistance in fungal cells (1,18). Even the most studied plant antifungal proteins, chitinase (PR-3) and β-(1,3)-glucanase (PR-2), which act as cell wall degrading enzymes, are not uniformly active against all fungi (17) that contain substrates for these enzymes as important cell wall components. This differential activity is not understood and specific genetic factors that condition sensitivity or resistance to antifungal enzymes have not been identified. Knowledge of the bases for this selectivity would be very helpful in determining strategies to overcome the resistance of important pathogens. The resistance of fungi to these toxic proteins could be the result of the nature of interacting targets present on the cell wall or plasma membrane of fungi as was shown for killer toxins of yeast (2,12). If these targets could be identified, structural modifications to the antifungal proteins might be engineered to improve their specific toxicity against insensitive fungi.

In order to study the bases for the specificity and the mechanism of toxicity of PR-5 antifungal proteins we began a search for a biological system that would allow the genetic identification of determinants governing resistance and sensitivity to PR-5 proteins. We report here i) the existence of genetic variants of S. cerevisiae with increased sensitivity to tobacco PR-5. ii) cell wall components are important osmotin resistance determinants and that

differential resistance among yeast strains with plasma membranes targeted by osmotin was determined by variations in the architecture of the cell wall. iii) osmotin stimulates a mitogen-activated protein kinase (MAPK) signal system in yeast to induce changes in the cell wall that enhance cytotoxicity of this antifungal protein. It has been suggested earlier that the cytotoxic action of plant antifungal proteins could involve activation of signaling cascades, based on the ability of G protein inhibitors to block the cytotoxic effect of plant defensin (15). Our results confirm these suggestions and, for the first time, provide details of genes and pathways involved. Our most unusual finding is that the protein toxin utilizes a signal transduction pathway to increase the susceptibility of a target fungus to its cytotoxic effects. This could represent a general mechanism of action of many plant antifungal proteins.

RESULTS

I. Differential Sensitivity to Osmotin among Yeast Strains.

With the aim to use the yeast *S. cerevisiae* as a model to identify determinants of resistance-/sensitivity to antifungal proteins, several yeast strains were surveyed for their sensitivity to tobacco osmotin, an antifungal protein of the PR-5 family. Most laboratory strains that were tested had various degrees of resistance to osmotin, but strain BWG7a displayed a uniquely high sensitivity to tobacco osmotin. Addition of as little as 10 μg/mL (~ 0.4 μM) of osmotin to the medium prevented the growth of BWG7a cells (IC50 ~ 3 μg/mL), whereas a saturating concentration of osmotin (240 μg/mL) only partially inhibited the growth of the highly tolerant strain GRF167 (IC50 ~ 200 μg/mL) (data not shown). Treatment of BWG7a cells with tobacco osmotin for various lengths of time, followed by dilution and plating, showed that the cytotoxic effect of osmotin in sensitive yeast cells was irreversible, as demonstrated by the dramatic decrease in viable counts after one hour in the presence of osmotin (Fig. 2). The exceeding sensitivity of BWG7a cells is specific to osmotin purified from tobacco cells since the homologous osmotin-like proteins A8 and A9 purified from *Atriplex nummularia* cells had little or no effect, respectively, on BWG7a up to a concentration of 100 μg/mL, the maximum concentration tested (Fig. 2). However, the same batches of proteins were active against other fungal species tested, such as *Veticillium dahliae* and *Trichoderma longibrachiatum* (data not shown).

