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Metabolism of Pisatin Stereoisomers by *Ascochyta rabiei* Strains Transformed with the Pisatin Demethylase Gene of *Nectria haematococca* MP VI

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The chickpea pathogen *Ascochyta rabiei* was transformed with plasmid pUCH1/PDA containing the hygromycin B resistance gene as a selectable marker and the pisatin demethylase gene of *Nectria haematococca* mating population VI, a pathogen of pea. The rate of transformation was approximately 2 transformants per µg of DNA. While untransformed *A. rabiei* is inhibited by hygromycin B at 50 µg/ml, transformants were able to grow on 300 µg of this antibiotic. Transformants demethylated the pea phytoalexin (+)pisatin to (+)6a-hydroxymaackiain (6a-HMK) within 2 to 4 h. The product was not further metabolized and accumulated in the medium. Untransformed strains failed to show any significant metabolism of (+)pisatin to (+)6a-HMK within a given interval of 24 h. One of the transformants, when tested for metabolism of (–)pisatin, produced a demethylation product that only transiently accumulated in the medium. One subsequent degradation product was tentatively identified as 3,7,2′-trihydroxy-4′,5′-methylenedioxy isoflavan. These results support the hypothesis that the enzymes of *A. rabiei* involved in the metabolism of pterocarpan phytoalexins are specific for (–) isomers such as (–)maackiain and (–)medicarpin produced by the natural host of this fungus.

Additional keywords: phytopathogenic fungi, host–parasite interaction, ascomycetes.

A well-studied defense reaction of plants against a pathogenic microorganism is the accumulation of low molecular weight compounds called phytoalexins (Dixon 1986; Ebel 1986). The degradation of these substances to less toxic products has long been discussed as one means for a plant pathogen to overcome this defense barrier of the host (VanEtten et al 1989; Weltring 1992). The best studied example for the importance of phytoalexin degradation for virulence of a plant pathogen is the demethylation of the pea phytoalexin (+)pisatin by *Nectria haematococca* Berk. & Broome mating population (MP) VI, a pathogen of pea (*Pisum sativum*) (Fig. 1; VanEtten et al. 1975). Pathogenic isolates of this fungus demethylate the phytoalexin to the less toxic product (+)6a-hydroxymaackiain (6a-HMK) (VanEtten et al. 1980; Tegtmeier and VanEtten 1982). The enzyme involved in this reaction has been characterized as a microsomal NADPH-dependent cytochrome P-450 monooxygenase (Matthews and VanEtten 1983; Desjardins et al. 1984; Desjardins and VanEtten 1986). Pisatin demethylase (PDA) can be independently conferred by one of at least six *PDA* genes identified so far (Kistler and VanEtten 1984; Mackintosh et al. 1989; Miao and VanEtten 1992). One *PDA* gene, designated *PDAT9,* was cloned by expression in *Aspergillus nidulans* from a cosmid library of a pisatin-metabolizing isolate of *N. haematococca* MP VI (Weltring et al. 1988). The *PDA* gene was also expressed in *Cochliobolus heterostrophus* (Schäfer et al. 1989), which confirmed previous indications that the cytochrome P-450 hemoprotein encoded by *PDAT9* can be reduced by a reductase present in filamentous fungi other than *N. haematococca* MP VI (Scala et al. 1988).

Ascochyta rabiei (Pass.) Labrousse is the causal agent of Ascochyta blight of chickpea (*Cicer arietinum*). Resistant plants accumulate high amounts of the pterocarpan phytoalexins (–)medicarpin and (–)maackiain (Fig. 2) in response to infection by the fungus (Jaques et al. 1987). *A. rabiei* is able to degrade the chickpea phytoalexins completely (Höhl et al. 1989; Tenhaken et al. 1991). As the first step of degradation, both pterocarpans are simultaneously converted to the corresponding 2′-OH isoflavans and the 1a-OH pterocarpdiens (Fig. 2; Kraft et al. 1987). The catalyzing enzymes, a reductase and a hydroxylase, are substrate specific and are expressed constitutively in all isolates tested to date (Höhl and Barz 1987; Tenhaken et al. 1991). Enzymatic studies on the reductase and hydroxylase indicate that these enzymes of *A. rabiei* can only convert pterocarpans with a (–) stereochemistry, as found in chickpea (Fig. 2). Another example for this type of stereospecific metabolism of phytoalexins is the 6a-hydroxylation of the (–) isomers of medicarpin and

