INDUCTION OF CYTOCHROME P450 GENES BY ETHANOL IN MAIZE

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SUMMARY

Ethanol treatment of etiolated maize seedlings led to a marked enhancement in metabolism of the herbicides metolachlor and prosulfuron. cDNA clones representing eight genes that encode putative cytochrome P450 enzymes were isolated from maize. They fall into three families and are designated CYP71C5, CYP73A6, A7, and A8, and CYP81A1, A2, A3, and A4. Ethanol treatment induced the CYP81A subfamily at the mRNA level in both roots and shoots of etiolated seedlings.

KEY WORDS

herbicide metabolism, detoxification, *Zea mays*

INTRODUCTION

The complex microsomal mixed function oxidase system provides intermediates for a large number of metabolic pathways and has been shown to oxidize xenobiotics in a variety of organisms. As available molecular information about the cyt P450 superfamily in plants increases, the effects of xenobiotics on the induction of metabolic enzyme systems can be investigated at the transcriptional level, bypassing the need for purification of proteins often present only in small amounts.

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Application of various organic compounds, including herbicide safeners /1-3/ and ethanol, has been shown to enhance herbicide oxidation in monocots. In higher plants the inductive action of ethanol was first reported by Reichart *et al.* /4/ to increase total cyt P450 levels in Jerusalem artichoke tubers. In subsequent studies with wheat, ethanol did not increase cyt P450 concentration, but did enhance the rate of hydroxylation of the herbicides diclofop, triasulfuron, and chorsulfuron /5/. In maize, ethanol treatment also enhanced the activity of flumetsulam hydroxylase /6/. Here, we show that in maize, ethanol treatment increases metabolism of endogenous and exogenous substrates and also increases levels of several cyt P450 mRNAs.

MATERIALS AND METHODS

First strand cDNA was synthesized from mRNA isolated from leaves of 6-day old maize hybrid Blizzard seedlings by phenol/ chloroform extraction followed by lithium chloride precipitation /7/. cyt P450s CYP71C5 and CYP73A7 were amplified using primers 1 and 2 (Fig. 1) which were designed to correspond to regions of similarity between avocado CYP71A1 /8/ and mung bean cinnamate 4-hydroxylase (C4H) CYP73A2 /9/. Primer 1 was based on the amino acid consensus sequence YGE(H/Y)WR and primer 2 on the sequence PFG(A/V)GR in the heme-binding region.

For induction studies, captan-treated maize seeds (Pioneer hybrid 3245) were sown on germination paper which was folded and placed upright in 1 l beakers containing 400 ml 0.5-strength Hoagland's solution. Seeds were germinated in the dark at 28°C for 72 hours. Where used, ethanol was applied by subirrigation as a 10% solution 24 hours before the tissue was harvested. Treatments were divided, with half of the roots and shoots frozen in liquid nitrogen for later RNA extraction and the remaining shoots harvested for preparation of microsomes. Isolation of microsomes, measurements of cytochromes P450, P420, and protein concentrations, enzyme assays, and metabolite identifications were made following procedures detailed previously /10/.

Using a method similar to that described by Meijer *et al.* /11/, CYP81A1 and CYP81A2 PCR fragments were amplified from first strand cDNA from the ethanol-treated roots described above using a primer based on the heme-binding domain consensus sequence and an oligo dT primer. These fragments and the CYP71C5 and CYP73A7

fragments were labeled with the random priming DNA labeling system (Life Technologies, Gaithersburg, MD) and used to probe a maize hybrid Blizzard etiolated shoot Lambda ZAP cDNA library (Stratagene, La Jolla, CA). Duplicate plaque lifts were made with nitrocellulose filters (Schleicher & Schuell, Keene, NH) and hybridized and washed at 50°C /12/. Plasmids were *in vivo* excised and sequenced using fluorescent dideoxy terminators (Applied Biosystems, Foster City, CA). Sequence alignments were performed using the GAP and PILEUP programs of GCG /13/.

