Maize Ribosome-Inactivating Protein Inhibits Normal Development of *Aspergillus nidulans* and *Aspergillus flavus*

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The abundant maize kernel ribosome-inactivating protein 1 (RIP1) was tested for antifungal activity against *Aspergillus nidulans* and *Aspergillus flavus*. A microculture assay was developed to monitor fungal growth and development after treatment of conidia with RIP1 or control proteins. A striking decrease in hyphal proliferation was observed when conidia of *A. nidulans*, a genetically well-characterized nonpathogenic species, were treated with RIP1 protein. Treatment with a RIP1 mutant protein that lacked enzymatic ribosome-inactivating activity caused no observable effects. RIP1 treatment of conidia from the maize pathogen *A. flavus* resulted in increased hyphal branching. Examination of the branched hyphae after Congo red staining revealed only one growing hyphal tip per conidium. These results indicate that both fungi were affected by RIP1 treatment, but the lysis seen with treatment of *A. nidulans* was apparently avoided by *A. flavus*. A developmental time course revealed that both fungal species were affected by RIP1 at the postdivisional growth stage. The inhibitory activity of RIP1 against normal fungal growth is consistent with a biological function to protect the seed from fungal invasion.

Additional keywords: b-32, plant defense, seed protein, *Zea mays* L.

Fungi are a highly versatile group of eukaryotic organisms that occupy most natural habitats. Of the approximately 100,000 known species of fungi, less than 10% are able to colonize plants (Knogge 1996), yet cause crop yield losses of almost 20% annually (Knight et al. 1997). As a result, phytopathogenic fungi, especially those that produce mycotoxins, are a serious economic factor. Fungicides that maximize crop yields and benefit food quality by controlling fungi often fail to meet the criteria of benign environmental profiles and low toxicity to humans and wildlife that is demanded by the public (Knight et al. 1997). Therefore, the search for alternatives such as plant-produced antifungal compounds is becoming increasingly important.

Several proteins such as chitinases, glucanases, and protease inhibitors that show activity against fungi in vitro already

have been identified. Only a few of these proteins, however, have a definitive role in plant defense (Kauffmann et al. 1987; Kombrink et al. 1988; Mauch et al. 1988; Nasser et al. 1988; Roberts et al. 1988; Schlumbaum et al. 1986; Walker-Simmons and Ryan 1986). Other plant proteins that may have defense functions are the ribosome-inactivating proteins (RIPs), a group of N-glycosidases that irreversibly block protein synthesis by modifying the large rRNA so that it is not recognized by translational elongation factors (Hartley et al. 1996; Lord and Roberts 1998; Stirpe et al. 1992).

RIPs are widely distributed throughout the plant kingdom and are active against ribosomes from different species, although the level of activity depends on the source of the RIP and of the ribosome (Bass et al. 1992; Battelli et al. 1984; Harley and Beevers 1982; Stirpe et al. 1992). Three classes of RIPs have been identified (Mundy et al. 1994). Type 1 RIPs have a single polypeptide chain that contains the ribosome-inactivating activity. Type 2 RIPs have a polypeptide chain (A chain) that contains the ribosome-inactivating activity and a second lectin chain (B chain) that promotes uptake by the cell. Type 3 RIPs are synthesized as single-chain proenzymes that require processing events to produce two noncovalently linked chains equivalent to a type 1 RIP. The mode of uptake of types 1 and 3 RIPs by cells is unknown. Although the enzymatic activities of RIPs have been shown in vitro, their role in plant defense is less clearly defined.

At present, the availability of plant-derived compounds with antifungal activities sufficient to make them feasible for agronomic use in disease control remains inadequate. Transgenic plants expressing various RIPs, chitinases, and glucanases have increased resistance to fungal infection in greenhouse tests (Jach et al. 1995; Maddaloni et al. 1997; Zoubenko et al. 1997), although broad resistance against agronomically important fungal pathogens has not yet been reported. One strategy developed on the basis of data showing that a few of these compounds may interact synergistically in plant defense (Jach et al. 1995; Leah et al. 1991; Mauch et al. 1988) is the use of multiple compounds in combination to increase their toxicity to fungi. Implementation of such a strategy, however, would first require a better understanding of the mode of action of these compounds and their interactions.

