Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6349–6354, June 1996
Agricultural Sciences

Construction of an improved mycoinsecticide overexpressing a toxic protease

RAYMOND J. ST. LEGER, LOKESH JOSHI, MICHAEL J. BIDOCHKA, AND DONALD W. ROBERTS

Boyce Thompson Institute, Cornell University, Tower Road, Ithaca NY 14853

Communicated by John H. Law, University of Arizona, Tucson, AZ, March 1, 1996 (received for review October 31, 1995)

ABSTRACT **Mycoinsecticides are being used for the control of many insect pests as an environmentally acceptable alternative to chemical insecticides. A key aim of much recent work has been to increase the speed of kill and so improve commercial efficacy of these biocontrol agents. This might be achieved by adding insecticidal genes to the fungus, an approach considered to have enormous potential for the improvement of biological pesticides. We report here the development of a genetically improved entomopathogenic fungus. Additional copies of the gene encoding a regulated cuticle-degrading protease (Pr1) from *Metarhizium anisopliae* were inserted into the genome of *M. anisopliae* such that Pr1 was constitutively overproduced in the hemolymph of *Manduca sexta*, activating the prophenoloxidase system. The combined toxic effects of Pr1 and the reaction products of phenoloxidase caused larvae challenged with the engineered fungus to exhibit a 25% reduction in time of death and reduced food consumption by 40% compared to infections by the wild-type fungus. In addition, infected insects were rapidly melanized, and the resulting cadavers were poor substrates for fungal sporulation. Thus, environmental persistence of the genetically engineered fungus is reduced, thereby providing biological containment.**

Current widely publicized problems with synthetic chemical insecticides have given rise to a sense of urgency in the development of biological control agents as supplements or alternatives to these chemicals. Although widely used in some less developed nations, entomopathogenic fungi have only recently assumed importance in agriculture and household entomology in the United States. This is in spite of the fact that entomopathogenic fungi are key regulatory factors in pest insect populations, are considered very promising biological control agents, and provide the only practical microbial control of insects that feed by sucking plant or animal juices, and for the many acridid (grasshoppers and locusts) and coleopteran pests which have no known viral or bacterial diseases (1–4). However, traditional biopesticides (viruses, bacteria, and fungi) are commonly perceived to perform poorly compared with chemical pesticides. A major deterrent to the development of entomopathogenic fungi has been that it can take 5–10 days after application to kill an insect pest, during which time the infected insect can cause serious damage to the crop. A key aim of much recent work has been to increase the speed of kill and so improve commercial efficacy. To date, fungi have been improved as pathogenic agents by developing the technologies required for optimizing production, stability, and application of the inoculum (1, 2). Improvements to reduce the lag time before feeding cessation might also be achieved by adding insecticidal genes to the fungus, an approach considered to have enormous potential for the improvement of biological pesticides (5).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Until recently, lack of information on the molecular basis of fungal pathogenicity precluded genetic engineering to improve control potential. We have developed molecular biology methods to elucidate pathogenic processes in the commercially important entomopathogen *Metarhizium anisopliae* and have cloned several genes that are expressed when the fungus is induced by starvation stress to alter its saprobic growth habit, develop a specialized infection structure (i.e., the appressorium), and attack its insect host (6). One of these genes encodes a subtilisin-like protease (designated Pr1) which solubilizes the proteinaceous insect cuticle, assisting penetration of fungal hyphae, and providing nutrients for further growth (7, 8). Pr1 production by *M. aniosopliae* is under dual control of a general carbon catabolite repression/derepression mechanism and a carbon source (cuticle) specific induction mechanism (6–10). As penetration involves the induced expression of Pr1, we hypothesized that constitutive overexpression of this gene could provide a direct strategy for engineering enhanced virulence. Here we report that the efficacy of *M. anisopliae* as a biological control agent can be substantially improved by transforming multiple copies of the *Pr1* gene into the genome, under control of a constitutive promoter, to generate strains which secrete Pr1 into the insects hemolymph. We also show that the early cessation of feeding is due, at least in part, to the toxic effects of Pr1-induced activation of the hosts prophenoloxidase system.

MATERIALS AND METHODS

Strains and Media. *M. anisopliae* strain 1080 (an American strain held by the Agricultural Research Service Collection of Entompathogenic Fungi (ARSEF) collection, U.S. Department of Agriculture, Ithaca, N.Y.) was originally isolated from a lepidopterous larva (*Heliothis zea*). Media, growth conditions, and storage of *M. anisopliae* have been described (8).

DNA Manipulations. Standard molecular methods were used (11). pAN52-1 vector (obtained from Peter Punt of the Netherlands Central Organization for Applied Scientific Research Medical Biology Laboratory, Rijswijk, The Netherlands) was used to produce pMAPR-1 (Fig. 1) for the cloning and expression of Pr1 cDNA. pAN52-1 comprises the *gpd* promoter and the terminator region of the *trp C* gene (both from *Aspergillus nidulans*) separated by *Bam*HI and *Nco*I sites (12). A full-length cDNA clone of Pr1 in a Bluescript vector (7) was amplified by reverse transcription–PCR with the forward primer 5′-AAGGATCCCGTACTAGAATTTGGAATCA-TG-3′, containing the ATG codon, and the reverse primer 5′-GTAAAACGACGGCCAGT-3′ (universal M13–20 primer). The amplified cDNA was cloned into vector pCRII (Invitrogen) and subsequently excised by digestion with *Bam*HI, which cuts at sites incorporated into the forward primer and 19 nucleotides downstream of the Pr1 insert in the amplified portion of the Bluescript vector. The 1.2-kb *Bam*HI fragment was cloned into *Bam*HI-cut pAN52–1. The resulting

Abbreviations: IEF, isoelectric focusing; AFC, 7-amino-4-trifluoromethyl coumarin.