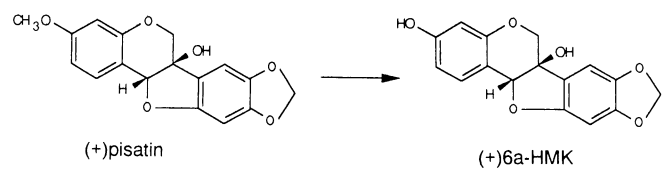

Fig. 1. Demethylation of (+)pisatin to (+)6a-HMK by *Nectria haematococca* MP VI.

maackiain by *N. haematococca* MP VI isolate T-95, which does not metabolize the (+) isomers of these compounds (VanEtten et al. 1983).

Because of the great resemblance of (+)6a-HMK, the demethylation product of pisatin produced by *N. haematococca* MP VI (Fig. 1), to maackiain, we wanted to investigate whether the (+)6a-HMK is further metabolized by *A. rabiei,* or the proposed natural specificity of the metabolizing enzymes of this fungus for the (–) isomers prevents further degradation of this compound. To avoid direct feeding and to assure that the (+)6a-HMK, which is not a natural substrate, is available to the degrading enzymes at the correct intracellular location, we wanted to use strains of *A. rabiei* transformed with the *PDAT9* gene of *N. haematococca* MP VI. For this purpose, a transformation system was developed based on hygromycin B resistance (*hygB*) as a selectable marker. In addition to the *hygB* gene, plasmid pUCH1/PDA, used for transformation, contained the *PDA* gene of *N. haematococca* MP VI (Turgeon et al. 1987; Weltring et al. 1988). Transformants were compared for metabolism of (+)pisatin, (+)6a-HMK, and the corresponding (–) isomers.

Our results show that even though transformed strains of *A. rabiei* carrying the *PDAT9* gene became capable of metabolizing (+)pisatin to (+)6a-HMK, the (+)6a-HMK was not further metabolized. In contrast, (–)6a-HMK produced from (–)pisatin by one transformant accumulated only transiently in the medium. These results support the hypothesis that the enzymes of phytopathogenic fungi involved in phytoalexin metabolism are adapted very closely to the stereochemistry of the phytoalexins of their host plants.

RESULTS

Transformation with plasmid pUCH1/PDA.

Three isolates of *A. rabiei,* referred to as strains 8, 9, and 10, were transformed with plasmid pUCH1/PDA (Fig. 3), which contains the *hygB* gene as a selectable marker fused to a *C. heterostrophus* promoter (Turgeon et al. 1987). In addition, the plasmid includes a 3.2-kb fragment of *N. haematococca* MP VI genomic DNA containing the entire *PDAT9* gene, including 5′ and 3′ regulatory sequences (Weltring et al. 1988; Maloney and VanEtten 1994).

Transformation rates of up to 2 transformants per µg of DNA were obtained. Five transformants of each strain were tested for hygromycin B resistance. All grew in the presence of 200 µg of hygromycin B per ml in the medium, and 10 transformants even tolerated 300 µg of the antibiotic per ml. In contrast, growth of the untransformed strains was completely inhibited by 50 µg/ml. The resistant phenotype was stable under nonselective conditions, although some transformants seemed to lose their hygromycin B resistance gradually when grown on nonselective medium for several passages. Nevertheless, these transformants were still able to grow on 150 µg of hygromycin B per ml.

Southern analysis.

Three transformants of strain 8 and two each of strains 9 and 10 were subjected to Southern analysis. DNA of transformants and untransformed strains was digested with *Bam*HI, which cuts twice in plasmid pUCH1/PDA and gives rise to a 3.2-kb fragment and a 5.6-kb fragment. In some cases, these fragments were visible on ethidium bromide stained gels within the smear of restriction fragments of the genomic DNA (data not shown). This suggested that the plasmid was integrated into the genome of these transformants in high copy numbers in a head-to-tail arrangement. Another possible explanation of a plasmid replicating autonomously was disproved by Southern analysis of uncut DNA of the transformants. In all cases, no free plasmid was detectable (data not shown).