For mRNA induction analysis, samples (10 μg) were separated electrophoretically through formaldehyde agarose gels and capillary blotted to nylon membrane (Genescreen Plus, NEN, Boston, MA) as described /14/. Hybridization and washing was at 65°C according to Church and Gilbert /12/. Induction of mRNA levels was measured using a Betascope blot analyzer (Betagen, Waltham, MA).

RESULTS

We investigated the effect of ethanol treatment on metabolism of six substrates by microsomal membranes from etiolated maize shoots. Identification of the metabolites was confirmed by cochromatography with authentic standards. If standards were not available, identities were postulated from reports in the literature. Cinnamic acid was aryl hydroxylated forming p-coumaric acid and a mixture of hydroxylated products was formed from lauric acid (C-8 through C-12) which remain to be resolved. Of the two herbicides, metolachlor was O-demethylated and prosulfuron (CGA-152005) was hydroxylated at the C-5 position of the phenyl ring. Two metabolites were formed from each of the organophosphate insecticides (diazinon and isazofos): the phosphorothioate moiety was oxidatively desulfurated to the corresponding oxon and the thiophosphate ester oxygen was cleaved (Table 1).

The rate of metabolite formation by microsomes isolated from shoots of untreated and ethanol-treated seedlings is shown in Table 1. Metabolism of the five substrates other than cinnamic acid was enhanced from 2.4- to 15.7-fold with the greatest effect being reflected in the metabolism of metolachlor.

Ethanol did not affect the concentrations of cytochromes P450 and P420 in microsomal fractions. The concentrations of the two cytochromes were approximately 111 and 7 pmol/mg protein, respectively, in preparations from both untreated and ethanol-treated tissue.

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          1                                                            50
CYP73A7   .......... .......... .......... ..MDLALLEK ALLGLFAAAV
CYP73A6   .......... .......... .......... . MVLLFVEK LLVGLLASVM
CYP73A8   MAVSAARMAV ATAVSLAVHW LLRSFLQAQH PALGLLFPTA VFIGIAASGN
CYP81A2   .......... .......... ........MD KAYVAVLSFA FLFVLHYLVG
CYP81A3   .......... .......... .......... .......... ..........
CYP81A4   .......... .......... .......... .......... ..........
CYP81A1   .......... .......... .......... .......... ..........
CYP71C5   .......... .......... .......... .......... ..........

          51                                                           100
CYP73A7   VAIAVAKLTG KRYRLPPGPP GAPVVGNWLQ VGDDLNHRNL MAMAKRFGDI
CYP73A6   VAIAVSKIRG RKLRLPPGPV PVPVFGNWLQ VGDDLNHRNL AALSRKFGDV
CYP73A8   VAGAANDA.. .....PPGPP AVPVFGNWLQ VA.RLNHRFL ARLSARYGPV
CYP81A2   RAGRKGNGKG KGTQRLPPSP PAVPFLGHLH LVKTPFHEAL AGLAARHGPV
CYP81A3   .......... .......... .......... .......... ..........
CYP81A4   .......... .......... .......... .......... ..........
CYP81A1   .......... .......... .......... .......... ..........
CYP71C5   .......... .......... .......... .......... ..........

          101                                                          150
CYP73A7   FLLRMGVRNL VVVSTPELAK EVLHTQGVEF GSRTRNVVFD IFTGKGQDMV
CYP73A6   LLLRMGQRNL VVVSSPPLAR EVLHTQGVEF GSRTRNVVFD IFTDKGQDMV
CYP73A8   FRLRLGVRNL VVVSDPRLAT EVLHTQGVEF GSRPRNVVFD IFTANGADMV
CYP81A2   FSMRMGSRRA LVVSSPDCAK ECFTEHDVAF ANRPRFATQD LVSFGGAALA
CYP81A3   .......... .......... .......... .......... ..........
CYP81A4   .......... .......... .......... .......... ..........
CYP81A1   .......... .......... .......... .......... ..........
CYP71C5   .......... .......... .......... .......... ..........