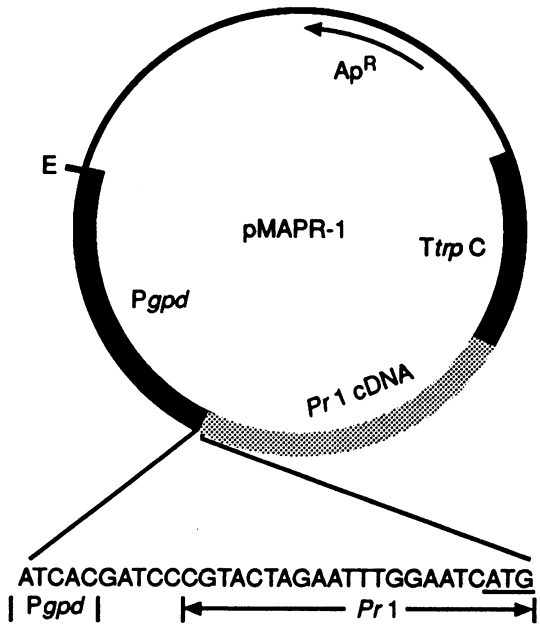

FIG. 1. Diagram of pMAPR-1. The cDNA coding for the *M. anisopliae* Pr1 proteinase is inserted 3′ to the *A. nidulans gpd* promoter sequence and 5′ to terminator sequences from the *A. nidulans trp C* gene. The sequence preceding the *Pr1* start codon is shown at the bottom. ApR, ampicillin resistance gene. Details of plasmid construction are given in the experimental protocol.

vector was digested with *Nco*I, blunt ended with Mung bean nuclease to remove the *gpd* ATG translation start codon, and religated. Sequence analysis confirmed the orientation of the insert in the recombinant vector and that the Pr1 initiator ATG was in-frame with the *gpd* promoter. Isolation of genomic DNA from *M. anisopliae,* transfer of DNA from gels, and filter hybridizations with ^{32}P-labeled probes have been described (7).

Transformation and Screening. The expression cassette was introduced into *M. anisopliae* 1080 by cotransformation at 20-fold excess with the *A. nidulans* benomyl resistance plasmid pBENA3 using our standard procedures (13). Transformants were selected for resistance to 5 μg ml^{-1} of benomyl and for constitutively expressed Pr1 by plating on a nutrient-rich Difco nutrient agar plus 1% *N*-acetylglucosamine (GlcNAc) medium incorporating insoluble elastin (0.08%, wt/vol), a Pr1 substrate (10).

Shake Cultures. Standardized mycelial inocula (5 g wet weight) from 48-h Sabouraud dextrose broth cultures (14) were incubated with shaking (100 rpm) for 10 h in 100 ml basal media (0.02% KH_2PO_4/0.01% $MgSO_4$, pH 6) supplemented with cockroach cuticle (14), or GlcNAc at 1% (wt/vol). Pr1 protease activity in culture filtrates was assayed against the synthetic peptide succinyl-(alanyl)$_2$-prolyl-phenylalanine (*p*-NA; *p*-nitroanilide) (Sigma) as described (8). Activities are expressed as μmol *p*NA min^{-1} ml^{-1}.

Plasmid Copy Number. The integration vector copy number was estimated by slot-blot analysis (15) and quantified by scintilation counting. Serial dilutions of equal amounts of genomic DNA from strains 1080, *gpd*-Pr1-1, *gpd*-Pr1-2, *gpd*-Pr1-3, and *gpd*-Pr1-4 were spotted on nitrocellulose membrane and hybridized against the ^{32}P-labeled vector. The Pr1 sequence is known to be a single copy gene in *M. anisopliae* (7).

Insect Bioassays. *Manduca sexta* larvae from a laboratory colony were reared on an artificial diet according to standard procedures (16). Ten newly emerged 5th instar larvae (mean weight, 1.4 ± 0.13 g) per dose were inoculated by dipping into conidial suspensions and transferred to containers at 25°C, 70% relative humidity to follow the course of the infection (17). The eight doses were determined by haemocytometer counts and ranged from 5.1 × 10^5 to 7 × 10^7. Mortality was recorded at 8-h intervals, and the data analyzed by probit analysis with standard software (18). LC_{50} values and ST_{50} values were considered significantly different if their 95% fiducial limits did not overlap. The time mortality data was also analyzed by the VISTAT time program (19). All insects were given a preweighed piece of diet 12 h after inoculation. Uneaten food was removed at the time of death and dried to a constant weight at 60°C. A sample of new food was dried in the same way to determine its water content and to allow an estimate of food consumption.