The pattern of hybridizing fragments in the genomic DNA of the transformants (Fig. 4) showed that, with the exception of strain 8-1-1, all had integrated several copies of plasmid pUCH1/PDA into the genome. The DNA of strains 8-7-2, 9-8-3, 9-9-1, and 10-4-1 contained the 3.2-kb and 5.6-kb *Bam*HI fragments of pUCH1/PDA, indicating that these transformants integrated several copies of the plasmid in a head-to-tail order. While transformant 9-8-3 seemed to have integrated these copies at one location, the large number of additional hybridizing fragments in the other transformants suggested that integration of multiple copies of pUCH1/PDA might have taken place at several locations in the genome.

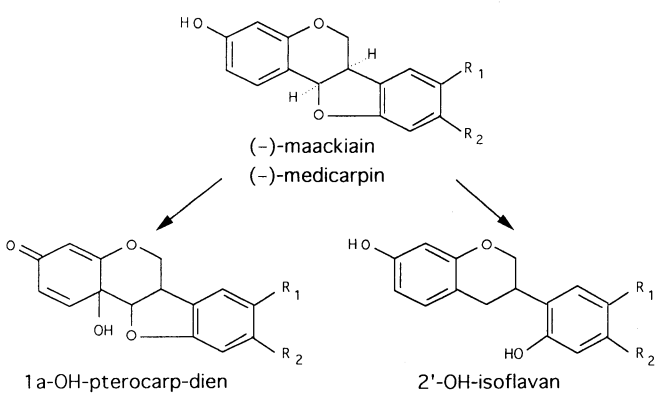

Fig. 2. Initial steps of metabolism of (–)maackiain and (–)medicarpin by *Ascochyta rabiei*. Medicarpin: R_1 = H, R_2 = OCH_3, maackiain: $R_1;R_2$ = OCH_2O.

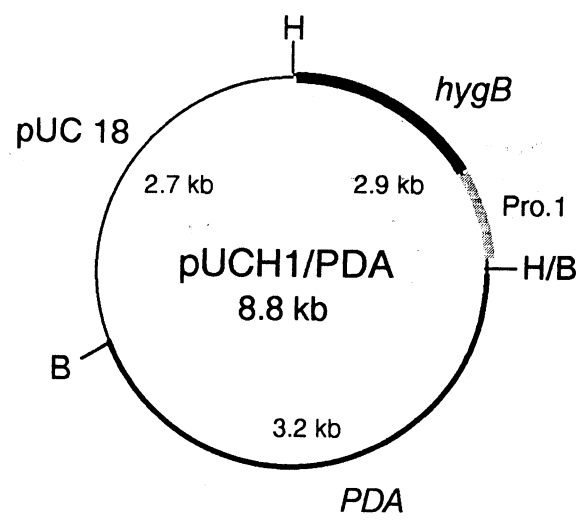

Fig. 3. Restriction map of plasmid pUCH1/PDA. H = *Hin*dIII, B = *Bam*HI, Pro.1 = Promoter 1 of *Cochliobolus heterostrophus, hygB* = hygromycin B resistance gene, *PDA* = pisatin demethylase gene *PDAT9* of *Nectria haematococca.*

The latter is true also for transformants 8-14-1 and 10-3-2, but in these cases integration of only one copy of the plasmid at different locations had occurred. The lack of a hybridizing 3.2-kb *Bam*HI fragment containing the *PDA* gene could indicate that this fragment was involved in the recombination event leading to integration of the plasmid into the genome. In this case, the site of recombination must be located outside of the coding region of the *PDA* gene, because both transformants are able to demethylate pisatin (see below). Transformant 8-1-1 integrated only one copy of the plasmid, with no *Bam*HI site left. A possible explanation for this loss of the restriction sites is the deletion of the PDA fragment of pUCH1/PDA, which is flanked by the *Bam*HI sites. This would also explain why this transformant was not able to metabolize pisatin (see below).