            Primer 1
          151------>                                                   200
CYP73A7   FTVYGDHWRK MRRIMTVPFF TNKVVAQNRA GWEEEARLVV EDVRKDPEAA
CYP73A6   FTVYGDHWRK MRRIMTVPFF TNKVVQQYRH GWEAEAAAVV DDVRLDPKAA
CYP73A8   FTEYGDHWRR MRRVMTLPFF TARVVQQYRG MWEAEMDAAV SDVASAAQRA
CYP81A2   RASYGPYWRN LRRVATVQLL SAHRVACMSA VVAAEVRAMA RRMGRAAAAA
CYP81A3   .......... .......... .......... .......... ..........
CYP81A4   .......... .......... .......... .......... ..........
CYP81A1   .......... .......... .......... .......... ..........
CYP71C5   ...YGEHWRQ ARKLVTAHLF TVKRVHSYRR ARKEEVRLVV AKVREAAVAG

          201                                                          250
CYP73A7   AGGVVLRRRL QLMMYNDMFR IMFDRRFDSE HDPLFNKLKA LNAERSRLSQ
CYP73A6   TDGIVLRRRL QLMMYNNVYR IMFDRRFESM DDPLFLRLRA LNGERSRLAQ
CYP73A8   A.GLVVRRRL QLMLYNIMYG MMFDARFGSV DDPMFVEATR FNSERSRLAQ
CYP81A2   PGGAARVQLK RRLFEVSLSV LMETIARTKT SRAEADADSD MSPEAHEFKQ
CYP81A3   .......... .......... .......... .......... ..........
CYP81A4   .......... .......... .......... .......... ..........
CYP81A1   .......... .......... .......... .......... ..........
CYP71C5   TATDMSLAMN TFANDIISRA VSGKFFRAEG RNKLFRELVE ANSALFGGFN

          251                                                          300
CYP73A7   SFEYNYGDFI PVL....... ..RPFLRGYL NRCHDLKTRR MKVFEDNFVQ
CYP73A6   SFEYNYGDFI PIL....... ..RPFLRGYL RVCKEVKETR LKLFKDFFLE
CYP73A8   SFDYNYGDFI PVL....... ..RPFLRGYL ARCKDLQARR LAFFNSNYVE
CYP81A2   IVDEIVPHLG TANLWDYLPV LRWLDVFGVR NKITAAVGRR DAFLRRLIDA
CYP81A3   .......... .......... .......... .......... .....RLIDA
CYP81A4   .......... .......... .......... .......... ..........
CYP81A1   .......... .......... .......... .......... ..........
CYP71C5   PGKDYFPGLA RALGF....L SRRLLFLRSR RRVQETHRRW DELLETILSD
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          301                                                        350
CYP73A7   ERKKV....M AQTGEIR... .CAMDHILEA ERKGEINHDN VLYIVENINV
CYP73A6   ERKKLASTKA TDSNGLK... .CAIDHILEA QQKGEINEDN VLFIVENINV
CYP73A8   KRRKVMDA.P GDKGKLR... .CAIDHVLQA EKSGEITPEN VIYIVENINV
CYP81A2   ERRSLDDGGG DGDSDSDKKS MIAVLLSLQK SEPEVYTDTM IMALCGNLFG
CYP81A3   ERRRM..... DGDGDGEKKS MIAVLLSLQK SEPELYTDTM IMALCGDLFG
CYP81A4   .......... ........KS MIGVLLSLQK SEPEVYTDTM IMALCSSMFA
CYP81A1   .......... .......... .......... .......... ......NLLA
CYP71C5   HEGRRGSVSV DGGGD..... FTDVLLSVQT EYG..MTRDH LKAILVDMFG

          351                                                        400
CYP73A7   AAIETTLWSI EWGIAELVNH PAIQHKLREE LASVLGAGVP .VTEPDLERL
CYP73A6   AAIETTLWSI EWAVAELVNH PEIQQKLRQE LDTVLGPGHQ .ITEPDTHNL
CYP73A8   AAIETTLWSI EWALAEVVNH PAVQRKVRDE IKAVVADHEP .ITESTIHRL
CYP81A2   AGTETTSTTT EWAMSLLLNH PEALKKAQAE IDAVVGTSR. LLAAEDVPRL
CYP81A3   AGTETTSVTT EWAMSLLLNH PEALKKAQSE IDAVVGSSR. LITADDVPRL
CYP81A4   GGSETTATTA EWAMSLLLSH PDVLKKAQAE IDASVGHSR. LLGADDLPRL
CYP81A1   AGTETTSTTL EWAMSLLLNH PDVLKRAQEE IESNVGRDR. LLDKNDLPRL
CYP71C5   AGTDTSSLVL ELAMAELMRN PQQMAKLQAQ VRRHTPEGQE TVEEENLSDM