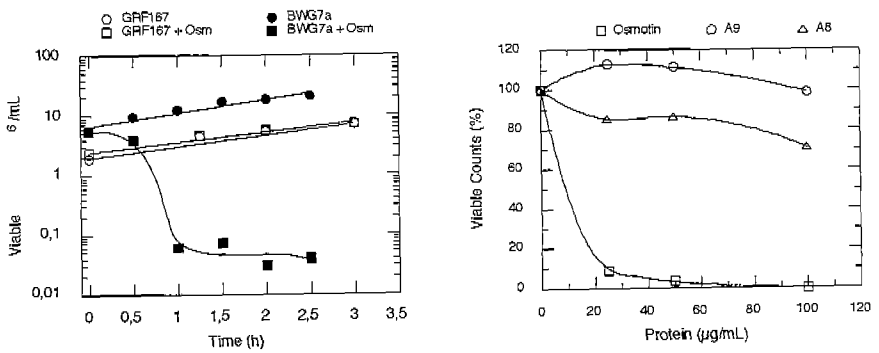

Fig. 2. Differential sensitivity of yeast strains to the cytotoxic effect of osmotin-like proteins. Upper panel, cells of strains GRF167 and BWG7a were incubated in YPD medium with and without 50μg/mL of purified tobacco osmotin for the times indicated. Subsequently, cells were diluted and plated, and the number of viable counts was determined after incubation at 30℃ for 2 days. Lower panel, ~6 $\times 10^5$ cells/mL of strain BWG7a were incubated at 30℃ for 1 hr. in YPD containing the indicated concentrations of tobacco osmotin and the osmotin-like proteins A8 and A9 purified from cultured cell suspensions of A. nummularia. Viable counts were determined as indicated above and are shown normalized to the value without added proteins.

II. Cell wall barriers determine osmotin resistance.

Our first approach to clone determinants of osmotin resistance was to complement the sensitive strain BWG7a with a genomic DNA library from the highly resistant yeast strain GRF167 in a high copy plasmid and select for acquired resistance to osmotin. Three types of genes were obtained, *MATα2*, *PIR2* and *SSD1*. MATα2 is a transcriptional regulator of mating type- and haploid-specific genes. It protects the cells against osmotin action in a cell wall-dependent manner by a mechanism that is unknown yet. The most frequently isolated gene encoded a protein, designated PIR2, a member of a gene family encoding stress glycoproteins induced by heat and nitrogen limitation.

Over-expression of PIR proteins increased resistance to osmotin, whereas simultaneous deletion of all *PIR* genes in a tolerant strain resulted in sensitivity. PIR proteins were immunolocalized to the cell wall and overexpression of any PIR isoform protected cells against osmotin in a cell wall dependent manner (19).

SSD1 is a protein of unknown function that interacts with several regulatory networks in the yeast *S. cerevisiae*, most importantly with the cell division cycle and cell wall morphogenesis. Slight homology of SSD1 protein with *E. coli* exoribonuclease II and bacterial vacB proteins has led to the suggestion that SSD1 modulates activity of RNA polymerases or regulates gene expression by a post transcriptional mechanism. The *SSD1* locus is polymorphic among laboratory strains of *S. cerevisiae*. Resistance to osmotin was found to correlate with the expression of a functional *SSD1*-v allele, which encodes a protein with an apparent mass of 170 kDa. Deletion of *SSD1*-v in resistant strains results in high sensitivity to osmotin, whereas deletion of the *ssd1*-d allele (80 kDa polypeptide) in the Osm^S strain BWG7a has only marginal effects on sensitivity. *ssd1*-d is recessive for osmotin tolerance when coexpressed with *SSD1*-v. SSD1 operates in osmotin resistance through a mechanism involving the cell wall because acquired resistance mediated by *SSD1*-v is abrogated by enzymatic digestion of the cell wall. The sensitivity of Δ*ssd1*-v cells is partially suppressed by the overexpression of cell wall localized PIR proteins but sensitivity acquired by simultaneous deletion of all *PIR* genes is not suppressed by overexpression of *SSD1*-v. Deletion of *SSD1*-v results in distinct morphological changes suggestive of softening of the cell wall, as well as biochemical changes, the most significant of which is a reduction of the alkali soluble glucans. It also results in the absence of immunodetectable PIR proteins in the cell wall, and in the absence of PIR protein-filled sorting vesicles in budding cells (unpublished result). However, deletion of *SSD1*-v has no effect on *PIR* mRNA abundance and PIR proteins are secreted to the medium in Δ*ssd1*-v cells, suggesting that *SSD1*-regulated sorting or anchoring of PIR proteins to the cell wall is an important determinant of osmotin tolerance. Overexpression of PIR proteins in Δ*ssd1*-v mutants results in the appearance of PIR proteins in sorting vesicles and in the cell wall by a mechanism that is not understood at this time.

The increased sensitivity to osmotin by simultaneous detection of all PIR genes or by deletion of *SSD1*-v in a tolerant strain was accompanied by a small but significant increase in osmotin binding to the cell wall as determined by immunolocalization (unpublished result), consistent with the idea that the cell wall of osmotin tolerant yeast strains prevent osmotin from reaching the plasma membrane, the primary site of osmotin action. Taken together with the observations that *SSD1*-regulated sorting or anchoring of PIR proteins to the cell wall is an important determinant of osmotin tolerance, the results also suggest that proper anchoring of PIR proteins in the cell wall is necessary to prevent acess to the plasma membrane. *PIR2* mRNA is

induced by osmotin trearment (19) suggesting that it is the end product of a defensive signal transduction pathway induced by osmotin insult.