The number of hybridizing fragments and their intensity in the DNA of the transformants was not correlated with hygromycin B tolerance. For example, transformants 8-1-1 and 9-9-1 were equally tolerant to the antibiotic, although the latter transformant contained many more copies of the plasmid (Fig. 4).

Pisatin demethylation.

A. rabiei strains 8, 9, and 10 and their transformants were analyzed for their ability to demethylate (+)pisatin. For this purpose, mycelium of each strain was incubated with 5×10^{-5} M (+)pisatin as substrate, and the metabolism was measured by high-pressure liquid chromatography (HPLC) using extracts of aliquots taken at different time intervals. As shown in Figure 5, pisatin disappeared from the medium of untransformed strains within 10 to 24 h. As sole metabolite, (+)6a-HMK, the demethylation product of pisatin, was detectable in trace amounts. This metabolite was identified by its UV spectrum and by comparison of the HPLC retention time with authentic (+)6a-HMK.

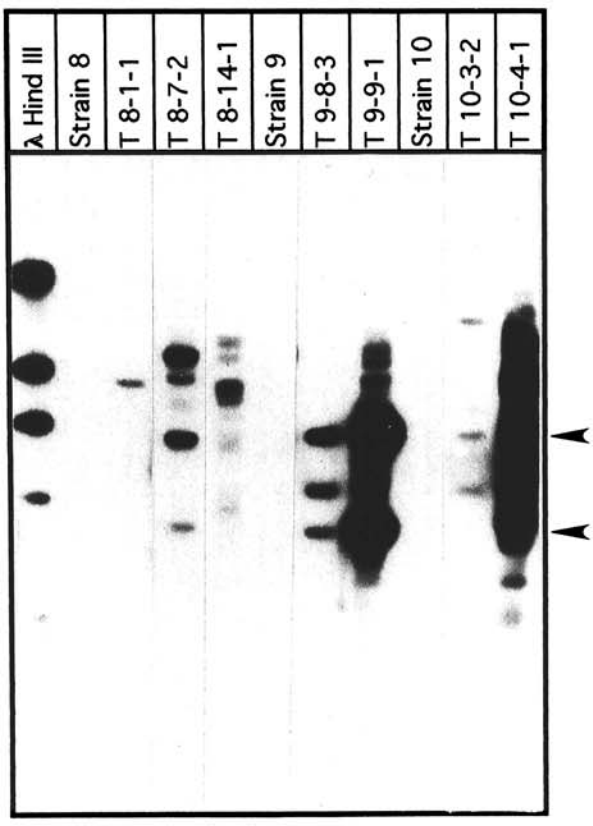

Fig. 4. Southern blot analysis of DNA of strains 8, 9, and 10 and transformants 8-1-1, 8-7-2, 8-14-1, 9-8-3, 9-9-1, 10-3-2, and 10-4-1. DNA was cut with *Bam*HI and hybridized to radioactively labeled pUCH1/PDA. The arrows indicate the positions of the 3.2-kb and 5.6-kb *Bam*HI fragments of plasmid pUCH1/PDA (Fig. 3).

In contrast to the wild-type strains, all tested transformants with the exception of strain 8-1-1 rapidly converted (+)pisatin to the demethylation product (+)6a-HMK, which was not further metabolized over the test period of 24 h. The conversion usually occurred within approximately 2 to 4 h with no detectable lag phase (Fig. 5, e.g., transformant 8-14-1) except for transformant 10-3-2, which needed around 12 h (Fig. 5). In any case, the demethylation rate of the transformants was much faster than the rate of disappearance of (+)pisatin from the medium of untransformed strains.

No difference was found between mycelium preincubated with (+)pisatin for 18 to 20 h and untreated mycelium (data not shown). This indicates that the *PDA* gene is constitutively expressed in *A. rabiei* essentially as in *C. heterostrophus* and *A. nidulans* (Schäfer et al. 1989; Weltring et al. 1988). This constitutive expression is in contrast to *N. haematococca* MP VI, in which expression is induced by the substrate (VanEtten and Matthews 1984).

