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One Enzyme Makes a Fungal Pathogen, But Not a Saprophyte, Virulent on a New Host Plant

W. SCHÄFER,* D. STRANEY,† L. CIUFFETTI, H. D. VAN ETTEN,‡ O. C. YODER

Certain genes of *Nectria haematococca*, a fungal pathogen of pea (*Pisum sativum*), encode pisatin demethylase (pda), a cytochrome P-450 monoxygenase that detoxifies the phytoalexin pisatin. Because pda is required by *N. haematococca* for pathogenicity on pea, pisatin helps defend pea against *N. haematococca*. The possibility that pisatin is a general defense factor—that is, that pda can confer pathogenicity to fungi not normally pathogenic on pea—was investigated. Genes encoding pda were transformed into and highly expressed in *Cochliobolus heterostrophus*, a fungal pathogen of maize but not of pea, and in *Aspergillus nidulans*, a saprophytic fungus, neither of which produces a significant amount of pda. Transformants contained at least as much pda as did wild-type *N. haematococca*. Recombinant *C. heterostrophus* was normally virulent on maize, but it also caused symptoms on pea, whereas recombinant *A. nidulans* did not affect pea. Thus, phytoalexins can function in nonspecific resistance of plants to microbes; saprophytes appear to lack genes for basic pathogenicity.

ONE HYPOTHESIS CONCERNING the cause of plant disease is that the successful pathogen can prevent or disarm plant defenses. Among the best known plant responses to a stress such as microbial attack is the synthesis of antimicrobial secondary metabolites called phytoalexins. Fungal detoxification of a plant's phytoalexins has been suggested as a general mechanism for circumventing phytoalexin-mediated plant defense (*1*).

The filamentous ascomycete *Nectria haematococca* Berk. and Br. mating population VI (anamorph: *Fusarium solani*) is a pathogen of pea and uses the detoxification strategy, since it can degrade the pea phytoalexin pisatin to a less toxic compound (*1*). This detoxifying activity is due to a substrate-inducible cytochrome P-450 monoxygenase called pisatin demethylase (pda). Data, based on analyses of progenies in which Pda is segregating, confirm that pda is necessary for pathogenicity (*1*). Moreover, when a cloned gene (*PDA-T9*) encoding pda (*2*) is transformed into a nonpathogenic Pda$^-$ strain of *N. haematococca*, the recombinant strain can attack pea (*3*). These results indicate that pda is required by *N. haematococca* for pathogenicity on pea and that pisatin itself is a plant defense factor.

We designed experiments to assess the role of pisatin in defense of pea against fungi not normally pathogenic to pea. Genes encoding pda (*2, 4–8*) were transformed (*7–10*) into *Cochliobolus heterostrophus*, a pathogen of maize but not pea, and *Aspergillus nidulans*, a saprophyte, by means of vectors (*4–8*) and strains (*11–14*) designed for this purpose. To achieve overexpression of pda, we chose transformants with high copy numbers of the transforming plasmid in their genomes (*15–17*). A *C. heterostrophus* transformant (strain C2-P) and an *A. nidulans* transformant (strain UCD1-P), which carried 18 and 12 copies of the transforming plasmid (*16, 18, 19*), respectively, were used for further experiments. Control *C. heterostrophus* transformant C2-V and *A. nidulans* transformant UCD1-V each contained comparable numbers of the respective vectors alone (*15–17*).

Rates of pisatin demethylation by transformants C2-P and UCD1-P were equal to or greater than those of the highly virulent, rapidly demethylating *N. haematococca* wild-type strain N77-2-3 (Figs. 1 and 2). Strains C2, C2-V, UCD1, and UCD1-V had background pda levels 0 to 7% of those of

Department of Plant Pathology, Cornell University, Ithaca, NY 14853.

*Present address: Institut fur Genbiologische Forschung Berlin GmbH, Ihnestrasse 63, 1000 Berlin 33, Federal Republic of Germany.
†Present address: Department of Botany, University of Maryland, College Park, MD 20742.
‡Present address: Departments of Plant Pathology and Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721.

induced strain N77-2-3. In strain N77-2-3, pda was inducible by pisatin as previously reported (*20*), but was constitutively produced in strains C2-P and UCD1-P. It appears that either the fragment (*4–8*) of *N. haematococca* DNA encoding pda lacks cis-acting sequences for pda regulation or that *C. heterostrophus* and *A. nidulans* lack trans-acting factors present in *N. haematococca*. Glucose repressed pda in strain UCD1-P (Fig. 2), as it does in strain N77-2-3 (*20*), but not in strain C2-P (Fig. 1).

