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Alterations in gene expression in sorghum induced by the host-specific toxin from *Periconia circinata*

(pathogenesis/phytotoxin/milo disease/protein synthesis)

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ABSTRACT **Susceptibility of sorghum to the fungal pathogen *Periconia circinata* and sensitivity to its host-specific toxin are determined by the semidominant allele at the *pc* locus. Pretreatment of susceptible seedlings with cycloheximide or cordycepin for 4 hr before treatment with the toxin protected the seedlings against toxin-induced loss of electrolytes and prevented development of disease symptoms. *In vivo* incorporation of [^{3}H]leucine into protein was inhibited 91% and 47% by cycloheximide and cordycepin, respectively, but was not affected by the toxin. Gel electrophoresis and fluorography of *in vivo*-labeled proteins extracted from nontreated and toxin-treated root tips of near-isogenic susceptible and resistant lines revealed a selective increase in radioactivity of a protein band at M_r 16,000 only in preparations from toxin-treated susceptible root tips. Two-dimensional gel electrophoresis separated the M_r 16,000 band into four proteins and confirmed the increased rate of synthesis. Products of *in vitro* translation were substantially enriched with the four M_r 16,000 proteins when total RNA from toxin-treated susceptible root tips was used in a cell-free protein-synthesizing system. Because the proteins that increase are common to both susceptible and resistant genotypes, the toxin apparently interferes with a regulatory function, perhaps a function of the *pc* locus, and thereby alters gene expression in the susceptible genotype. The data suggest but do not establish that phytotoxicity results from the increased rate of synthesis of the specific proteins.**

Many plant pathogenic fungi produce metabolites that are directly involved in pathogenesis and reproduce the characteristic visible and biochemical symptoms of disease only in host species or genotypes that are susceptible to the fungus (1). These phytotoxins comprise a class of compounds known as host-specific toxins (2) or selective pathotoxins (3). Isolates or mutants of the species that are unable to produce the toxins are nonpathogenic (4, 5); thus, the term "pathogenicity factors" also has been suggested (6).

Pathogenic isolates of *Periconia circinata* (Mangin) Sacc. cause milo disease, a root and crown rot of sorghum [*Sorghum bicolor* (L.) Moench] (7), and produce two similar, acidic, low molecular weight toxins (designated PC toxins 1 and 2) with host-selective activity (8). Susceptibility to the pathogen and sensitivity to PC toxin are both determined by the semidominant allele at the same genetic locus, designated *pc* (9). Genotypes homozygous recessive at the *pc* locus are insensitive to the toxin at concentrations at least 2,000-fold greater than those affecting susceptible genotypes (8). Thus, the essential molecular events in milo disease appear to involve the interaction between a single metabolic product of the pathogen and a single genetic locus or gene product of the host.

Although many biochemical and physiological responses to host-specific toxins have been studied, the precise mechanism or site of action has not been determined (6). One of the most common effects of the toxins is an alteration in membrane permeability (10). However, attempts to establish that the cell membrane is the primary site of action or that interaction of the toxin with a membrane-bound receptor results in development of disease symptoms have not been convincing (6, 11).

Previously, we determined that symptom development in sorghum seedlings treated with PC toxin was not a direct consequence of electrolyte loss (11). Under certain conditions toxin-treated seedlings developed disease symptoms without a significant loss of electrolytes, and, conversely, a nonselective loss of electrolytes was induced by fusaric acid or citrinin without symptom development. Furthermore, electron microscopic examination of toxin-treated roots revealed no detectable ultrastructural alterations in the plasma membrane (12). These observations and the results of studies on the effects of the host-specific toxin produced by *Helminthosporium victoriae* (HV) on oats (13) led to the conclusion that the determinative or lethal site of toxin action is within the cell (11, 13).