III. Osmotin resistance is also controlled by subversion of a signaling pathway .

To investigate conferring osmotin sensitivity, we isolated osmotin resistant mutants from the sensitive strain BWG7a. In a preliminary screen, we isolated 11 spontaneous *ore* mutants (for osmotin resistance) that were able to grow on medium containing 2 μM purified tobacco osmotin. Of these, two (*ore9* and *11*) were found to be *STE20* mutants (Fig. 3), two others (*ore 4* and *14*) to be *SIR4* mutants and the remainder (*ore 6,7,10* and *13*) to be *SIR3* mutants (not shown). All of these mutants were sterile or exhibited severe mating deficiencies. The three fertile mutants (*ore 5,8* and *12*) fell into different complementation groups, in dicating that the mutagenesis was not performed to saturation.

SIR3 and *SIR4* are genes required for transcriptional silencing at the HM-mating locus and teleomers (10). The role of these genes in osmotin tolerance/sensitivity is unknown. STE20 is protein kinase functioning in the mating pheromone response pathway in yeast (Fig. 3). Briefly, mating of haploid cells is triggered by binding of pheromone to a cell-type specific receptor (STE 2/ STE3 on MATa/ MATα cells respectively). The signal thus initiated is transmitted by a heterotrimeric guanosine triphosphate-binding protein (G protein) encoded by GPA1 (G_α), Ste4 (G_β), and STE18 (G_γ). The G protein β and γ subunits, through STE20 protein kinase, stimulate a MAP kinase module (STE11, STE7 and FUS3/KSS1) whose components constitute a signal complex by association with the scaffolding protein STE5. Downstream of the MAP kinase cascade, STE12 activates the transcription of genes which are involved in arresting cell division in G1, formation of projections, agglutination and fusion of mating partners and ultimately, nuclear fusion (14). We found that deletion mutations in other

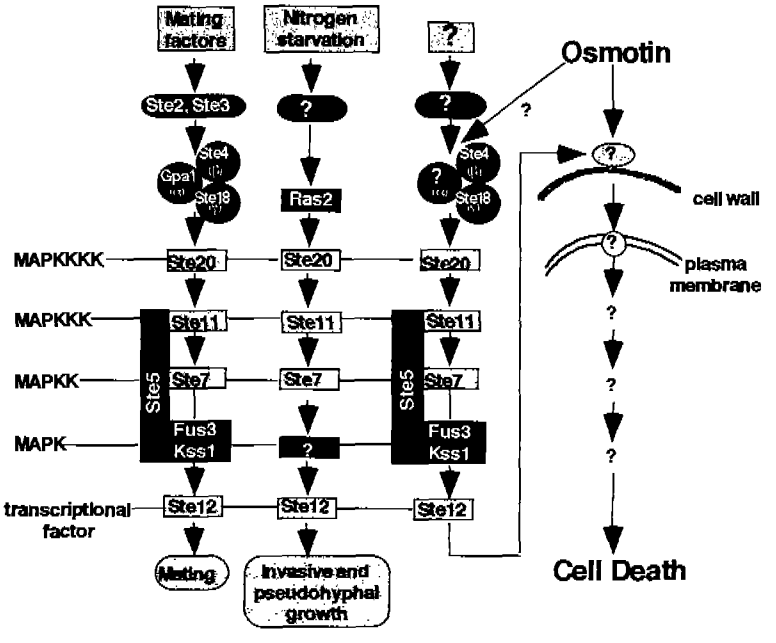

Fig. 3. Model for osmotin action. In this model, osmotin, in order to enhance its intoxicating ability, activates a MAPK cascade whose stimulation results in changes in the cell wall that facilitate osmotin access to the plasma membrane. Since mutation of the genes encoding the G-protein β and γ subunits, the MAPK module, and STE12 increased resistance to osmotin but did not block cell death completely, and spheroplasts of the MAPK cascade mutants are as sensitive to osmotin as isogenic wild type yeast, it is proposed that cell death ultimately results from a different set of interactions of osmotin with the plasma membrane (Roberts and Selitrennikoff, 1990;Abad et al., 1996).