The objective of our research was to investigate the toxicity of maize ribosome-inactivating protein 1 (RIP1) toward fungi. We and others have found that RIP1 has ribosome-inactivating

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activity in vitro against nonplant ribosomes such as those from insects, fungi, and mammals but little activity against plant ribosomes (Dowd et al. 1998; Hey et al. 1995; Krawetz and Boston 2000). Maize RIP1 is produced in seeds as an inactive zymogen proRIP1, which was originally called b-32 on the basis of its 32-kDa apparent molecular weight (Soave et al. 1981). ProRIP1 is proteolytically cleaved into α and β subunits that interact to produce the active RIP1 protein (Bass et al. 1992; Walsh et al. 1991). Because maize is an important food crop, RIP1 is expected to have a benign environmental profile. The high activity of maize RIP1 against nonplant ribosomes, however, suggests that RIP1 may function in plant defense and has potential application as an antifungal agent.

We developed a microculture assay that enabled us to monitor the cellular growth and morphology of fungi upon addition of purified RIP1. We found that active RIP1 proteins altered the growth and morphology of *Aspergillus flavus*, a corn fungal pathogen, and *Aspergillus nidulans*, a nonpathogen. Our data suggest that the ribosome-inactivating activity of RIP1 is required for the observed effects. Thus, we conclude that maize RIP1 has antifungal activity and is most likely a resistance component in maize seeds.

Fig. 1. Light micrographs of *Aspergillus nidulans* hyphae after treatment of conidia with bovine serum albumin (BSA) or MOD1 protein. Conidia were treated with 1 mg per ml of the control protein BSA or the active ribosome-inactivating protein, MOD1, and cultured for 18 h. **A**, Brightfield image of hyphal growth after treatment with BSA. **B**, Brightfield image of hyphal growth after treatment with MOD1. Scale bars = 10 μ m.

RESULTS

RIP1 treatment of *A. nidulans* conidia decreased growth.

Conidia were mixed with protein and observed microscopically at various times during germination and hyphal elongation for altered growth or morphology. Bovine serum albumin (BSA) was used for a control, and recombinant proteins produced in *Escherichia coli* were used for the treatments. Use of protein synthesized in *E. coli* allowed us to avoid possible contamination problems from a second RIP present at low levels in maize kernels (Bass et al. 1992). Prior to expression in *E. coli*, the proRIP1 clone was modified to produce a derivative that lacks the internal 25 amino acid coding region that normally is removed during proteolytic activation (Krawetz and Boston 2000; Walsh et al. 1991). The resulting protein, MOD1, was enzymatically active in vitro without further proteolytic processing (Hey et al. 1995; Krawetz and Boston 2000).

Conidia treated with BSA had normal hyphal growth (Harris 1997) that led to the formation of a mycelial mat by 18 h after inoculation (Fig. 1A). In contrast, conidia treated with MOD1 showed reduced hyphal growth, resulting in a decrease in the size of the mycelial mat by 18 h after inoculation (Fig. 1B).

To determine whether the amount of growth was dependent on the type and concentration of protein in the assay, we treated *A. nidulans* conidia with increasing concentrations of MOD1 or BSA. In addition, we used site-directed mutagenesis to introduce an amino acid substitution that rendered the MOD1 protein enzymatically inactive in vitro. The mutant protein, MOD1mut, served as an additional control to confirm that the effect of MOD1 was a result of its enzymatic activity. After propagation by microculture, the hyphae were fixed and imaged to determine the total hyphal growth on the slides. In this analysis, densitometry was used to define areas of growth and an integrated morphometry analysis program was used to calculate the area

Fig. 2. Morphometric analysis of *Aspergillus nidulans* hyphal growth in response to increasing concentrations of ribosome-inactivating protein 1 and control proteins. Conidia were treated with bovine serum albumin (●), MOD1 (□), or MOD1mut protein (▲) at concentrations varying from 0 to 1.0 mg per ml, cultured for 18 h, fixed, and imaged. Hyphal growth, as measured by the coverage of the slide surface, was quantified by integrated morphometry analysis. Error bars = standard error from three replicates.

occupied by the hyphae (Angata et al. 1997; Li 1997; Matter and Laurie 1994). Figure 2 shows the amount of hyphal coverage as a function of protein concentration. As the concentration of MOD1 was increased, fungal growth decreased. This concentration effect fit a linear regression model with the equation $y = 88.0 - 86.9x$ ($R^2 = 0.63$, $P < 0.0001$). In contrast, no effect of protein concentration on the growth of the fungus was observed for either BSA or the MOD1mut protein. An analysis of covariance revealed a significant difference between MOD1 protein treatment and both BSA and MOD1mut protein treatments ($P < 0.0001$).