Sensitivity of oat (14) and sorghum (15) tissues to the HV and PC toxins, respectively, can be abolished by pretreatment of the tissues with cycloheximide for 12–18 hr before treatment with toxin. Gardner and co-workers (14, 15) concluded, therefore, that the toxin receptor or sensitive site is a protein with a rapid turnover rate. However, other interpretations are equally tenable. For example, PC toxin may induce or regulate the synthesis of a protein (or proteins) that is required for expression of toxicity rather than for toxin recognition. The objective of this study was to determine whether PC toxin selectively affects transcriptional and translational activities in sorghum seedlings susceptible to the pathogen.

MATERIALS AND METHODS

Host and Pathogen. Seeds of near-isogenic lines (9) of sorghum cv. Colby, susceptible or resistant to *P. circinata*, were germinated in moist paper toweling for 2 days at 25°C. Seedlings with roots 2–3 cm long were transferred to 25-ml beakers containing nutrient solution (16) and grown in continuous light at 25°C for 5 days.

A single-spore isolate of *P. circinata* was obtained from an infected root of susceptible sorghum and maintained on potato dextrose agar (17). Culture medium and conditions for production of toxin were described previously (8).

Toxin Purification and Bioassay. The host-specific toxin was purified (8) by deproteinization of culture filtrates with meth-

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Abbreviations: PC and HV toxins, toxins from *Periconia circinata* and *Helminthosporium victoriae*, respectively.

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anol, adsorption of toxin to activated charcoal, chromatography on QAE-Sephadex, preparative thin-layer electrophoresis, and thin-layer chromatography. Toxin 1 was subjected to preparative high-performance liquid chromatography on a Beckman Instruments model 322 liquid chromatograph. The toxin (fraction 1a) was separated from a chemically similar nontoxic compound (fraction 1b) on a reversed-phase, C_8 column (4.6 × 250 mm; Ultrasphere-octyl; Beckman) by isocratic elution with 14% acetonitrile/0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The solvents were removed by repeated evaporation *in vacuo* at 30°C, and the toxin was dissolved and stored in deionized water. The final product satisfied all analytical and biological criteria of purity defined earlier (8).

Activity of the toxin preparation was determined by the root growth inhibition bioassay and by the effect on seedling growth (18). Toxin-induced loss of electrolytes was determined as described (11).

Leucine Incorporation. Terminal 1-cm segments were excised from roots of 3-day-old seedlings and aerated for 1–2 hr in nutrient solution containing 1% (wt/vol) sucrose and 200 μg of chloramphenicol per ml (incubation medium). Duplicate sets of 10 root tips (*ca.* 30 mg fresh weight) were incubated with aeration in 1 ml of incubation medium without or with PC toxin (500 ng/ml) for various periods, and [^{3}H]leucine (110 or 122 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) was added to give 5 μCi/ml. After a 1- or 2-hr incubation period, the root tips were homogenized in a conical, glass homogenizer with 1.5 ml of cold (4°C) buffer [50 mM Tris·HCl, pH 7.0/700 mM sucrose/2 mM dithiothreitol/100 mM KCl/5 mM Na_2EDTA] and 1.5 ml of 80% (wt/vol) aqueous phenol. The homogenate was centrifuged to separate the phases, and 5 vol of methanol containing 100 mM ammonium acetate was added to 0.75 ml of the phenol phase. The proteins were precipitated overnight at −20°C and collected by filtration on Whatman GF/C filters. The filters were washed with 5–10 ml of cold methanol, air dried, and placed in scintillation vials with ACS (Amersham). Radioactivity was determined by liquid scintillation spectroscopy.