Phenoloxidase Activation Studies. Gypsy moth (*Lymantria dispar*) larvae were kindly provided by Ann Hajek (Cornell University) from a laboratory colony. Larvae were injected ≈48 h after ecdysis into the 5th instar with affinity-purified preperations of subtilisin-like (Pr1) or trypsin-like (Pr2) proteases from *M. anisopliae* (8) in a volume of 10 μl of TC-100 (GIBCO) tissue culture medium. Control insects were injected with autoclaved Pr1 or tissue culture medium only. To investigate the mode of action of Pr1 on melanization, some insects were injected with Pr1 (in 10 μl of fluid) supplemented with 50 ng of phenylthiourea (an inhibitor of phenoloxidase) or 5 μg of leupeptin (an inhibitor of trypsins).

Analysis of Hemolymph Samples. Hemolymph was collected from a puncture in a proleg and centrifuged (12,000 × *g* for 5 min), and proteins in the supernatant were precipitated with trichloroacetic acid (5% final concentration). SDS/PAGE of hemolymph proteins (15 μg total protein per lane) was performed under reducing conditions on 4–20% gradient gels, and proteins were stained with Coomassie blue or electroblotted. Immunoblots were performed by using Pr1-specific antibodies (20) and alkaline phosphatase-conjugated anti-rabbit secondary antibodies (Promega).

Immunoprecipitations were performed at various times up to 120 h postinoculation with wild-type or *gpd*-Pr1-4. Hemolymph samples were taken 10 h after insects were injected with 0.15 mCi ^{35}S-methionine (Trans ^{35}S-label; ICN), and the samples added to 20 volumes of immunoprecipitation buffer (1% skimmed milk/1.1% Triton X-100/100 mM Tris−HCl, pH 7.8/100 mM NaCl/10 mM EDTA/1 mM phenylmethylsulfonyl fluoride/0.1 mM aprotinin/0.02 mM tosyl-lysyl-chloroketone/0.02 mM leupeptin). Immunoprecipitation procedures and autoradiography were described (21). Phenoloxidase activity in 10 μl of diluted hemolymph was assayed against L-DOPA (5 mM) in 90 μl 50 mM potassium phosphate buffer (pH 6.0) in a microtiter plate. Plates were read at 490 nm in a microtiter reader. One activity unit was defined as the amount of enzyme that in 5 min gives a change of 0.01 in absorbance. Hyphal bodies (blastospores and short hyphal lengths) within the hemolymph were examined by light microscopy, and counts performed by quantitative culturing (serial dilution plate counts) as described (22).

Insect Trypsins. Proteinases present in hemolymph were separated by analytical polyacrylamide isoelectric focusing (IEF). Centrifuged samples (2 μl) containing about 3 μg protein were applied to gels (5% polyacrylamide, 2% 3–10 ampholytes). Immediately following IEF (100 V for 15 min, 200 V for 15 min, 450 V for 1 hr), protease activity was detected by gelatin zymography by using gelatin-coated X-ray film and with enzyme overlay membranes impregnated with fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC) substrates (Enzyme Systems Products, Livermore, CA) as described (14). To further examine specificity, some films were preincubated for 10 min in the trypsin inhibitor leupeptin at 20 μg/ml.

RESULTS

Construction and Characterization of a Series of *M. anisopliae* Recombinants Expressing *Pr1* under the Transcriptional Control of the *gpd* promoter. *M. anisopliae* strains producing Pr1 under the *A. nidulans* promoter were constructed by

placing 1.2 kb of the PCR-amplified *Pr1* gene downstream of the *gpd* promoter in the pAN52-1 vector to produce pMAPR-1 (Fig. 1). The *M. anisopliae* strain 1080 was transformed with pBENA3 (Ben$^+$, control) or cotransformed with pBENA3 and pMAPR-1, and transformants were selected on the basis of benomyl resistance. Forty transformants per transformation were streaked twice on selective plates and screened for Pr1 production in nutrient-rich conditions that repress Pr1 synthesis by the wild-type strain. Twelve pMAPR-1 transformants expressed levels of Pr1 clearly detectable by the screening method used, indicative of the constitutive expression of Pr1. No Pr1 activity was detected in transformants carrying pBENA3 alone. The transformants expressing Pr1 were purified through single spore isolation and were once more tested for Pr1 production on selective plates. Four strains (designated *gpd*-Pr1[-1, -2, etc.) that were morphologically stable showed wild-type levels of growth and conidiation and produced large elastin clearing zones were again single spore isolated and cultured for DNA isolation (7). Copy numbers of Pr1 cDNA ranged from three to six in transformants (Table 1).

Shake Flask Cultivations. To assay for constitutive production of Pr1, the wild-type strain and the four transformants, *gpd*-Pr1-1 to -4, were grown for 5 h in shake flasks on GlcNAc-containing media or cockroach cuticle-containing media (Table 1). All the strains produced Pr1 during growth on cuticle, which induces Pr1 synthesis (9), but only the transformants produced Pr1 while cultured on GlcNAc, which represses Pr1 synthesis under native control (10), indicating expression of the heterologous promoter and synthesis and secretion of Pr1.