          401                                                        450
CYP73A7   PYLQAIVKET LRLRMAIPLL VPHMNLNDGK LAGFDIPAES KILVNAWFLA
CYP73A6   PYLQAVIKET LRLRMAIPLL VPHMNLHDAK LGGYDIPAES KILVNAWYLA
CYP73A8   PYLQAVIKET LRLHSPIPLL VPHMNLEEAK LGGYTIPKGS KVVVNAWWLA
CYP81A2   GYLHRVISET LRMYPAAPLL LPHESSADCK VGGYDVARGT LLIVNAYAIH
CYP81A3   GYLHCVINET LRMYPAAPLL LPHESAADCK VGGYDVPRGT LLIVNAYAIH
CYP81A4   GYLHCIVSET LRLYPVVPTL VPHESTADCT VGGHRVPSGT MLLVNVYAIH
CYP81A1   PYLHCIISET LRLYPPTPML LPHEASTDCK IHGYDVPAGS MVLVNAYAIH
CYP71C5   PFLRAVVKET LRLHPPAPLL VPHLSLADCV VDGYHVPSGT RVIINAWALG

                                                    Primer 2
          451                                                        500
CYP73A7   NDPKRWVRPD EFRPERFLEE EKSVEAHGN. ..DFRFVPFG VGRRSCPGII
CYP73A6   NNPDSWRRPE EFRPERFLEE EKHVEANGN. ..DFRYLPFG VGRRSCPGII
CYP73A8   NNPELWDKPE EFRPERFLGE EKSVDATVGG KVDFRFLPFG VGRRSCPGII
CYP81A2   RDPLVWEDPD EFRPERFEDG ........KA EGRL.LMPFG MGRRKCPGET
CYP81A3   RDPAVWEDPG RFVPERFEDG ........KA EGRL.LMPFG MGRRKCPGET
CYP81A4   RDPAIWADPA AFRPERFEDG ........RA DGLF.VMPFG MGRRKCPGEA
CYP81A1   RDPAMWEDPE EFRPERFELG ........RA EGKF.MMPFW VGRRRCPGEN
CYP71C5   RDPGSWEKPE EFLPERFMDG GSAAGVDIKG NHFH.LLPFG AGRRICPGLN

          501                                                        550
CYP73A7   LALPIIGITL GRLVQNFQLL PPPGLDKIDT T.EKPGQFSN QIAKHATIVC
CYP73A6   LALPILGITI GRLVQNFELL PPPGQDKVDT T.EKGGQFSL HILKHSTIVC
CYP73A8   LALPILALIV GKLVRSFEMV PPPGVEKLDV S.EKGGQFSL HIANHSVIAF
CYP81A2   LALRTISLVL GTLIQCFDWD ...RVDGHEI DMAAGGGLTL PKAVPLEATC
CYP81A3   LALRTVGLVL GTLLQCFDWD ...TVDGAEV DMTESGGLTM PRAVPLEAMC
CYP81A4   LALRTLGLVL GTLIQCFDWD ...TVGGAEV DMAEGGGITL PRAVPLEAIC
CYP81A1   LAMRTMGLVL GALLQCFDWT ...RVGDREV DMATATGTIM SKAVPLEAQC
CYP71C5   FGMATVEIML ANLVYCFDWQ LPMGMEEKDI DMTEVFGLTV HPKEKLMLVP

          551                                                  593
CYP73A7   KPLEA*
CYP73A6   KPRTL*
CYP73A8   HPVSA*
CYP81A2   KPRAAMRHLL LEL*
CYP81A3   KPRAAMCDVL REL*
CYP81A4   KPRHAMLGVL KGL*
CYP81A1   KPRANMSAVL QKI*
CYP71C5   KVARVLQLLV NNSIVQVADR RDSRSIMHVY GLFGVENCMQ RV*
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Fig. 1: Alignment of predicted protein sequences of the maize cyt P450 cDNAs. PCR primers are indicated by arrows.