As mentioned above, transformant 8-1-1 was an exception insofar as it showed the same demethylation phenotype as untransformed strain 8. This transformant was not able to metabolize pisatin within 24 h, and it accumulated only trace amounts of (+)6a-HMK. This suggests that 8-1-1 does not contain a functional *PDA* gene. Transformant 10-3-2 also seemed to have a reduced demethylation activity, but in this case the accumulation of (+)6a-HMK proves that it contained a functional *PDA* gene.

A possible explanation for the quantitative accumulation of (+)6a-HMK by the transformants and of only trace amounts of this compound by the untransformed strains is the (+)configuration of this pterocarpan, which is the opposite stereoisomer of the configuration found in the phytoalexins maackiain and medicarpin of chickpea, the natural host of *A. rabiei* (Jaques et al. 1987). This implies that the enzymes of *A. rabiei* for maackiain and medicarpin degradation are specific for the (–) configuration (Tenhaken et al. 1991). To obtain further evidence for this hypothesis, (–)pisatin was used as substrate. Since pisatin demethylase of *N. haematococca* was shown to be nonstereospecific, this stereoisomer of pisatin is readily converted to (–)6a-HMK by the enzyme (VanEtten et al. 1989).

As shown in Figure 6, transformant 9-9-1 was able to further metabolize the demethylation product, (–)6a-HMK. This proves that the enzymes of *A. rabiei* are indeed specific for the (–) configuration of chickpea pterocarpans (Tenhaken et al. 1991). A second product, which accumulated transiently at the expense of (–)6a-HMK, was tentatively identified by its UV spectrum and its retention time during HPLC as 3,7,2′-trihydroxy-4′,5′-methylenedioxy isoflavan. In a parallel experiment with transformant 9-9-1 using (+)pisatin as substrate, this isoflavan was not detectable and (+)6a-HMK accumulated in the medium (Fig. 6). Therefore, the slow metabolism of (+)pisatin by *A. rabiei* remains to be elucidated.

DISCUSSION

This paper reports the successful transformation of the chickpea pathogen *A. rabiei*. We chose the *hygB* gene as a selection marker because *A. rabiei* is very sensitive to the antibiotic and the vector containing the *PDA* gene carried the corresponding resistance gene. The tolerance of all transformants to large amounts of hygromycin B proves that the promoter from *C. heterostrophus* fused to the *hygB* gene functions in *A. rabiei* essentially as shown for other filamentous