Insect Bioassays. We assessed the biological activity of the wild-type and recombinant strains by inoculating 5th instar tobacco hornworm (*M. sexta*) larvae with LC_{100} doses (5×10^6 conidia). Infection with the wild-type fungus did not cause extensive discoloration of larvae, nor did it alter the profile of hemolymph proteins (Fig. 2 *A* and *B*). No differences were observed between the wild-type strain versus strain Ben$^+$ (control transformant containing multiple copies of the pBENA3 plasmid alone) either in culture or in insects. By contrast, recombinant strains that received pMAPR-1 caused extensive blackening of larvae and partial hydrolysis of hemolymph proteins (Fig. 2 *A* and *B*). These insects contained sufficient amounts of Pr1 in the hemolymph for Pr1 detection by Western blot analysis (Fig. 2*C*), indicating that transgenic strains continued to produce Pr1 in the hemocoel of caterpillars following penetration of the cuticle. All four transformants tested (*gpd*-Pr1-1 to -4) constitutively expressing Pr1 gave the same result in insect tests, thus allowing us to use *gpd*-Pr1-4 to represent the entire group. Precipitation of hemolymph samples with Pr1-specific antibody revealed that the wild-type strain at 60h postinoculation produced 50-fold less Pr1 than *gpd*-Pr1-4 (Fig. 3 *A* and *B*).

Table 1. Pr1 production by *M. anisopliae* wild type and transformants in samples from 10-hr shake cultures

Strain	Approximate copy no.	Expression of Pr1 in cockroach cuticle medium (μmol pNA $min^{-1} \cdot ml^{-1}$)	Expression of Pr1 in 1% GlcNAc medium (μmol pNA $min^{-1} \cdot ml^{-1}$)
Wild type	NA	0.22 ± 0.03*	0
Transformant			
gpd-Pr1-1	3	0.28 ± 0.03	0.08 ± 0.02
gpd-Pr1-2	3	0.30 ± 0.04	0.11 ± 0.03
gpd-Pr1-3	5	0.35 ± 0.03	0.14 ± 0.03
gpd-Pr1-4	6	0.38 ± 0.04	0.17 ± 0.03

*Mean Pr1 activity (vs. Suc-Ala-Ala-Pro-Phe-pNA) of three replicates ± SD.

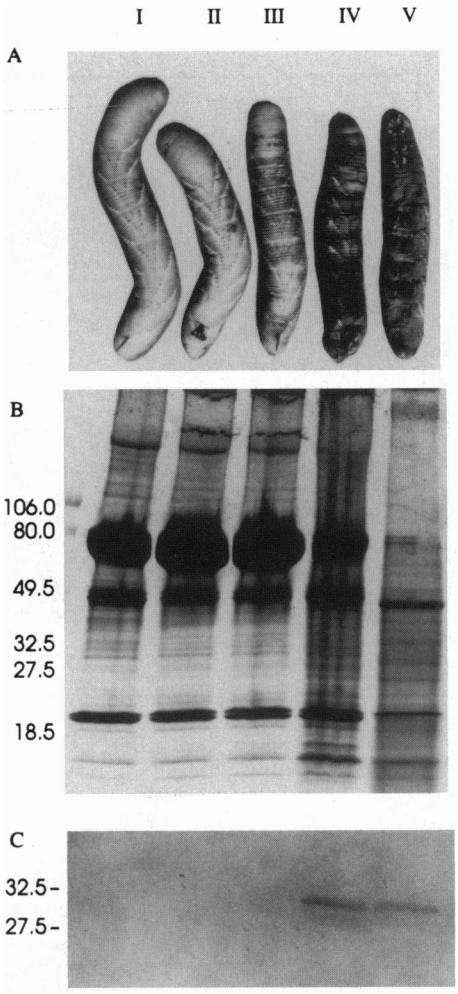

FIG. 2. Constitutive production of Pr1 causes partial hydrolysis of hemolymph proteins and melanization of infected insects. (*A*) The appearance, from left to right, of (I) an uninfected fifth instar *M. sexta* larva compared with larvae 90 h after dipping in conidial suspensions (1×10^6 ml^{-1}) of (II) wild-type *M. anisopliae* (strain 1080); (III) control transformant Ben$^+$ which contains four copies of the pBENA3 plasmid alone; (IV) *gpd*-Pr1-3; and (V) *gpd*-Pr1-4 (constitutive overproducers of Pr1 containing multiple copies of pMAPR-1), which are dark colored due to melanization of the blood. The wild-type and Ben$^+$ strains, in contrast, cause only localized melanization in the cuticle at sites of penetration. The lanes on the accompanying Coomassie-blue-stained SDS/PAGE gel of hemolymph proteins (*B*) are aligned with the caterpillars to show that the extensive blackening of larvae infected with *gpd* strains was associated with partial degradation of hemolymph proteins. The presence of Pr1 (28.5 kDa) in the hemolymph of these insects was demonstrated by immunoblotting with Pr1-specific antibodies (*C*). There were five to nine replicate insects per treatment with control and recombinant strains of *M. anisopliae*. The experiment was done four times with differences in symptoms which were consistant and obvious as shown in *A*. The only variation was in the amount of melanization in the cuticle, which varied as much within treatments as between treatments.