Fig. 3. *Aspergillus flavus* hyphal branching in response to different proteins. Conidia were treated with 0.5 mg per ml of the control proteins bovine serum albumin (BSA), lactalbumin, or myoglobin; the enzymatically inactive ribosome-inactivating protein, proRIP1; the active RIP1 proteins, or MOD1. After 18 h in culture, branched and unbranched hyphae for each protein treatment were imaged and counted. **A to D**, Differential interference contrast images of typical *A. flavus* conidial growth after protein treatment. **A**, BSA treatment showing germ tubes, each with a single septum. **B**, Active RIP1 treatment showing multiple hyphae arising from a single conidium. **C**, BSA treatment showing a germ tube with two septa. **D**, Active RIP1 treatment showing two hyphal tips on a single germ tube. Arrowheads indicate septa. Scale bars = 10 μm. **E**, Plot of the mean percent branching for the protein treatments. The two groups identified by Tukey's "Honestly Significantly Different" test are designated by black or white bars ($R^2 = 1.0$, $P < 0.0001$). aRIP = papain-activated kernel RIP1. pRIP = proRIP1; LA = lactalbumin; MG = myoglobin. Error bars = standard error from 25 replicates.

BSA and MOD1mut protein treatments, however, did not differ from each other in their effects on *A. nidulans*.

RIP1 treatment of *A. flavus* conidia increased hyphal branching.

To determine whether RIP1 contributes to fungal resistance of maize seed, we examined its effect on the maize pathogen *A. flavus*, which is not an aggressive maize pathogen but has a major economic impact because of its production of aflatoxin. Figure 3 shows the result of treating conidia with either BSA (Fig. 3A and C) or purified maize proRIP1 that had been proteolytically converted to active RIP1 by limited proteolysis with papain (Fig. 3B and D). The typical phenotype of *A. flavus* 18 h after the addition of BSA was the production of a single germ tube with one or two septa (Fig. 3A and C, respectively). In contrast to conidia treated with BSA, conidia treated with active RIP1 often produced a branching phenotype that showed either one germ tube with two hyphal tips (Fig. 3D) or two germ tubes (Fig. 3B).

The amount of branching in response to active RIP1, proRIP1, or control proteins was quantified (Fig. 3E). A Tukey's "Honestly Significantly Different" (HSD) test was performed to determine whether treatments had statistically different amounts of branching. No significant difference was observed in the amount of branching in samples treated with control proteins with no known ribosome-inactivating activity (BSA, lactalbumin, and myoglobin) or the inactive proRIP1. Therefore, maize proRIP1 does not induce the branching phenotype. The amounts of branching observed after treatment with active RIP1 (proteolytically activated maize kernel RIP1 or MOD1), however, were significantly greater than those seen after treatment with the control proteins but were not different from each other. Thus, we can deduce that the differences between the native and recombinant proteins do not affect the observed phenotype. In a separate experiment, we tested MOD1mut and saw no increase in branching, above that produced by control proteins (data not shown).

RIP1 altered hyphal growth in *A. flavus*.

Because the branched phenotype was not limited strictly to those conidia that had been treated with RIP1 (Fig. 3E), we performed a more stringent comparison with the selective stain Congo red. Congo red binds to the chitin in fungal cell walls (Kisko et al. 1997; Matsuoka et al. 1995; Wall and Solomon 1999; Yoshioka et al. 1996). Areas with active hyphal tip growth have little chitin deposition and thus have reduced Congo red staining. When cell growth is inhibited, Congo red staining increases at the nongrowing tip. Because an increase in staining is reflective of chitin deposition (and no further growth) at the tip, Congo red can determine active hyphal tip growth (Matsuoka et al. 1995). Moreover, Congo red staining can be used in living cells and thus provides an advantage over other techniques that require cell fixation.

Germ tubes from conidia in samples treated with active RIP1 showed distinct variations in Congo red staining patterns with one hyphal tip in a state of active growth (Fig. 4A) and the other having chitin depositions (Fig. 4D) that indicate that the tip was not actively growing. Figures 4B and E show differential interference contrast images to confirm that sections were in focus. The Congo red staining patterns can clearly be seen in individual confocal sections (Fig. 4A and D) but are

obscured in an extended focus of the confocal stack (Fig. 4C) as a result of the conical nature of the hyphal tip.