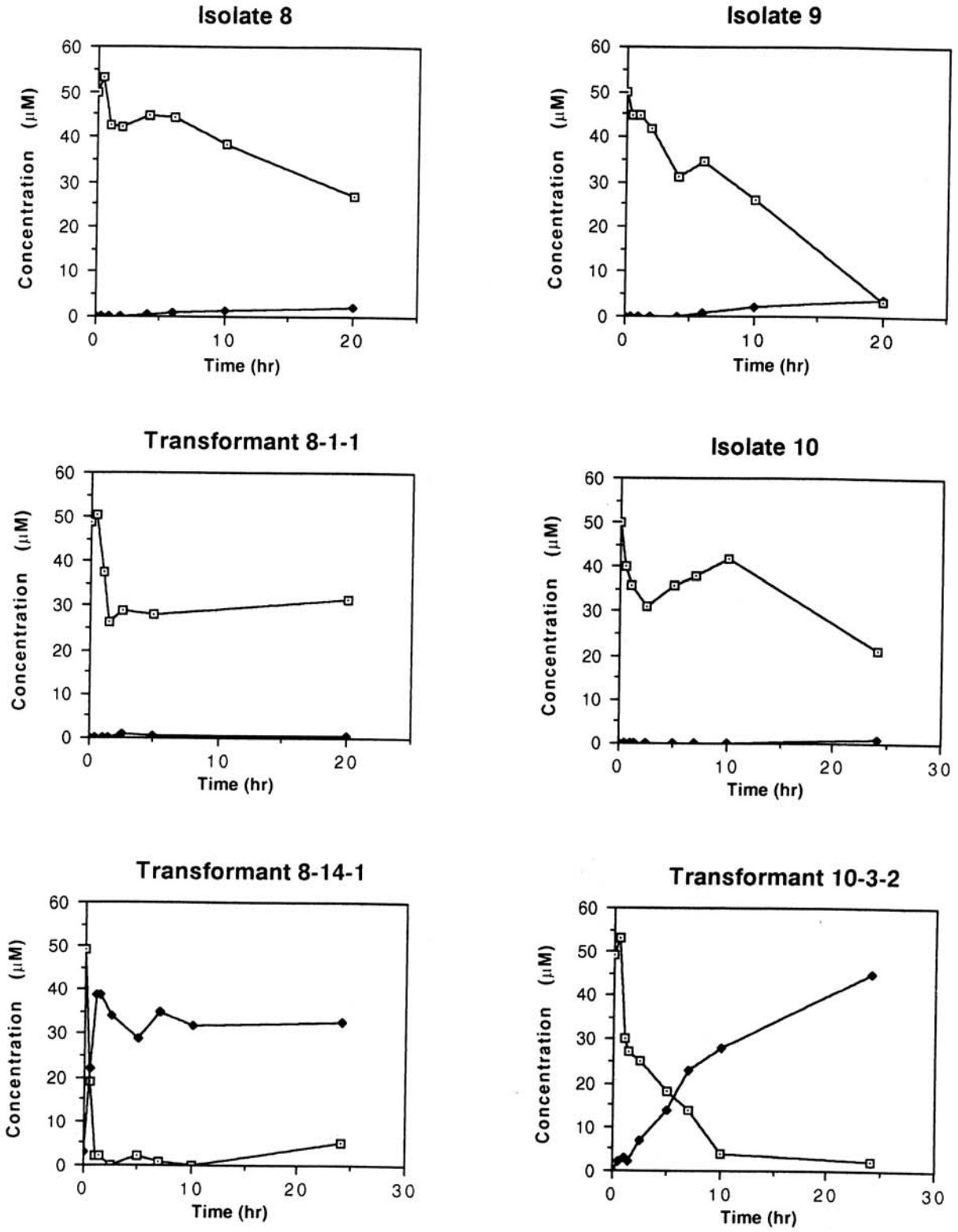

Fig. 5. Time course of (+)pisatin demethylation by *Ascochyta rabiei* strains 8, 9, and 10, and some representative transformants. —□— (+)pisatin, —•— (+)6a-HMK

fungi (Turgeon et al. 1987). On the other hand, this heterologous promoter is possibly one explanation for the low transformation efficiency of up to 2 transformants per µg of DNA. The use of a strong homologous promoter, for example of the *Gpd* gene, or the use of the *nia*D selection system would probably improve the transformation rate, as shown for *Aspergillus* (Punt et al. 1987; Campbell et al. 1989; Unkles et al. 1989).

With one exception, the plasmid pUCH1/PDA was integrated into the genome of the transformants in several copies with no obvious effect on the degree of resistance to hygromycin B. For example, transformant 8-1-1, which contains only one copy of the *hygB* gene (Fig. 4), is as resistant to the antibiotic as transformant 9-9-1, which contains many copies. This result has also been found with other fungi (Rodriguez and Yoder 1987).

The ability of the transformants to demethylate (+)pisatin indicates that the *PDA* gene is functional in *A. rabiei.* This suggests that the transcription signals and the splicing of the four introns of this gene of *N. haematococca* MP VI (Maloney and VanEtten 1994) are recognized and correctly processed in *A. rabiei.* Since this gene is also active in *A. nidulans* and *C. heterostrophus* (Schäfer et al. 1989; Weltring et al. 1988), our experiment provides an additional demonstration that genes of filamentous fungi are interchangeable among these organisms.