To evaluate the potential pathological significance of high pda activity in *C. heterostrophus* and *A. nidulans*, we assessed virulence on pea plants by using the standard assay developed for *N. haematococca* (*21*). *Cochliobolus heterostrophus* strain C2-P caused lesions 60 to 350% larger than those produced by the control strain C2-V or by *A. nidulans* strains UCD1-P or UCD1-V (Fig. 3). Thus, high pda production alone allowed *C. heterostrophus* to cause significant damage to pea stems, although the lesions were smaller (20 to 30% of the size of N77-2-3 lesions), more shallow, and lighter brown than those caused by strain N77-2-3. In contrast, *A. nidulans* strain UCD1-P made no significant lesions, like control strains UCD1-V (Fig. 3) and UCD1.

The foregoing experiments allowed us to evaluate the effect of a fungal pathogenicity gene product in a foreign host. High pda production alone appeared to enhance the ability of the maize pathogen *C. heterostrophus* to attack pea, under our experimental conditions, while not affecting virulence toward its own host (*22*). But high production of pda was not sufficient to alter the phenotype of an auxotrophic strain of the saprophyte *A. nidulans* on pea plants. This lack of pathogenicity is probably not due to glucose repression of pda in strain UCD1-P, since pda is also glucose repressible in *N. haematococca* and other pea pathogens (*1, 20*). Rather it seems that *C. heterostrophus* has basic genes (as yet unknown) for pathogenicity that the saprophyte *A. nidulans* lacks. This study and others (*1*) indicate that high virulence of *N. haematococca* on pea requires several gene products, one of which is pda. These conclusions demonstrate the utility of heterologous gene expression in molecular analysis of the plant-microbe interaction, shown also for *Rhizobium* plant systems (*23*).

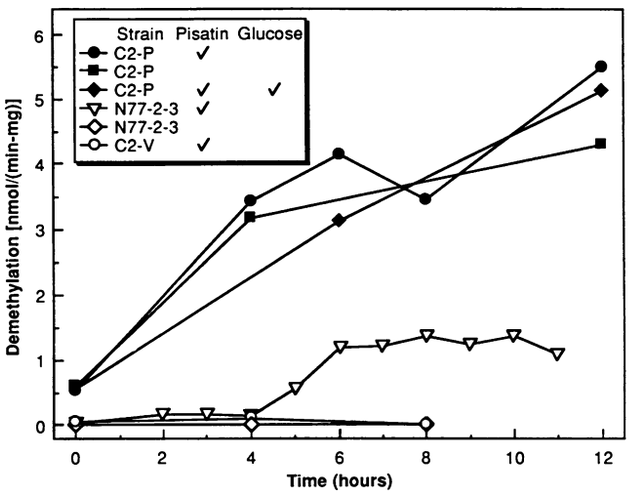

Fig. 1. Pisatin demethylase (pda) production by *N. haematococca* and transformed *C. heterostrophus*. Mycelial suspensions (30 mg/ml, fresh weight) in 50 m*M* potassium phosphate buffer (*p*H 6.5) were treated for the times indicated with unlabeled pisatin (0.1 m*M*) or glucose (or both) in 0.5% dimethyl sulfoxide (DMSO) or 0.5% DMSO. The rate of demethylation of 0.1 m*M* [3-*O*-*methyl*-^{14}C] pisatin over a 20-min interval was then assayed (*24*).