In fungi treated with control proteins, we noticed that tips of branched hyphae exhibited the reduced Congo red staining that is indicative of growth (data not shown). To obtain a quantitative comparison of growth from conidia treated with MOD1 or BSA, we counted hyphal tips that were stained with Congo red. The results of this assay are summarized in Table 1. Branched hyphae that showed staining at only one of the hyphal tips accounted for 94% of the MOD1 samples but only 3% of the BSA samples. The rest of the branched hyphae (97%) in the BSA samples exhibited growth at multiple hyphal tips. In those fungi that had no branching, MOD1 and BSA treatments also had a differential effect, with 32% of the MOD1 samples being stained at the tip and thus having arrested growth compared with 9% of the BSA samples being arrested.

Comparison of *A. nidulans* and *A. flavus* growth inhibition by RIP1.

Deviation from the normal developmental patterns was not observed until several hours after treatment of *A. nidulans* or *A. flavus* with active forms of RIP1. To compare the developmental stage(s) when an effect of RIP1 treatment could be observed, we examined growth of *A. nidulans* and *A. flavus* over time. Preliminary growth experiments in the absence of added RIP1 protein showed that *A. nidulans* generally initiated germination 6 h after treatment and reached the postdivisional growth stage with one septum after 12 h. *A. flavus*, however, usually initiated germination within 8 h and reached the postdivisional growth stage with one septum after 20 h (data not shown). Because of the temporal differences in development of the two fungi, we subdivided the developmental processes into four morphologically distinct stages, schematically shown in Figure 5A. In stage 1, the conidium had not germinated. In stage 2, the conidium had germinated or swelled but had not produced a hypha. In stage 3, the conidium had germinated and produced a hypha shorter than four lengths of the conidium but lacked septa. In stage 4, the conidium had germinated and had either a hypha longer than four lengths of the conidium but no septum or a hypha of any length with a single septum.

Replicate slides were analyzed by counting conidia in each developmental stage at various times after treatment, as noted in Figure 5. For hyphae at stages 3 and 4, the amount of branching also was monitored. Results among stages and between the two species were compared by normalizing the RIP1 treatments relative to BSA controls, which remained fairly constant (see below). When conidia were observed after RIP1 treatment, little difference was seen among stages 1 through 3. At stage 4, however, *A. nidulans* showed a striking decrease and *A. flavus* showed a slight increase in the number of conidia (Fig. 5B). When we looked at branching, we found that *A. flavus* had a large amount at stage 4 but little in earlier stages. In the case of *A. nidulans*, we saw little branching in any of the stages (Fig. 5C).

DISCUSSION

We used a microculture assay to visualize changes in fungal appearance in response to protein treatments and found that

the growth and morphology of *A. nidulans* and *A. flavus* were altered after treatment with active forms of maize RIP1. The most striking effect was the lysis of hyphae observed with *A. nidulans* (Figs. 1 and 5B). The initial numbers of conidia in active RIP1 and control protein treatments were equivalent and remained so until the germinated conidia underwent the transition from pre- to postdivisional growth. Delayed hyphal lysis has precedence in fungal systems. For example, the *A. nidulans* developmental mutants *flbA* and *fadA*^{G42R} undergo autolysis in the middle of colonies as the colonies mature (Adams et al. 1998; Weiser et al. 1994; Yu et al. 1996). The exact pathway leading to this autolysis has not been completely defined. It has been shown, however, to involve sig-

Fig. 4. Congo red staining of *Aspergillus flavus* conidium treated with ribosome-inactivating protein. Conidia were treated with 0.5 mg per ml of MOD1 and cultured for 18 h prior to being stained with Congo red and examined by confocal microscopy. **A**, Congo red staining for an enlarged image of a single section showing conidium and hyphal tip in the plane of focus. **B**, Differential interference contrast (DIC) image of the conidium and hyphal tip shown in **A**. **C**, Extended focus of the 32-section confocal stack showing conidium and germ tubes. **D**, Congo red staining for an enlarged image of a single section with the other hyphal tip in focus. **E**, DIC image of the hyphal tip shown in **D**. White boxes denote areas enlarged for **A**, **B**, **D**, and **E**. Scale bars = 10 μ m.

naling through a heterotrimeric G-protein (Adams et al. 1998). The autolysis of *flbA* and *fadA*^{G42R} mutants occurs later in fungal development than the lysis of germlings we saw with RIP1 treatment, although developmental regulation appears to be involved in both cases.