Quantitative Evaluation of Fungal Virulence. Constitutive expression of Pr1 speeds up the disease process and improves insecticidal activity of the recombinants as demonstrated by a comparison of time-mortality data (Fig. 4); the median survival time of *gpd*-Pr1-4-infected larvae (ST_{50} = 93 h) is 25% shorter than those infected with the wild-type strain (ST_{50} = 120 h) (Table 2). Hyphal bodies (blastospores and short hyphal lengths of 3–10 cells) of the wild-type strain proliferated within the hemolymph, increasing by 50-fold between 90 and 120 h up to 2.3×10^7 colony forming units per ml (Fig. 3*D*). This coincided with a 31-fold increase in Pr1 and a 7-fold reduction

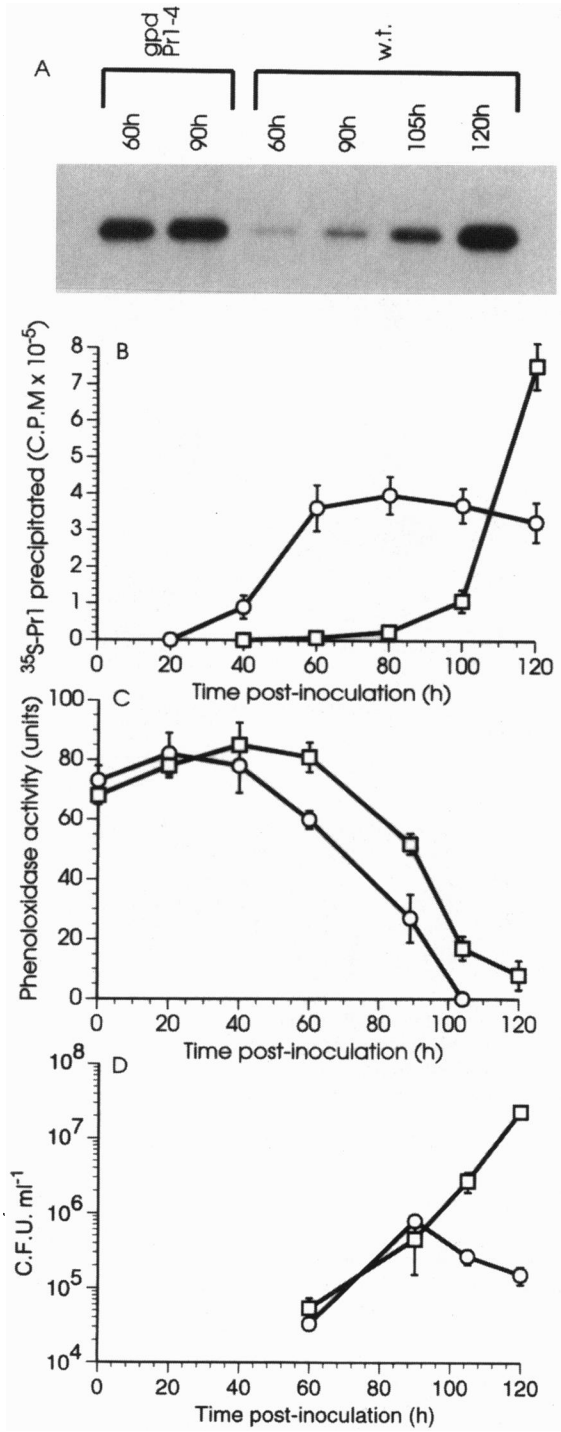

FIG. 3. Time course of Pr1 production and Pr1 effects on phenoloxidase activity and propagation of *M. anisopliae* in the hemolymph. (*A*) Pr1 production. Larvae infected with the wild-type or *gpd*-Pr1-4 were radiolabeled with ^{35}S-methionine for 10 h. Immunoprecipitations by a Pr1-specific antibody were analyzed by SDS/PAGE and autoradiography. (*B*) Radiolabeled Pr1 bands immunoprecipitated as in *A* were excised from SDS/PAGE gels and quantified with a scintillation counter [wild-type 1080 (□); *gpd*-Pr1-4 transformants (○)]. (*C*) To compare phenoloxidase activities, hemolymph from larvae infected with wild-type or *gpd*-Pr1-4 were assayed against L-DOPA, which showed a reduction in activities, as (*D*) the number of fungal propagules (colony forming units, C.F.U.) in the hemolymph increased during infection. Results are the mean of five determinations ± SEM.

in phenoloxidase activity in hemolymph during the same time period (Fig. 3 *B* and *C*). Thirty-fold fewer propagules were present in *gpd*-Pr1-4-infected insects at ST_{50} (=93 h) compared

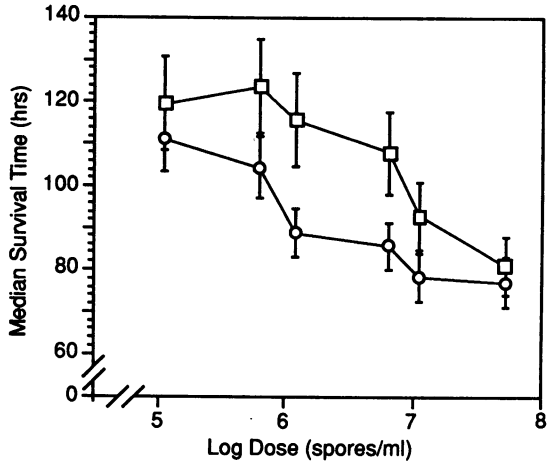

FIG. 4. The relationship between dose and median survival time (ST_{50}) for the wild-type *M. anisopliae* (□) and recombinant strain *gpd*-Pr1-4 (○) infecting 5th instar *M. sexta* larvae (20 larvae per dose; bars delimit 95% confidence limits for ST_{50} values). A spore concentration of 6.1×10^6 was optimal for this experiment. At the lowest inoculum load (5.1×10^5 ml^{-1}), more than 50% of insects pupated before death occurred. At the highest spore concentration (7.7×10^7 ml^{-1}), differences between the wild-type and *gpd*-Pr1-4 treated insects were less apparent with time because of early mortality.