The results obtained with the *PDA* transformants support the hypothesis that the enzymes involved in maackiain metabolism are specific for the stereoisomers (Tenhaken et al. 1991; VanEtten et al. 1983). This is the best explanation for the result that the (+)6a-HMK produced by the transformants is not further metabolized. The detection of 3,7,2′-trihydroxy-4′,5′-methylenedioxy isoflavan shows that (–)6a-HMK is mainly metabolized via the reductive cleavage of the dihydrofuran ring (Fig. 2). These results are supported by enzymatic studies on the reductase and 1a-hydroxylase involved in the metabolism of maackiain and medicarpin (Fig. 2; Höhl and Barz 1987; Tenhaken et al. 1991) and by recent experiments by R. Tenhaken and W. Barz (*unpublished*), who showed that *A. rabiei* is able to metabolize (–)6a-HMK, which was obtained by bioconversion of (–)maackiain by *N. haematococca* MP VI isolate T-200 (Lucy et al. 1988).

Similar results with regard to stereospecific detoxification of (+) and (–) isomers of maackiain and differential sensitivity to these isomers were obtained with *N. haematococca* MP VI (VanEtten et al. 1983) and pathogens of red clover (Delserone et al. 1992), respectively. In the first case, *N. haematococca* MP VI isolate T-95 6a-hydroxylated the (–) isomer of maackiain but failed to metabolize the (+) isomer. In the second study, five out of seven fungal pathogens of red clover were inhibited by (–)maackiain, which is the isomer produced by this plant. These results and the data presented in this paper provide the basis for experiments suggested by VanEtten et al. (1989). Plants like pea or chickpea are to be transformed with genes coding for biosynthetic enzymes that control the stereochemistry of pterocarpan phytoalexins. The result would be transgenic plants that produce phytoalexins with the opposite stereochemistry. These plants should be more resistant to pathogens that depend on the ability to metabolize the phytoalexins of their host plant in order to be pathogenic.

MATERIALS AND METHODS

Strains and plasmids.

The *A. rabiei* strains 8, 9, and 10 were supplied by M. C. Saxena, ICARDA, Syria, and have been described previously (Höhl et al. 1989). The fungi were grown on solid chickpea extract medium (Kraft et al. 1987) and in liquid modified Richard's medium (Tenhaken et al. 1991).

Escherichia coli strain HB101 was used for propagation of pUCH1/PDA. This plasmid was constructed by ligating a 3.2-kb fragment of *N. haematococca* MP VI genomic DNA (Weltring et al. 1988) containing the *PDA* gene into plasmid pUCH1 containing promoter 1 of *C. heterostrophus* in front of the *hygB* gene (Turgeon et al. 1987).

Chemicals.

Hygromycin B, ampicillin, restriction endonucleases, and random primed labeling kit were purchased from Boehringer, Mannheim, Germany; Novozym 234 from Novo Nordisk A/S, Copenhagen, Denmark; ß-glucuronidase from Sigma Chemical Co., St. Louis, MO; $CaCl_2$, polyethylene glycol 4000, and sodium dodecyl sulfate (SDS) from Merck Darmstadt, Germany; Biodyne A membrane from Pall, Dreieich, Germany; and ^{32}P dATP from Amersham Buchlev, Braunschweig, Germany.