In contrast to the lytic effect observed in *A. nidulans*, the effect of treating *A. flavus* conidia with active RIP1 did not lead to a decrease in the total number of conidia; instead it produced a subtle, yet reproducible, branching phenotype. Although branching was seen occasionally after treatment of conidia with proteins lacking ribosome-inactivating activity, treatment with RIP1 increased the frequency at which the branching phenotype appeared. Moreover, only the branching phenotype associated with RIP1 treatment also was associated with an inhibition of tip growth. Congo red staining, as a measure of active growth, revealed tip growth in multiple hyphal branches in the control treatments but active growth of only one hyphal tip from conidia treated with RIP1. Thus, RIP1 appears to inhibit hyphal elongation but does not necessarily prevent the initiation of new hyphal tips.

Interestingly, the effect of RIP1 on *A. nidulans* and *A. flavus* was dependent on the developmental stage of the fungus. Even though RIP1 was present at the beginning of the assays for both fungi, it had no effect on germination or initiation of hyphal growth. In fact, no phenotypic effects of RIP1 were observed until the fungi began the transition from pre- to postdivisional growth. These observations suggest that either the fungal cell was not susceptible to RIP1 before this transition or that the cellular effects of RIP1 did not lead to an observable phenotype until this stage.

The mechanism(s) involved in the toxicity of RIP1 to fungi is unknown. Our data from the enzymatically inactive MOD1 mutant treatment argue that the effect of RIP1 on *A. nidulans* and *A. flavus* requires the catalytic ribosome-inactivating activity of the protein. Because the responses of these two fungi differed, however, it is unclear if the pathway leading from the initial catalytic activity to the resulting phenotype is the same in both species. The observation that both phenotypes first appeared at the same developmental stage is consistent with both fungi having a similar response pathway to RIP1 treatment.

Table 1. Hyphal tip growth determination on the basis of Congo Red staining after treatment of *Aspergillus flavus* conidia with MOD1 or bovine serum albumin (BSA)^a

Type of growth	Treatment	
	MOD1	BSA
Branched phenotype		
One growing tip per conidium	67 (94%)	1 (3%)
Multiple growing tips per conidium	4 (6%)	30 (97%)
Unbranched phenotype		
No growth per conidium	73 (32%)	26 (9%)
One growing tip–conidium	158 (68%)	249 (91%)
Total	302	306

^a Conidia were treated with 0.5 mg per ml of either MOD1 or the control protein BSA. After 18 h incubation, conidia were stained with Congo red and examined by confocal microscopy. Each conidium was examined for Congo red staining of its hyphal tip(s) and classified as branched or unbranched. Within each of these groups, conidia were examined for growth at one or more hyphal tips per conidium. Growth at the hyphal tip was defined as a lack of Congo red staining and no growth at the tip was defined as the presence of Congo red staining.

RIP1 is a relatively abundant protein in mature maize kernels where it accumulates to approximately 1 mg per gram of tissue (Dowd et al. 1998). Because RIP1 induced lysis in *A. nidulans* and branching in *A. flavus* at concentrations of less than 1 mg per ml, our bioassay should represent levels of RIP1 that fungi might encounter in vivo. In kernels, however, it is the zymogen proRIP1 form that accumulates during seed maturation and dormancy prior to activation during germination by endogenous proteases (Bass et al. 1992; Walsh et al. 1991). In our assay, proRIP1 did not show any antifungal activity against *A. flavus*. These data suggest that the protein must be activated to have antifungal activity but do not rule out activation occurring in a number of ways in vivo. For example, *A. flavus* has been shown to lyse and degrade cells at the fungal invasion front (Smart et al. 1990), presumably by the action of proteases and other degradative enzymes secreted by the fungus. Proteases stored intracellularly might also be released from plant cells that were lysed by the invading fungus.

Indirect evidence for the role of RIP1 in plant defense has been obtained from transgenic proRIP1 tobacco plants that showed increased resistance to *Rhizoctonia solani* (Maddaloni et al. 1997). Insect feeding studies showed that proRIP1 and active RIP1 have insecticidal activity toward certain groups of insects (Dowd et al. 1998). Within the seed itself, *A. flavus* colonizes germ tissue in preference to endosperm tissue, where high levels of proRIP1 accumulate (Jones et al. 1980; Marsh and Payne 1984; Payne et al. 1988; Smart et al. 1990). This apparent preference of the fungus for tissues that lack proRIP1 also is evident with *opaque-2* maize mutants that have very low levels of proRIP1 (Soave et al. 1981). Kernels from *opaque-2* mutants are more susceptible to ear-rotting fungi than those from corresponding normal lines (Loesch et al. 1976). Taken together with our evidence that RIP1 has antifungal activity, these observations provide support for a role for maize RIP1 in defending the endosperm from fungal invasion.