with the growth achieved by the wild-type strain at ST_{50} (=120 h). This is consistent with the earlier mortalities produced by *gpd*-Pr1-4 being caused by toxicosis rather than disruption of host issues by fungal growth (23). The median lethal concentration (LC_{50}) values for wild type and transformants were not significantly different. As LC_{50} values are based on the efficiency of infection (5), this is evidence that attachment and penetration are not effected by constitutive overexpression of Pr1.

We measured the weight of food consumed to determine whether infection of *M. sexta* with *gpd*-Pr1-4 could reduce feeding, the critical commercial parameter of pesticides. Insects infected with *gpd*-Pr1-4 consistently ate 40–45% less diet compared to individuals infected with the wild type-strain and 70% less than uninfected controls (Table 2).

Yield of Conidia from Infected Larvae. It was of interest to determine if the constitutive expression of Pr1 effects the production of infectious propagules (conidia) in insect larvae since this would influence the persistance of the fungus in a field population. The blackened cadavers resulting from infection by the transformants were found to support very little sporulation.

Toxicity by Injection of Pure Pr1. Blackening of host insects suggests that the recombinants were activating the enzyme (phenoloxidase) responsible for synthesis of melanin, a key component in arthropod immunity and wound healing (24). Activation of prophenoloxidase in the hemolymph plasma normally involves proteolytic cleavage during a cascade of trypsin-like enzyme reactions (24). To determine if some of the toxic effects of Pr1 involve melanization, 200 ng of Pr1 per larva was injected into the hemocoel of 10 5th instar larvae of gypsy moth (*Lymantria dispar*) and 10 5th instar larvae of *M. sexta*. Blackening and immobilization of all larvae occurred within 60 min. These effects were blocked by injection of 50 ng phenylthiourea per larva, indicating that the Pr1-induced melanization is insecticidal. Injection of leupeptin (5 μg), an inhibitor of trypsins with no effect on Pr1 (8), also inhibits prophenoloxidase activation by Pr1, indicating that Pr1 most likely acts indirectly by activating trypsins involved in the cascade rather than prophenoloxidase itself. Since prophenoloxidase activation seems to be affected by hemolymph trypsins activated by Pr1, we also tested the effect of Pr2, a

Table 2. Dose-mortality response of larval *M. sexta* infected with wild-type or recombinant fungus

Fungus	LC_{50} conidia $ml^{-1} \cdot g^{-1}$ (95% fiducial limit)	Slope	ST_{50} h^{-1} (95% fiducial limits)	Slope	Dry weight of food consumed, g ± SEM
None	—	—	—	—	6.25 ± 0.095
Wild-type	3.14×10^5 ($2.03–5.54 \times 10^5$)	1.87	128 (120–136)	17.2	3.41 ± 0.085
gpd-Pr1-3	2.98×10^5 ($1.85–5.50 \times 10^5$)	2.15	96 (92–106)	12.3	2.14 ± 0.078
gpd-Pr1-4	2.75×10^5 ($1.81–5.32 \times 10^5$)	2.21	93 (88–102)	11.7	1.95 ± 0.073

Ten newly emerged 5th instar larvae per dose were inoculated by dipping into conidial suspensions. The weight of food consumed by insects infected with wild type was significantly greater than that consumed by insects infected with either of the recombinants ($P < 0.025$) as determined by the Mann–Whitney Wilcoxon test.

trypsin-like enzyme produced by *M. anisoplia* (8). Pr2 caused negligible activation when mixed with hemolymph or injected in generous amounts (up to 3 μg per larvae) into either *M. sexta* or *L. dispar*. We were not able to obtain reproducible results in terms of number and quantity of endogenous trypsin activities in *M. sexta* hemolymph activated by Pr1, perhaps because of trypsin inhibitor activity in the hemolymph (24). By contrast, four bands were consistantly distinguishable on IEF gels when protease activity in Pr1-activated *L. dispar* hemolymph was detected with a gelatin overlay (Fig. 5). All four bands were inhibited by leupeptin and showed optimum activity against Bz-Gly-Gly-Arg-AFC. Other trypsin substrates with bulkier residues replacing the glycine residues at P_2 and P_3 produced greatly reduced activities. By contrast, the trypsin-like Pr2 enzymes produced by *M. anisopliae* prefer a bulky residue (e.g., Phe or Val) at P_2 (8), which could account for their failure to activate prophenoloxidase. Pr1 probably has additional insecticidal effects besides inducing melanization as reflected in non-specific protein degradation (Fig. 2*B*). We also noted that Pr1 treated hemolymph lost the ability to clot (data not shown).