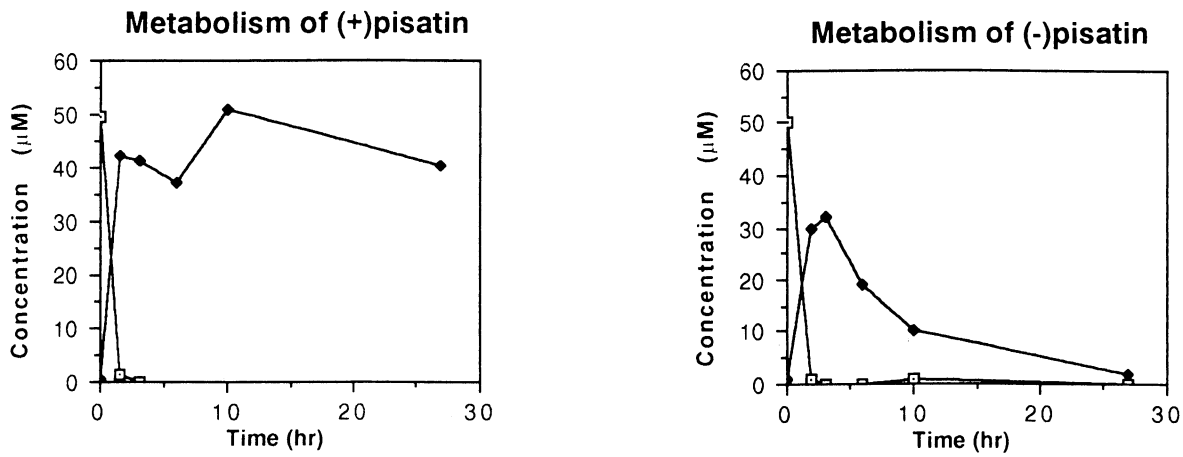

Fig. 6. Demethylation of (+) and (–)pisatin by *Ascochyta rabiei* transformant 9-9-1. —□— pisatin, —♦— 6a-HMK

A small amount of (–)pisatin was a gift of H. D. VanEtten. (+)Pisatin was isolated from etiolated pea germlings as described by VanEtten et al. (1980). (+)6a-HMK was obtained by incubating transformants of *A. rabiei* with (+)pisatin for 10 h, followed by an ether extraction of the product from the incubation medium (10 mM potassium phosphate buffer, pH 7.5). The ether was evaporated, and the residue was dissolved in methanol and submitted to HPLC (RP 18 column, Merck) for further purification. HPLC pure 6a-HMK was characterized by co-chromatography with authentic material on HPLC and thin-layer chromatography (TLC) in different solvent systems, and by using diagnostic reagents (Mabry et al. 1970).

DNA preparation.

Plasmid DNA was isolated according to Maniatis et al. (1982). Fungal DNA was prepared from lyophilized mycelium grown for 6 to 16 days in modified Richard's medium as described by Yelton et al. 1984.

Transformation.

A total of 5×10^8 spores was inoculated in 250 ml of modified Richard's medium for at least 24 h. Protoplasting of the germinated spores and transformation were carried out as described by Yelton et al. (1984) with the following modifications. Germinated spores were incubated in 10 ml of osmotic medium per g (fresh weight) containing 5 mg of Novozym 234 per ml and 0.4 ml of ß-glucuronidase for 3 h. After transformation, protoplasts were resuspended in 200 µl of STC (1.2 M sorbitol/10 mM Tris-HCl, pH 7.5/10 mM $CaCl_2$ divided into four aliquots. Each aliquot was embedded in 20 ml of chickpea medium containing molten agar and 1 M sucrose as osmoticum. After 16 h, the plates were overlaid with 10 ml of top agar containing 2.25 mg of hygromycin B (final concentration 75 µg/ml). Transformants were visible after 1 to 2 weeks. For further analysis, all tested transformants were purified by single sporing.

Southern analysis.

DNA was separated on 0.6% agarose gels, transferred to Pall Biodyne B membrane by capillary blotting, and hybridized under stringent conditions according to the supplier's manual. Plasmid DNA was labeled with a random primed labeling and detection kit (Boehringer) according to the manufacturer's instructions.

Metabolism assay.

Mycelium (5 g fresh weight [FW]) was incubated in 100 ml of potassium phosphate buffer (10 mM, pH 7.5) on a rotary shaker at 22°C, 150 rpm with 5×10^{-5} M (+)pisatin or 2×10^{-5} M (+)6a-HMK as substrate. At different times, 5-ml aliquots were taken and extracted two times with 3 ml of diethylether. The organic phase was evaporated. The residue was dissolved in 150 µl of methanol and analyzed by HPLC (Waters, Eschberg, Germany) on an RP 18 column (Merck) in a gradient of 40 to 90% acetonitrile in water within 15 min. Substrates and products of the extracts were detected by the Photo Diode-Array-System 990 (Waters), which allowed the UV spectral analysis of each compound in the extracts. Concentrations of pisatin and 6a-HMK were calculated from the HPLC signal measured at 309 nm.

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