Electrophoretic Analysis of Proteins. Root tips (25 per 5-ml vial; 70–80 mg fresh weight) were incubated in 1 ml of incubation medium with or without toxin for various times and then allowed to incorporate [^{3}H]leucine (50 μCi/ml) for 1.5–3 hr. Proteins were extracted from root tips as described above except that 2 ml each of homogenization buffer and 80% phenol were used. The precipitate was collected by centrifugation, washed with 2 ml of cold methanol, and dried *in vacuo*. Proteins were solubilized in $NaDodSO_4$ sample buffer [62.5 mM Tris·HCl, pH 6.8/2.3% (wt/vol) $NaDodSO_4$/5% (vol/vol) 2-mercaptoethanol] (19). Samples containing equal amounts of radioactivity (2 × 10^5 cpm) were boiled for 5 min, adjusted to *ca.* 15% sucrose, and subjected to electrophoresis on 12% polyacrylamide gels in the buffer system of Laemmli (20). Proteins were stained with Coomassie brilliant blue R-250 and detected by fluorography with EN^{3}HANCE (New England Nuclear) according to the manufacturer's procedure. Dried gels were exposed to Kodak XAR-5 film (Eastman-Kodak) at −80°C for 3 days.

For two-dimensional electrophoresis, urea and ampholytes were added to the samples (6 × 10^5 cpm) to give 9.5 M and 2% (vol/vol), respectively. The proteins were subjected to isoelectric focusing (IEF) as described by O'Farrell (19) in a gradient of pH 4.4–7.0 in the first dimension. The IEF gels, nonequilibrated with $NaDodSO_4$, were run in the second dimension on $NaDodSO_4$ gels with a gradient of 10–16% (wt/vol) acrylamide (19).

Extraction of RNA. Approximately 85 root tips (*ca.* 250 mg fresh weight), incubated in the presence or absence of PC toxin for 6 hr, were ground to a fine powder in a mortar with liquid nitrogen. RNA was extracted from the powder by the modified procedure of Lizardi and Engelberg (21) as described by Sachs *et al.* (22), with ethanolic perchlorate reagent prepared as described by Wilcockson (23). The final precipitate was collected by centrifugation, dried, solubilized in water to give 21 A_{260} units/ml, and stored at −20°C.

***In Vitro* Translation.** Cell-free protein synthesis with a wheat germ S23 extract was performed as described by Larkins and Hurkman (24). Reaction mixtures (50 μl) contained 19 unlabeled amino acids, 20 μCi of [^{3}H]leucine, and 0.42 A_{260} units of total RNA from toxin-treated or nontreated root tips. After incubation at 27°C for 60 min, the reaction mixtures were treated with RNase and DNase. Proteins were precipitated overnight with acetone, solubilized in $NaDodSO_4$ sample buffer, and analyzed by gel electrophoresis as described above.

Translation assays with rabbit reticulocyte lysate (Bethesda Research Laboratories) were performed in a total volume of 30 μl containing 19 unlabeled amino acids, 15 μCi of [^{3}H]leucine, and 0.33 A_{260} units of total RNA. Mixtures were incubated at 30°C for 60 min, and proteins were precipitated with acetone and analyzed by gel electrophoresis.

RESULTS

Within 24–36 hr after exposure to 500 ng of PC toxin per ml, 5- to 7-day-old seedlings of the susceptible sorghum genotype developed milo disease symptoms—a gray-green discoloration of the leaves, rolling and wilting of the leaves, and death of the seedlings. However, treatment of seedlings with cycloheximide (5 μg/ml) or cordycepin (50 μg/ml) for 4 hr before toxin treatment protected seedlings against symptom development and substantially reduced the toxin-induced loss of electrolytes, one of the earliest detectable effects of toxin (Table 1). Longer periods of pretreatment (up to 18 hr) with the antibiotics were also effective. But pretreatment times of 2 hr or less, including treatment with toxin and antibiotics concomitantly, had no effect. If the protected seedlings were transferred to water or nutrient solution without antibiotics, symptoms developed but were delayed. These treatments with antibiotics and toxin did not induce electrolyte leakage or influence growth of seedlings of the resistant genotype during 3 to 5 days of observation.