The phenotypic variation in the two closely related fungi after RIP1 treatment appears to reflect distinct responses. The nonpathogen *A. nidulans* has a dramatic lytic response to RIP1 treatment compared with the branching phenotype of the maize pathogen *A. flavus*. The branching phenotype of *A. flavus* was not seen in *A. nidulans* nor was the lytic phenotype observed in *A. flavus*. Whether or not the differential responses of these two fungi could affect their pathogenicity remains unknown. An alteration in growth patterns, as seen with *A. flavus*, would allow continued growth on the plant, whereas the cell death observed with *A. nidulans* would abolish growth on the plant. Regardless of the significance of the different responses to pathogenicity, understanding how RIP1 induces the observed phenotypes and defining components involved in producing these phenotypes could provide insight into fungal pathogenicity as well as allow better use of RIP1 in plant-defense strategies.

MATERIALS AND METHODS

Fungal strains and microculture.

A. nidulans strain A51 (biA1 wA3) was grown for 2 days at 37°C on *A. nidulans* complete medium (Barratt et al. 1965) supplemented with 0.82 µM biotin. Conidia were harvested in

0.05% (vol/vol) Triton X-100 and filtered through a 50- μ m screen to remove hyphal debris. The conidia were mixed with either RIP1 or control proteins to a final concentration of 2.5×10^5 spores and 1.0 mg of protein per ml. This conidial mixture was incubated on ice for 5 to 10 min before transfer of 1 μ l (250 conidia, 1 μ g of protein) onto a sterile microscope slide covered with an 0.5-mm layer of CM (*A. nidulans* complete medium, 0.35 mM CaCl₂, 0.10 mM MgSO₄, 0.5% [wt/vol] Phytagel [Sigma, St. Louis, MO, U.S.A.] supplemented with 0.82 μ M biotin. The inoculated slides were placed in a 100 $\times$ 100 $\times$ 15 mm petri dish that was covered with a damp paper towel and placed in a closed plastic bag. The slides were incubated at 28°C for 18 h. For imaging, the slides were covered with a coverslip and examined with an

Axiophot workstation (Carl Zeiss Inc., Thornwood, NY, U.S.A.) with 5 $\times$ objective. Images were captured by a Chalnicon camera (Hamamatsu Corp., Bridgewater, NJ, U.S.A.) and an Image 1 system (Universal Imaging, West Chester, PA, U.S.A.).

A. flavus strain 656-2 (FGSC#A1010, w leu-7 pyrG aflR) was cultured similarly to *A. nidulans*. The fungus was grown for 2 days at 37°C on potato dextrose agar (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 10 mM uracil. The plates were transferred to room temperature for 2 days to induce conidiation. Conidia were harvested with 0.05% (vol/vol) Triton X-100, diluted with dH₂O to a conidial concentration of 5×10^5 spores per ml, and mixed with either RIP1 or control proteins to give a final concentration of $2.5 \times$

Fig. 5. Time course of the effect of ribosome-inactivating protein (RIP) on *Aspergillus nidulans* and *Aspergillus flavus* development. Conidia were treated with 1.0 mg per ml of either MOD1 or the control protein bovine serum albumin (BSA), cultured for 7, 10, or 12 h for *A. nidulans* and 10, 15, or 20 h for *A. flavus*, and examined microscopically. Development was separated arbitrarily into five stages for quantification during the time course. **A**, Schematic diagram describing the five developmental stages. Nuclei represented by ●. Gray shading represent branching phenotypes observed with active RIP1 treatment. **B**, Comparison of the effect of MOD1 treatment on the total number of conidia in each developmental stage. **C**, Branching phenotype production in each developmental stage. * = values normalized against BSA controls, as described in text. Error bars = standard error from two replicates. N/A = samples where no branching phenotype was observed for controls or treatments.

10⁵ spores and 0.5 mg of protein per ml. The conidia mixture was incubated on ice for 5 to 10 min and deposited as a 1 µl (250 conidia, 0.5 µg of protein) drop onto a microscope slide coated with a 0.5-mm layer of Phytigel PDB (potato dextrose broth (Difco Laboratories), 0.68 mM CaCl₂, 0.2 mM MgSO₄, 0.5% [wt/vol] Phytigel) supplemented with 10 mM uracil.