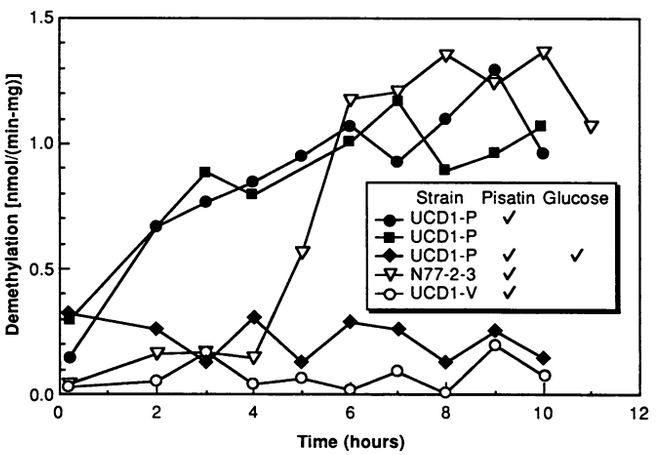

Fig. 2. Pisatin demethylase (pda) production by *N. haematococca* and transformed *A. nidulans*. Assays were performed as described in Fig. 1.

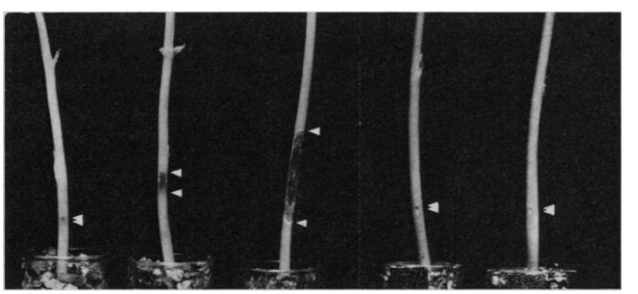

Fig. 3. Symptoms on unexcised stems of pea seedlings caused by *N. haematococca* and transformed strains of *C. heterostrophus* and *A. nidulans*. Cylinders (diameter, 4 mm) of agar medium bearing mycelium were placed on pin-prick wounds, and the plants were incubated 6 days in a moist chamber (*21*). From left to right: pea stems inoculated with strains C2-V (control), C2-P (high pda), N77-2-3 (high pda), UCD1-V (control), and UCD1-P (high pda); arrowheads delimit the extent of each lesion. There were five to eight replicate plants per treatment. The experiment was done six times with results similar to those shown here in five of them. In two of these assays, done independently (*25*), lesion lengths (millimeters) on pea stems were measured: the means were, in assay 1, C2 = 3.1^a, C2-V = 1.9^b, C2-P = 3.5^a, N77-2-3 = 18.0^c; in assay 2, C2 = 2.8^a, C2-V = 2.0^a, C2-P = 4.5^b, N77-2-3 = 17.6^c (within each experiment, numbers followed by different letters are significantly different from each other at the 0.05 level, by pairwise Student's *t* test). For unknown reasons, lengths of lesions produced by strain C2-P in assay 1 were not significantly different from those caused by strain C2; in all other assays the difference between sizes of lesions caused by strain C2-P versus strains C2 and C2-V was consistent and obvious as shown here.

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9. *Cochliobolus heterostrophus* and *A. nidulans* mycelia were grown overnight (*10*), cell walls were removed, and protoplasts were transformed (*7, 8*) with 10 μg of plasmid DNA per 10^7 protoplasts. After treatment with polyethylene glycol, each suspension of transformed protoplasts was diluted to 2.5 ml with osmoticum (*8*) and 50-μl portions were plated as described previously (*7, 8*).
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15. *Cochliobolus heterostrophus* transformants were selected on medium containing hygromycin B at 50 μg/ml. Two of these grew when exposed to hygromycin B at concentrations as high as 1 mg/ml, suggesting high copy numbers of inserted plasmids. DNA analysis (*16*) showed that strain C2-P had 18 copies of pUP1 and strain C2-V had 11 copies of pUCH1. *Aspergillus nidulans* transformants tend to have multiple copies of transforming plasmids (*17*); since strain UCD1-P was found (*16*) to have 12 copies, strain UCD1-V was assumed to have a comparable number, although copies were not actually counted.
16. Plasmid copy number was estimated by slot-blot analysis (*18*) and quantified by scintillation counting. For *C. heterostrophus*, serial dilutions of equal amounts of genomic DNA from strains C2-P, C2-V, and C2 were spotted on nitrocellulose membrane and hybridized against the *C. heterostrophus* promoter 1 sequence of pUCH1, known to be a single-copy sequence in the wild-type genome (*7*). For *A. nidulans*, serial dilutions of genomic transformant DNA and plasmid pD1 were spotted and hybridized to pD1. The most dilute concentration of plasmid was adjusted to represent one copy of *PDA1*/genome, given a genome size of 2.6×10^4 kb (*19*).
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Interleukin-1 Costimulatory Activity on the Interleukin-2 Promoter Via AP-1