DISCUSSION

Constitutive overexpression of Pr1 by *M. anisopliae* results in significant reductions in larval food consumption with concomitant reductions in larval weight gain during the course of fungal infection. The expression of Pr1 in the hemolymph of infected insects has no deleterious effect on the intrinsic infectivity of the transformed fungus as measured by LD_{50}. Thus, recombinant fungi expressing Pr1 should be useful in agricultural pest management, which requires rapid cessation of pest feeding to limit crop damage. Constitutive overexpression of Pr1 is also unlikely to alter host range because specificity is usually controlled by infection events at the level of the cuticle (6), which would be unaltered by the continuance of Pr1 production after cuticle penetration. Consistent with this, transformants resembled the parent strain in being pathogenic to gypsy moth larvae at massive doses only, and not displaying pathogenicity to beetle larvae (data not shown).

The development of genetically enhanced mycoinsecticides clearly offers an alternative to chemical pesticides. The registration of these products for environmental release will require special consideration of their reproductive potential and nature of the genetic enhancement (5). Melanized insects killed by transgenic strains were very poor substrates for fungal growth and reproduction. This is consistent with previous demonstrations of the toxicity of oxidized phenolics and melanin to *M. anisopliae* (17), and from a mycological point of view, may explain the tight control of Pr1 production during the infection process. The wild-type fungus only produces Pr1 in the hemolymph very late in infection, when levels of phenoloxidase are much reduced. *M. sexta* phenoloxidase is resistant to proteolysis by Pr1 (17), indicating that Pr1 is not involved in this suppression of phenoloxidase activity. The failure of a genetically engineered organism to sporulate is attractive from both a commercial and an environmental standpoint as it would presumably lead to the eventual extinction of transformants, reducing environmental impact and allowing repeat sales. It is also consistent with the recent emphasis on using mycopathogens as contact insecticides maximizing the kill from the initial application of the fungus in the same way as with a chemical insecticide (1). Furthermore, the development of strains of *M. anisopliae* that consti-

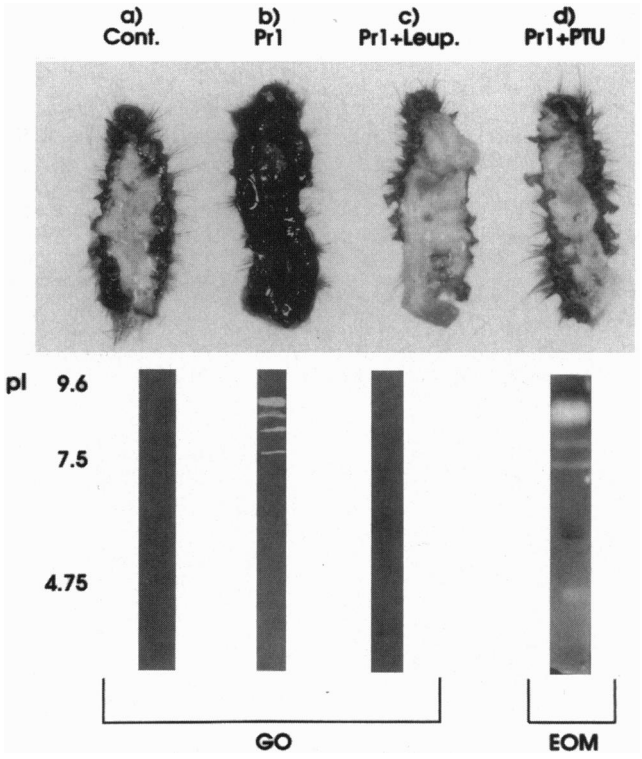

FIG. 5. Induced blackening of insects is caused by activation of endogenous trypsin-like enzymes. (*A*) Dissected 5th instar *L. dispar* larvae 60 min after injection with 10 μl of buffered insect saline containing 200 ng of autoclaved (inactive Pr1) or (B) active Pr1 with only the later showing massive internal melanization. No blackening was detected when Pr1 was amended with 5 μg of leupeptin (*C*) or 50 ng phenylthiourea (PTU) (*D*). The lanes on the accompanying analytical IEF (pH 3–10) gels are aligned with the caterpillars to show activation of proteases in insect hemolymph by treatment with Pr1. Gelatin zymograms (GO) show no active proteases in hemolymph from the insect injected with inactive Pr1 (*A*). Four basic bands could be distinguished in hemolymph samples treated with active Pr1 (*B*). No activity was detected in the presence of leupeptin (*C*), but phenylthiourea was without effect. Enzyme overlay membranes (EOM) were used to study the substrate specificity of individual proteases. Highest levels of activity were detected against Bz-Gly-Gly-Arg-AFC (*D*) with much reduced activity against Bz-Gly-Pro-Arg-AFC, Bz-Phe-Ala-Arg-AFC, or Bz-Val-Leu-Arg-AFC (data not shown).

tutively express Pr1 from plasmid pMAPR-1 provides an example of a strategy that, in using a homologous gene, is less likely to raise some of the public concerns faced by researchers working with another recently developed transgenic entomopathogen—i.e., baculoviruses expressing scorpion toxins (5).