Construction of MOD1 and MOD1mut.

MOD1 was created as a deletion construct of the proRIP1 clone pZmcRIP3 (Bass et al. 1992; Krawetz and Boston 2000). The MOD1 construct encodes a single polypeptide chain with RIP enzymatic activity equivalent to that of the proteolytically activated maize protein (Hey et al. 1995; Krawetz and Boston 2000). The final product contained DNA corresponding to the original proRIP1 clone (GenBank accession no. M83926) from nucleotides 36 to 505 and 587 to 949 and included an ATG and novel *Xho*I site. MOD1mut was created with site-directed mutagenesis by the Altered Sites II In Vitro Mutagenesis System (Promega Corp., Madison, WI, U.S.A.). The coding region of MOD1 was subcloned into the *Bam*HI–*Pst*I site of the pALTER-1 vector (Promega Corp.). For mutagenesis, thymidine 651 of MOD1 was changed to a cytosine with the mutagenic oligonucleotide 5′-GACAGGAT CcCCAAGGCGGC-3′ (Integrated DNA Technologies Inc., Coralville, IA, U.S.A.) to give pAM57mut. The nucleotide target of mutagenesis is shown above in lowercase. A 760-bp *Bcl*I fragment containing the mutation was removed from pAM57mut and inserted into the *Bcl*I sites of MOD1 to create MOD1mut. Mutation at the equivalent position in other ribosome-inactivating proteins previously was found to abolish their ribosome-inactivating activities in translational inhibition assays (Katzin et al. 1991). Likewise, recombinant MOD1mut lacked activity in translational inhibition assays (data not shown).

Protein expression, purification, and ribosome-inactivating activity.

ProRIP1 was purified from immature maize kernels, as described by Bass et al. (1992). ProRIP1 was activated by treatment with papain, a protease that removes the NH₂-terminal, COOH-terminal, and internal acidic domains of proRIP1 to yield the 16- and 8-kDa peptides that form the enzymatically active RIP1 (Walsh et al. 1991). Digestion buffer (10 mM cysteine-HCl; 10 mM NaPO₄, pH 7.0; and 5 mM EDTA) was added to give a final concentration of 2 mg of proRIP1 per ml. The protein sample was mixed with a papain agarose bead slurry (Pierce, Rockford, IL, U.S.A.) with a 10:1 volume ratio of protein to beads. Proteolysis was carried out at 37°C on a bidirectional rotator for 3 to 4 h. The reaction was stopped by subjecting the mixture to centrifugation and moving the supernatant containing the active RIP1 to a new tube.

Expression of the recombinant MOD1 and MOD1mut proteins was carried out by the Xpress Protein Expression System (Invitrogen Corp., Carlsbad, CA, U.S.A.). The MOD1 and MOD1mut DNA constructs were cloned into pRSET C and used to transform BL21(DE3)pLysS cells. Optimal protein production occurred when cells grown at 37°C in RICH medium (20 g of Bacto tryptone, 10 g of Bacto yeast extract, and 10 g of NaCl per liter) containing 75 µg of ampicillin and 35 µg of chloramphenicol per ml to an optical density at 600 nm of 0.4 to 0.6 were treated with 1 mM isopropyl-β-D-thiogalactopyranoside for 2.5 h.

Purification and characterization of MOD1 and MOD1mut proteins with nickel-agarose resin (Invitrogen Corp.) was performed as described in Krawetz and Boston (2000), except for the final dialysis, which occurred in 5 mM NaPO₄. Protein concentration was determined with the BCA Protein Assay Kit (Pierce).

When required, proRIP1 and MOD1 were concentrated with a Centricon-10 concentrator (Amicon Inc., Beverly, MA, U.S.A.). MOD1mut was concentrated with dialysis against polyethylene glycol. BSA, lactalbumin, and myoglobin were purchased in powder form (Sigma) and dissolved in 5 mM NaPO₄ to the desired concentrations.

Cell-free translational inhibition assays (Bass et al. 1992) were performed on proRIP1, proteolytically activated RIP1, MOD1, MOD1mut, and BSA. Only proteolytically activated RIP1 and MOD1 had detectable ribosome-inactivating activity (data not shown).

Quantification of *A. nidulans* growth in microculture.