TABLE 1

Comparative metabolism of six substrates by microsomal membranes isolated from etiolated shoots of untreated and ethanol-treated corn seedlings

Substrate	Treatment[a] None	Ethanol	Ratio[b]
Cinnamic acid	49.69	53.43	1.1
Lauric acid	2.15	5.07	2.4
Metolachlor	0.10	1.57	15.7
Prosulfuron	0.02	0.12	6.0
Diazinon	0.04	0.20	5.0
Isazofos	0.09	0.46	5.1

[a] Metabolite formation is quantified as nmol/mg protein/h.

[b] The concentration of metabolites formed by microsomes isolated from shoots of ethanol-treated seedlings was divided by the concentration of metabolites formed by microsomes isolated from untreated seedlings. A ratio > 1.0 identifies the extent of enhancement attributed to the ethanol treatment.

Data for those substrates in which multiple metabolites were formed (lauric acid, diazinon, and isazofos) are presented as the sum of the concentration of the individual metabolites.

We used molecular methods to study the effects of chemical treatment at the transcriptional level. To isolate novel maize cyt P450 cDNAs, we initially used primers complementary to conserved regions between avocado CYP71A1 /8/ and mung bean C4H CYP73A2 /9/ to PCR amplify three clones with features characteristic of cyt P450 sequences. Two of these clones, CYP71C5 and CYP73A7, were shown in preliminary experiments to be chemically inducible and are discussed in this paper.

The CYP73A7 fragment was used to probe a cDNA library at low stringency, and the full-length clone as well as two additional novel

cDNAs, CYP73A6 and CYP73A8, were isolated (Fig. 1). The maize CYP73 family members share greater than 60% protein identity with known cinnamate 4-hydroxylases from Jerusalem artichoke (CYP 73A1) /15/, mung bean (CYP73A2) /9/, and alfalfa (CYP73A3) /16/ (CYP73A6, approximately 82%; CYP73A7, approximately 75%; CYP73A8, approximately 62%). The three maize CYP73A mRNAs were induced two-fold in 10% ethanol-treated roots but were not induced significantly in maize shoots (Fig. 2). This is consistent with the observation that the metabolism of cinnamic acid by shoot microsomes was not significantly enhanced by ethanol (Table 1).

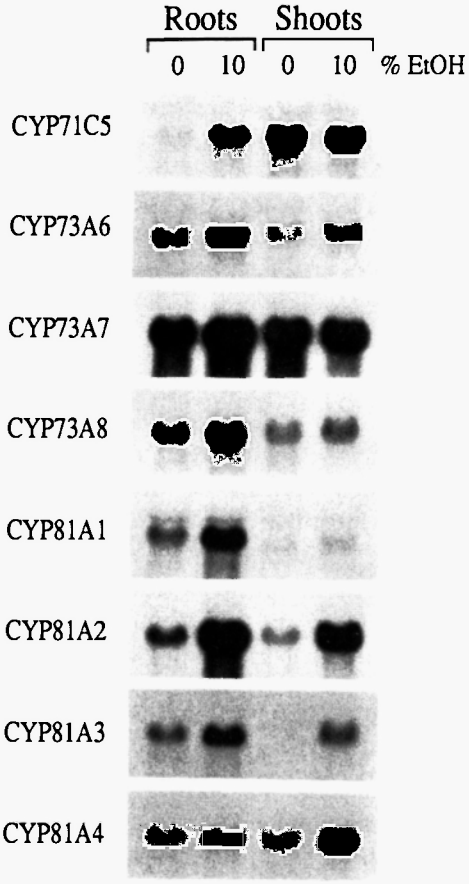

Fig. 2: Gel blot of total RNA isolated from etiolated maize shoots and roots treated with 0 or 10% ethanol, probed with the cyt P450 cDNAs indicated.