The protease itself provides a novel reagent for the pesticidal arsenal, including a unique mode of action (activation of prophenoloxidase) for combating potential resistance development in selected pests when expressed in an appropriate fungal or insect virus. Confirming this, we have produced a recombinant baculovirus that expresses Pr1 and shows greatly increased speed of kill (X. Huang, R.J.S.L., A. Wood, T. Davis, and P. Hughes, unpublished data). Novel features of Pr1, which distinguish it from proteases produced by saprophytes, include its resistance to proteinase inhibitors (serpins) in hemolymph (unpublished data) and its retention of activity in a melanizing milieu (17). Up to now, fungal genes have played little part in the implementation of biotechnology in crop protection, which is surprising given both the dazzling array of metabolites fungi produce and that the lack of useful pesticidal genes for transfer has been a major constraint on the development of this technology (25).

We thank A. Wood and A. Churchill for critically reading this manuscript and P. Hughes for performing the statistical analysis. The work was supported in part by the U.S. Department of Agriculture Competitive Research Grants Office.

1. Bateman, R. P. (1992) *Antenna* **16,** 6–13.
2. Prior, C. (1992) in *Biological Control of Locusts and Grasshoppers,* eds. Lomer, C. J. & Prior, C. (CAB, Wallingford, Oxon, U.K.), pp. 159–180.
3. Glare, T. R. & Milner, R. J. (1991) in *Handbook of Applied Mycology,* eds. Arora, D. K., Ajello, L. & Makerji, K. J. (Dekker, New York), pp. 547–612.
4. Roberts, D. W. & Hajek, A. E. (1992) in *Frontiers in Industrial Mycology,* ed. Leatham, G. F. (Chapman & Hall, New York), pp. 144–159.
5. Wood, H. A. (1995) in *Baculovirus Expression Systems and Biopesticides,* eds. Shuler, M. L., Wood, H. A., Granados, R. R. & Hammer, D. A. (Wiley, New York), pp. 91–102.
6. St. Leger, R. J. (1993) in *Parasites and Pathogens of Insects,* eds. Beckage, N. C., Thompson, S. N. & Federici, B. A. (Academic, San Diego), Vol. 2, pp. 211–229.
7. St. Leger, R. J, Frank, D. C., Roberts, D. W. & Staples, R. C. (1992) *Eur. J. Biochem.* **204,** 991-1001.
8. St. Leger, R. J., Charnley, A. K. & Cooper, R. M. (1987) *Arch. Biochem. Biophys.* **253,** 221–232.
9. Paterson, I. C., Charnley, Cooper, R. M. & Clarkson, J. M. (1994) *Microbiology* **140,** 3153–3159.
10. St. Leger, R. J., Durrands, P. K., Cooper, R. M. & Charnley, A. K. (1988) *Arch. Microbiol.* **150,** 413–416.
11. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
12. Punt, P. J.,. Oliver, R. P., Dingemance, M. D., Pouwels, P. H. & van den Hondel, C. A. M. J. J. (1987) *Gene* **56,** 117–124.
13. St. Leger, R. J., Shimizu, S., Joshi, L., Bidochka, M. J. & Roberts, D. W. (1995) *FEMS Microbiol. Lett.* **131,** 289–294.
14. St. Leger, R. J., Bidochka, M. J. & Roberts, D. W. (1994) *Arch. Biochem. Biophys.* **313,** 1–7.
15. Kafatos, F. C., Jones, C. W. & Efstratiadis, A. (1979) *Nucleic Acids Res.* **7,** 1541–1545.
16. Bell, R. A. & Joachim, F. G. (1976) *Ann. Entomol. Soc. Am.* **69,** 365–373.
17. St. Leger, R. J., Cooper, R. M. & Charnley, A. K. (1988) *J. Invertebr. Pathol.* **52,** 459–470.
18. Daum, R. J. (1970) *Bull. Entomol. Soc. Am.* **16,** 10–15.
19. Hughes, P. R. & Wood, H. A. (1985) in *The Biology of Baculoviruses,* eds. Granados, R. R. & Federici, B. A. (CRC, Boca Raton, FL), Vol. 2, pp. 1–30.
20. Goettel, M. S., St. Leger, R. J., Rizzo, N. W., Staples, R. C. & Roberts, D. W. (1989) *J. Gen. Microbiol.* **135,** 2233–2239.
21. St. Leger, R. J., Staples, R. C. & Roberts, D. W. (1991) *J. Gen. Microbiol.* **137,** 807–815.
22. St. Leger, R. J., Durrands, P. K., Charnley, A. K. & Cooper, R. M. (1988) *J. Invertebr. Pathol.* **52,** 285–293.
23. Samuels, R. I., Charnley, A. K. & Reynolds, S. E. M. (1988) *Mycopathologia* **104,** 51–58.
24. Sugumaran, M. & Kanost, M. R. (1993) in *Parasites and Pathogens of Insects: Parasites,* eds. Beckage, N. C., Thompson, S. N. & Federici, B. A. (Academic, San Diego), Vol. 2, pp. 317–342.
25. Gatehouse, A. M. R., Boulter, D. & Hilder, V. A. (1992) in *Plant Genetic Manipulation for Crop Protection,* eds. Gatehouse, A. M. R., Hilder, V. A. & Boulter, D. (CAB, Wallingford, Oxon, U.K.), pp. 155–182.