The above results suggested that transcriptional and translational processes are involved in toxin-induced damage to susceptible sorghum. However, when root tips of susceptible

Table 1. Effect of pretreatment with cycloheximide or cordycepin on the PC toxin-induced loss of electrolytes from near-isogenic sorghum seedlings

		Conductivity, μS		Symptoms	
Pretreatment	Treatment	S	R	S	R
None	None	3.5	0.8	0	0
None	Toxin	42.7	0.2	3	0
Cyc	Cyc	5.8	6.0	0	0
Cyc	Cyc + toxin	7.2	6.3	0	0
Cor	Cor	1.4	1.7	0	0
Cor	Cor + toxin	8.9	2.0	0	0

Roots of seedlings susceptible (S) or resistant (R) to *P. circinata* were incubated for 4 hr in water, in cycloheximide (Cyc) at 5 μg/ml, or in cordycepin (Cor) at 50 μg/ml before PC toxin was added to a final concentration of 500 ng/ml. Conductivity of the solution bathing the roots was measured 10 hr after addition of toxin. Values are the means from two separate sets of seedlings. Disease symptoms were rated at 24 hr on a scale of 0 (no symptoms) to 4 (dead seedlings).

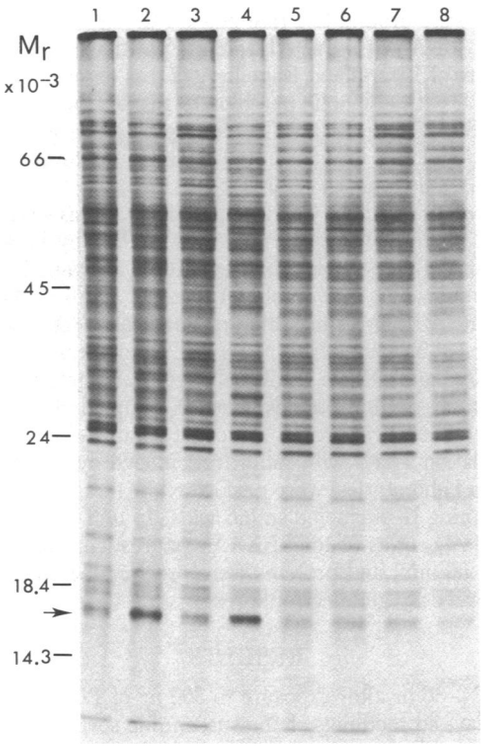

FIG. 1. Fluorograph of $NaDodSO_4$/polyacrylamide gel of proteins synthesized *in vivo* during a 1.5-hr pulse with [^{3}H]leucine. Root tips of the susceptible (lanes 1–4) or resistant (lanes 5–8) genotype were incubated in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of PC toxin for 3 hr (lanes 1, 2, 5, and 6) or 6 hr (lanes 3, 4, 7, and 8), and [^{3}H]leucine was added at 1.5 hr or at 4.5 hr, respectively. Arrow indicates the protein band at M_r 16,000 that increases in the toxin-treated susceptible root tips.

seedlings were treated with PC toxin for 2–8 hr, there was no detectable effect on incorporation of [^{3}H]leucine into protein. Root tips maintained their capacity to incorporate leucine into protein for at least 10 hr after excision. Further, PC toxin had no apparent effect on leucine uptake because the rate of incorporation was nearly constant during that time period and comparable to incorporation by nontreated root tips (data not shown). When root tips were incubated for 4 hr in cycloheximide or cordycepin before [^{3}H]leucine was added, the rate of incorporation into protein was inhibited 91% and 47%, respectively.

The potential effect of PC toxin on the synthesis of specific proteins was determined by electrophoretic analyses of proteins synthesized *in vivo* in the presence of [^{3}H]leucine. One-dimensional $NaDodSO_4$/polyacrylamide gels revealed no detectable differences in the proteins synthesized in root tips of non-toxin-treated susceptible, nontreated resistant, or toxin-treated resistant genotypes (Fig. 1). However, significant differences were evident in the proteins synthesized in toxin-treated susceptible root tips. The most obvious difference was a marked increase in the radioactivity associated with a band at M_r 16,000 (Fig. 1). Other, less pronounced differences resulting from toxin treatment, particularly with the longer treatment time, included a slight decrease in a protein band at M_r 76,000 (Fig. 1, lanes 2 and 4) and increases in proteins at M_r 43,000 (Fig. 1, lane 4) and at M_r 24,000–28,000 (Fig. 2*C*).