KATHRIN MUEGGE, THOMAS M. WILLIAMS, JEFFREY KANT, MICHAEL KARIN, ROBERT CHIU, ALBRECHT SCHMIDT, ULRICH SIEBENLIST, HOWARD A. YOUNG, SCOTT K. DURUM

Interleukin-1 (IL-1) is a major regulator of inflammation and immunity. IL-1 induces T lymphocyte growth by acting as a second signal (together with antigen) in enhancing the production of interleukin-2 (IL-2). An IL-1–responsive element in the promoter region of the human IL-2 gene was similar to the binding site for the transcription factor AP-1. IL-1 enhanced expression of c-*jun* messenger RNA, whereas the antigenic signal enhanced messenger RNA expression of c-*fos*. Thus, the two components of the AP-1 factor are independently regulated and the AP-1 factor may serve as a nuclear mediator for the many actions of IL-1 on cells.

IL-1 AFFECTS MANY CELL TYPES; IT INduces the acute phase response of hepatocytes, fever mediated by the hypothalamus, and fibroblast and T lymphocyte growth [reviewed in (*1*)]. An IL-1 receptor has been cloned and characterized (*2*), but little is understood of the intracellular mechanisms mediating the action of IL-1 (*3*).

We examined the nuclear basis of IL-1 action on T cell activation. The mouse T lymphoma LBRM-331A5 (LBRM) reflects the two-signal requirement of normal T cells (*4*) in the competence phase of lymphocyte activation. One signal is derived from antigen engaging the T cell receptor [mimicked by phytohemagglutinin (PHA)], the second signal is provided by IL-1 via a high-affinity receptor (*2*). These two signals result in IL-2 production, which is regulated at the level of transcription (*5*).

Activation of the IL-2 gene appears to be mediated through a transcriptional enhancer region between −548 and +52 upstream of the transcription start site [reviewed in (*6*)]. The enhancer region of the human IL-2 gene is 85% homologous with the mouse IL-2 gene, which permits expression in murine cells. To look for an IL-1–responsive element on the IL-2 promoter, we used the −548- to +39-bp region of the human IL-2 gene linked to the chloramphenicol acetyltransferase (CAT) indicator gene (*7*); this construct, termed "IL-2–CAT," was transiently transfected into LBRM cells. The IL-2–CAT construct responded to IL-1 plus PHA six- to sevenfold above controls of PHA alone or IL-1 alone (Table 1). Control experiments with plasmids that contained enhancer elements of the Rous sarcoma virus upstream of reporter genes (pRSV-luciferase or pRSV-CAT (*9, 10*) did not increase luciferase or CAT activity in response to IL-1 (*8*); thus, IL-1 had no nonspecific effect on viability, transfection efficiency, or stability of CAT mRNA or protein.

To identify the IL-1–responsive element within the −548- to +39-bp enhancer region, we constructed 5′ deletion mutants of IL-2–CAT and transiently transfected LBRM cells. The −218 to +39 fragment retained full IL-1 responsiveness (about six to seven times background PHA stimulation) (Table 1A). Therefore, the region 3′ to −218, which must harbor an IL-1–responsive element, was examined with internal deletion mutants. The deletions −107 to −76 or −83 to −42 contained one of the two recently defined T cell receptor-responsive elements (−93 to −63) (*11*), and had about 50% of the CAT activity of the full-length IL-2–CAT, although an IL-1 response was maintained; this was expected, since PHA delivers its signal via T cell receptors, as verified by antibodies against the CD-3 complex, which mimic the PHA effect on this cell (*8*). The deletion −218 to −176 abrogated the IL-1 effect (Table 1B),

K. Muegge, A. Schmidt, U. Siebenlist, H. A. Young, S. K. Durum, Laboratory of Molecular Immunoregulation, Program Resources Inc., and Laboratory of Biological Therapeutics, National Cancer Institute and National Institute of Allergy and Infectious Diseases, Frederick, MD 21701 and Bethesda, MD 20892.
T. M. Williams and J. Kant, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104.
M. Karin and R. Chiu, University of California San Diego, La Jolla, CA 20892.