STE genes (*STE4, STE18, STE20, STE5, STE11, STE7, FUS3* and *KSS1*, and *STE12*) functioning between pheromone reception (*STE2*) and transcriptional activation (*STE12*) in the BWG7a background also resulted in resistance, whereas mutation in the pheromone receptor *STE2* had no discernible effect on osmotin sensitivity. Disruption of *GPA1* (Ga subunit) is lethal in haploids but overexpression of GPA1, which ameliorates pheromone-induced cell cycle arrest, had no effect on osmotin sensitivity. SST2, a protein that enhances the intrinsic GTPase activity of G_α subunits, negatively regulates both osmotin toxicity and the response to mating pheromone but the participation of SST2 on osmotin activity is independent of GPA1. It was therefore concluded that full sensitivity to osmotin-induced cell death requires most of the known components of the yeast pheromone response pathway with the major differences arising at the point of signal perception, i.e. the receptor protein and the G_α subunit of the heterotrimeric G protein (Fig. 3). We further demonstrated that osmotin induces signal flux through the pheromone response pathway preceding any changes in cell vitality by measuring osmotin-induced phosphorylation of STE7 (MAPKK) (Fig. 4).

However, in contrast to pheromone treatment (5), osmotin did not induce expression of the *FUS1-lacZ* reporter, cause G1 arrest nor promote the formation of shmoo or cells with altered morphology. Distinctive morphological consequences of osmotin treatment, included increased vacuolation, increased vesiculation, membrane blebbing and in some cases, autophagy. Osmotin activation of the mating-specific MAPK signal transduction cascade therefore appears to lead to expression of a set of genes specific for the facilitation of osmotin induced cell death that is distinct from mating-specific genes. Enzymatic removal of the cell wall abrogated the osmotin tolerance conveyed by Δste7 mutation, Δ*ste20*, Δ*ste4* and other mutations (not shown). Similar results were obtained by other assays wherein spheroplast regeneration frequencies were measured after treatment with osmotin for short periods of time or in the presence of various amounts of osmotin in the embedding agar. Thus, mutations in the MAPK pathway produce changes in the cell wall that limit osmotin access to the plasma membrane and thereby cause osmotin resistance (Fig. 3) and increased signal flux through the pathway by osmotin- or osmotin-plus-pheromone-treatment produce cell wall changes that increase sensitivity to osmotin (20).

CONCLUSION

In order to study the bases for the specificity and the mechanism of toxicity of PR-5 antifungal proteins we began a search for a biological system that would allow the genetic

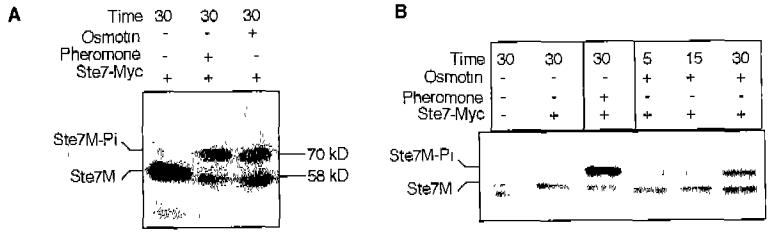

Fig. 4. Osmotin elicits signal flux through a MARK signal pathway. (A,B) Phosphorylation of STE7. Cells of strain BWG7a, without (-) or with (+) pNC267, which contains STE7 fused in-frame with a c-Myc epitope tag and under control of the CYC1-promoter (Zhou et al., 1993), were treated (+) with either α-factor (5 μM) or osmotin (A, 2 μM; B, 8 μM) for the indicated time periods at 28℃ (Cairns et al., 1992). Shown are immunoblots of total protein from cell extracts (30 μg per lane) separated by SDS-PAGE, reacted with (A) Mycl-9E10 antibody and (B) STE7 antibody polyclonal antibody, and developed by the ECL method (Cairns et al., 1992).

identification of determinants governing resistance and sensitivity to PR-5 proteins. We selected the unicellular Ascomycete, *Saccharomyces cerevisiae*, as a model target fungus to study the mechanism of osmotin action to accrue the advantages of vast genetic, molecular biology and biochemical tools as well as a completely sequenced genome. Recent studies have revealed that most of the ORFs of *Ashbya gossypii*, a filamentous phytopathogen of cotton, have homology to those of *S. cerevisiae*. At least a quarter of the clones in an *A. gossypii* genome bank contain pairs or groups of genes organized in the same order in their genome as the *S. cerevisiae* counterparts (4). These observations and the successful expression of fungal genes in yeast and vice versa (8,16,22) imply that results obtained with the model fungus (*S. cerevisiae*) could then be quickly tested for cross-functionality in phytopathogenic fungi.

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