Two-dimensional gel electrophoresis resolved the M_r 16,000 protein band into four distinct polypeptides with the same molecular weight but with different isoelectric points between pH 5.8 and 6.3 (Fig. 2). No polypeptides were detected that were unique to the toxin-treated tissue.

The increased synthesis of specific proteins induced by toxin was prevented by treatment of the root tips with cordycepin prior to toxin treatment (Fig. 3, lanes 3 and 4). This result and the protection against toxin-induced symptoms by cordycepin

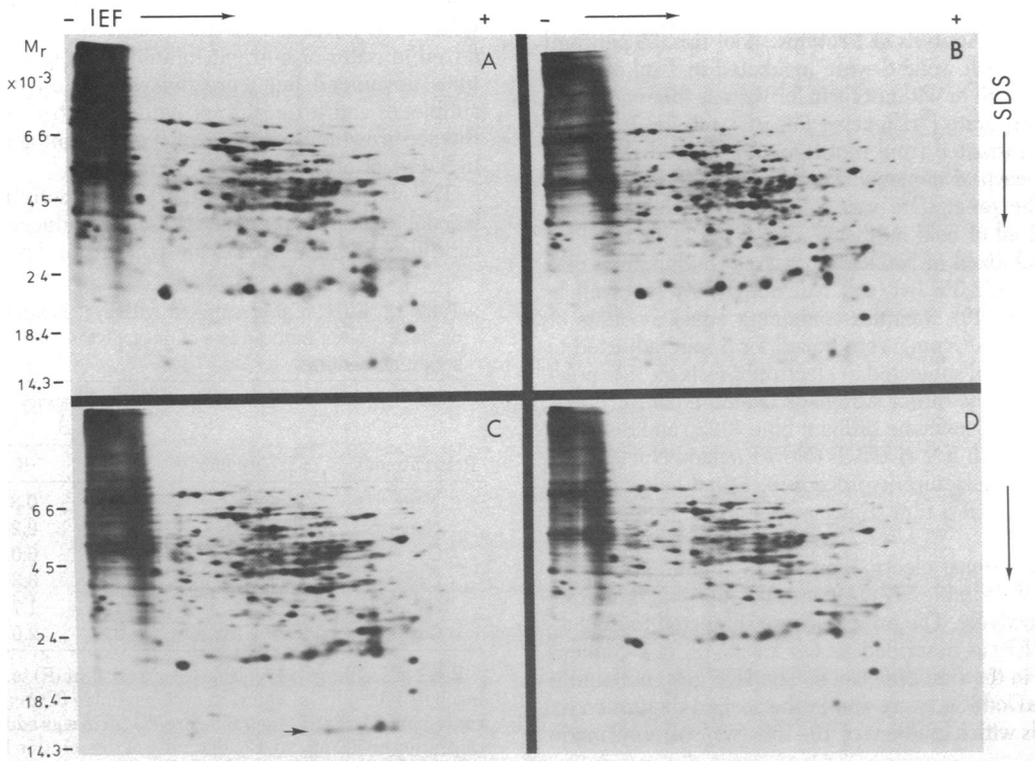

FIG. 2. Fluorographs of two-dimensional polyacrylamide gels of proteins synthesized *in vivo* by root tips of susceptible (*A* and *C*) or resistant (*B* and *D*) seedlings incubated in the absence (*A* and *B*) or presence (*C* and *D*) of PC toxin for 6 hr. The [^{3}H]leucine was added 4.5 hr after the beginning of toxin treatment. The arrow in *C* indicates proteins at M_r 16,000 that increase in the toxin-treated susceptible root tips.