The CYP71C5 fragment was also used in a low stringency cDNA library screen with the only isolated clone being a truncated CYP71C5. A partial deduced protein sequence of maize CYP71C5 is 46% identical to CYP71A1 from avocado; its mRNA was induced 5.4-fold in ethanol-treated roots (Fig. 2).

We isolated two additional cyt P450 cDNAs (CYP81A1 and A2) by PCR amplification using primers complementary to the conserved heme-binding domain and poly(A) tail /11/. The CYP81A2 PCR fragment was used as a probe in a low stringency screen to isolate a full-length CYP81A2 and partial sequences of CYP81A3 and CYP81A4. The deduced protein sequence of CYP81A3 has 85% identity to CYP81A2; CYP81A4 has 73% identity to CYP81A2. The maize CYP81A subfamily shares less than 30% protein sequence identity with other plant cyt P450 families. The CYP81A mRNAs were inducible in both etiolated seedling roots and shoots by ethanol treatment. CYP81A1, A2, A3, and A4 mRNAs were induced in roots 2.6-, 8.7-, 2.2-, and 2.5-fold, and in shoots, 1.3-, 6.1-, 7.9-, and 3.6-fold, respectively (Fig. 2). In similar experiments, CYP81A1 was inducible up to 45-fold in roots of 10% ethanol treated maize hybrid Atlet etiolated seedlings, and CYP81A2 was 90-fold inducible in roots and 40-fold inducible in shoots (data not shown).

DISCUSSION

Our results show ethanol treatment increases metabolism of multiple substrates by microsomes isolated from maize etiolated shoots. mRNA levels from three cyt P450 families also increase; one of these families, CYP81, is defined by the four novel CYP81A subfamily members described here. Of the genes analyzed in this study, this CYP81A group was the most inducible by ethanol. Whether these genes encode proteins that metabolize ethanol or other substrates is unknown.

In addition to the several cyt P450s described here, a variety of genes are induced by ethanol in etiolated seedling tissue (data not shown). These include the benoxacor-inducible GST27 encoding the 27 kDa subunits of glutathione S-transferase IV /17/, and other genes of unknown function, such as In2-2, an N-(aminocarbonyl)-2-chlorobenzenesulfonamide (2-CBSU)-inducible cDNA from roots /18/. Based on these results, we suggest that ethanol is perceived by plants

as a general chemical stress, in response to which diverse detoxification mechanisms are activated.

Similar responses are observed in animals, where treatment with polycyclic hydrocarbons, phenobarbital, or peroxisome proliferators is associated with induction of particular subsets of drug-metabolizing enzymes including cyt P450, glutathione transferase, UDP-glucuronosyltransferase, aldehyde dehydrogenase, and other enzymes /19/. Ethanol-inducible mammalian CYP2E1 metabolizes as many as seventeen endogenous and exogenous substrates /20/, including the metabolism of ethanol to acetaldehyde. Concentration of CYP2E1 may be substrate-induced by a variety of molecular mechanisms, including increased mRNA levels /21,22/.

Application of ethanol increased total cyt P450 levels in Jerusalem artichoke tuber slices, but the activity of C4H was not increased /4/. Our results in maize were similar, with total cyt P450 levels remaining steady and no significant increase in C4H activity in ethanol-treated etiolated maize shoots. The maize CYP73A subfamily members share a high degree of similarity with known cinnamate 4-hydroxylases, but it is unknown whether any or all of the maize genes encode a C4H activity. Although the maize CYP73A mRNAs were not induced in etiolated maize shoots, they were slightly inducible in roots of those seedlings. This induction in roots but not in shoots may be attributed to the subirrigation method of treatment.

While little is known about cyt P450 gene regulation in plants, in other organisms cyt P450 protein levels are induced primarily by increased gene transcription /23/. Our results show that ethanol treatment of maize leads to induction of a battery of cyt P450s, of which one, a subset, or all may be responsible for various detoxification activities. Although the physiological significance of the plant ethanol response is not clear, it nonetheless appears to be a useful tool for generating material rich in transcripts encoding enzymes related to known detoxification activities.

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