A. nidulans conidia were exposed to different levels of MOD1 and MOD1mut protein. The conidia were microcultured as described above, except that the desired amount of MOD1 or MOD1mut protein was mixed with an appropriate amount of BSA so that the final concentration of protein in the assay remained at 1.0 mg per ml. After 18 h of incubation, the conidia were fixed at room temperature for 2 h with Histochoice tissue fixative (Amresco, Solon, OH, U.S.A.). The conidia were then subjected to three 30-min washes with Tris-buffered saline, pH 7.5, at 25°C. The conidia were mounted with polyvinylalcohol–1,4-diazabicyclo[2.2.2]octane (PVA–DABCO) mounting medium (Banker and Goslin 1988) and placed at 4°C overnight. Conidia were imaged on an Axiovert 100 TV workstation (Carl Zeiss Inc.) with 5× objective. Images were collected with a 2.5-MHz cooled CCD camera (Princeton Instruments, Evry, France). Five nonoverlapping images of each slide were collected in a cross formation. Hyphal coverage in each image was measured by integrated morphometry analysis. Briefly, densitometry was used to define areas with hyphal growth. The area covered by hyphal growth was determined by integrated morphometry analysis (Universal Imaging). The hyphal coverage for the five images from each slide was summed to give the hyphal coverage for the sample. The effect of protein treatment and concentration on hyphal coverage for three experimental replicates was analyzed with ANCOVA Model JMP 3.2 software (SAS Institute, Raleigh, NC, U.S.A.) with a dummy-variable approach. The model was forced through a y-intercept of 88.07, the mean of all 0.0 protein treatments.

Quantification of branching phenotype.

The effect of protein treatment on *A. flavus* hyphal branching was determined. After microculture for 18 h, the slides (with approximately 250 conidia per slide) were covered with coverslips and examined with an AxioPhot workstation with 40× objective. All cells were scored for branching phenotype. Branching was defined as a conidium with multiple germ tubes or multiple hyphal tips on a single germ tube. Twenty-five replicates of each protein treatment were analyzed in a split-plot, balanced incomplete block design. The number of branched and unbranched hyphae were counted for the largest square area covered by hyphal growth. A Tukey's HSD test

was performed to determine whether the mean percent of branching between protein treatments was significantly different. This statistical analysis used the arcsine of the square root of the mean percent branching to stabilize the error between treatments.

Congo red staining.

Congo red staining was used on *A. flavus* conidia to allow visualization of chitin deposition at hyphal tips. After treatment with MOD1 or BSA followed by 18 h of growth in microculture, conidia were treated with 1 mM Congo red and incubated at 28°C for 30 min. The conidia were imaged with a confocal laser scanning microscope (Leica, Wetzlar, Germany) with 63× objective. The excitation wavelength was 500 nm and emission was measured at 615 nm.

Conidia in two fields of view on each of 15 replicate slides were examined for hyphal-tip growth. Growth at the tip was determined by lack of Congo red staining of the hyphal tip in the confocal section with the tip in focus. Tips with arrested growth had Congo red staining across the tip when the tip was in focus.

Developmental time course for *A. flavus* and *A. nidulans*.

Conidia were microcultured as described above, except that a final concentration of 1.25×10^5 conidia per ml was used so that approximately 125 conidia and 1 µg of protein were placed on each slide. Replicate slides were covered with a coverslip and examined microscopically with an Axiophot workstation with 63× objective at 7, 10, or 12 h for *A. nidulans* and 10, 15, or 20 h for *A. flavus*. The number of conidia and their branching phenotype in each developmental stage (defined in Fig. 5A) were determined at each time point and summed to give a total value for each developmental stage. For direct comparison of the total number of *A. flavus* and *A. nidulans* conidia, the values obtained from each MOD1 treatment replicate were divided by the mean value of the BSA treatment. The mean and standard error of the total number of conidia in the BSA treatments for *A. flavus* were: stage 1, 13.5 ± 3.5 ; stage 2, 160.5 ± 0.5 ; stage 3, 122.5 ± 13.5 ; stage 4, 87.5 ± 4.5 . For *A. nidulans*: stage 1, 53.5 ± 7.5 ; stage 2, 114.5 ± 4.5 ; stage 3, 97 ± 5 ; stage 4, 107 ± 5 . To compare directly the amount of branching observed between the two fungal species, the mean branching in BSA treatments was subtracted from each MOD1 treatment replicate. The mean and standard errors for branching in the BSA treatments for *A. flavus* were: stage 3, 0.5 ± 0.5 ; stage 4, 6 ± 1 . For *A. nidulans*: stage 3, 0.5 ± 0.5 ; stage 4, 3.5 ± 0.5 .

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