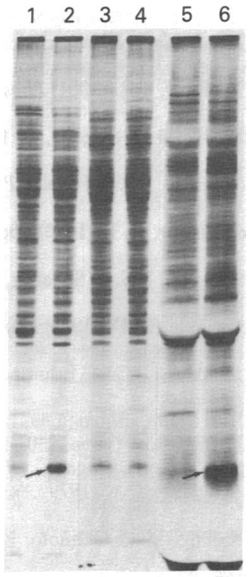

FIG. 3. Fluorographs of NaDodSO$_4$/polyacrylamide gels of proteins synthesized *in vivo* (lanes 1–4) by root tips of susceptible seedlings pretreated (lanes 3 and 4) or not pretreated (lanes 1 and 2) with cordycepin for 4 hr and incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of PC toxin for 6 hr and pulse labeled with [^{3}H]leucine during the last 2 hr; and fluorographs of gels of the *in vitro* translation products (lanes 5 and 6) of a cell-free protein-synthesizing system from rabbit reticulocyte lysate with RNA extracted from root tips of susceptible seedlings incubated for 6 hr in the absence (lane 5) or presence (lane 6) of PC toxin. Arrows indicate the protein band at M_r 16,000 that increases in both the *in vivo* and *in vitro* products.

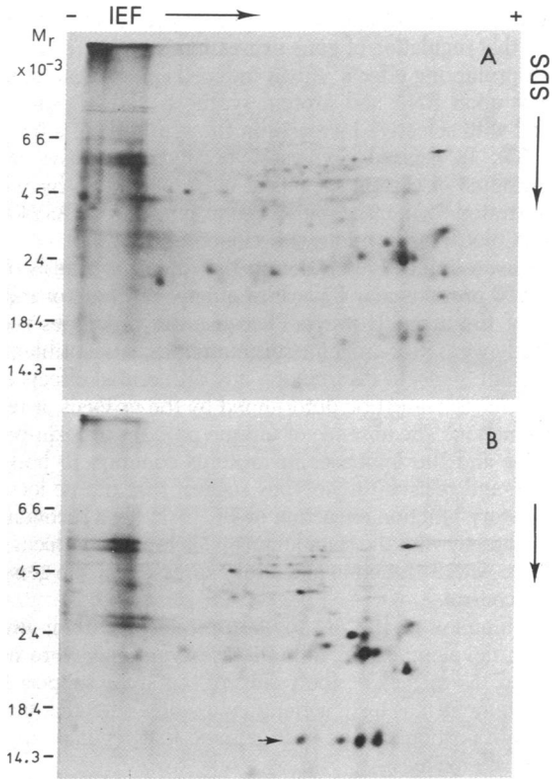

FIG. 4. Fluorographs of two-dimensional polyacrylamide gels of *in vitro* translation products of RNA from susceptible seedlings incubated for 6 hr in the absence (*A*) or presence (*B*) of PC toxin. *In vitro* translation was performed in a cell-free protein-synthesizing system from wheat germ.

suggested that PC toxin altered the rate of synthesis of selected RNAs. Thus, RNA extracted from root tips of nontreated or toxin-treated susceptible seedlings was translated in a cell-free protein-synthesizing system from wheat germ or rabbit reticulocyte lysate. Electrophoretic analyses of the *in vitro* translation products of RNA extracted from toxin-treated root tips indicated an increase in the M_r 16,000 proteins compared with those of RNA from nontreated root tips (Fig. 3, lanes 5 and 6). Two-dimensional electrophoretic analysis of the products confirmed the presence of four distinct polypeptides (Fig. 4) and suggested that post-translational modification of a single M_r 16,000 protein was not responsible for the four polypeptides. Increases in the proteins at M_r 24,000–28,000 were also apparent in the two-dimensional electrophoretograms of translation products.

DISCUSSION

Our results suggest that PC toxin alters gene expression or influences regulation of gene expression only in the sorghum genotype that is susceptible to *P. circinata*. Treatment with the toxin selectively increased the rate of synthesis of four polypeptides with M_r of 16,000 and, from results of *in vitro* translation, increased the amount of translatable RNAs encoding those proteins. Cordycepin, an inhibitor of RNA synthesis, reduced incorporation of leucine into protein and prevented the increased rate of synthesis of the M_r 16,000 proteins. In addition, pretreatment with cycloheximide or cordycepin protected seedlings against toxin-induced symptoms. These observations suggest that the effects of toxin on protein synthesis are responsible for milo disease symptoms. However, the inability to identify a function of the proteins precludes definitive conclusions regarding their role and essentiality in symptom development.

The increased radioactivity in certain proteins synthesized *in vivo* was apparently not a consequence of proteolytic activity, because *in vitro* translation products of RNA extracted from toxin-treated susceptible root tips were considerably enriched in those proteins. Moreover, if [^{3}H]leucine was incorporated into proteins and then chased with unlabeled leucine before treatment with PC toxin, an increase in radioactivity of the M_r 16,000 band was not detected (data not shown). Thus, PC toxin may act on the sorghum genome to influence patterns of protein synthesis.

Similarities between microbial toxins and plant hormones in altering plant growth responses, cellular metabolism, and membrane permeability and in inducing tissue proliferation have been described (25, 26). The effects of PC toxin on protein synthesis in sorghum are similar to those of gibberellic acid on barley aleurone cells and of auxins on elongating soybean hypocotyls. Treatment of barley aleurone layers with gibberellic acid induces the *de novo* synthesis (27) of α-amylase and other hydrolases (28) that are secreted from the aleurone cells into the endosperm. The poly(A)$^+$ RNA for α-amylase increases rapidly after gibberellic acid treatment and becomes the predominant translatable RNA (29). Cordycepin prevents the formation of α-amylase (30), suggesting that gibberellic acid acts at the level of transcription. Among other polypeptides whose *in vivo* synthesis may be regulated by gibberellic acid, the most notable, with respect to considerations of PC-toxin action, are those with M_rs of 16,000 and 24,000 (31). Interestingly, one of the early cytological effects of PC toxin is the disappearance of starch grains from plastids in root cap cells of susceptible sorghum (12), and one of the proteins that increases slightly in toxin-treated root tips has a M_r of 43,000 similar to the M_r of α-amylase from barley (32).

Recent studies on the mechanism of action of the auxins,

indoleacetic acid and 2,4-dichlorophenoxyacetic acid, likewise suggest that regulation of gene expression is associated with the growth-promoting effects. Auxin-induced cell elongation is dependent upon RNA and protein synthesis (33, 34) and is associated with selective increases in the synthesis of a few proteins (35). In excised soybean hypocotyls, increases in the concentration of certain species of messenger RNA have been demonstrated by *in vitro* translation of poly(A)$^+$ RNAs (36) and by RNA blot hybridization with cloned cDNAs (37).

The toxin-induced alteration in the rate of synthesis of the M_r 16,000 proteins may be a consequence of direct or indirect action of the toxin. However, because the genotypes used in this study were near-isogenic sorghum lines, susceptible (*PcPc*) or resistant (*pcpc*) to *P. circinata*, any differential effects of PC toxin ultimately must be determined by the *pc* locus or related to its function. The absence of unique proteins in toxin-treated root tips and the increases in proteins common to both susceptible and resistant genotypes suggest that the *pc* locus has a regulatory function. Reaction of PC toxin (or a secondary effector) directly with the semidominant allele at the *pc* locus could interfere with its function and alter expression of the genes under its control.

The function of the M_r 16,000 proteins and their involvement in development of milo disease symptoms were not established. Nevertheless, the results of this study support Daly's (26) statement that toxin action may involve selective interference with portions of the host genome that regulate synthesis of critical enzymes.

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