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Regulation of the Cinnamate 4-Hydroxylase (CYP73A1) in Jerusalem Artichoke Tubers in Response to Wounding and Chemical Treatments

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***trans*-Cinnamate 4-hydroxylase (C4H) is a plant-specific cytochrome (P450) that is encoded by the gene *CYP73A* and catalyzes the second step of the multibranched phenylpropanoid pathway. Increases in C4H activity in response to physical and chemical stresses have been well documented, but the mechanism of these increases has never been studied in detail. This paper reports on the regulatory mechanism controlling C4H activity in Jerusalem artichoke (*Helianthus tuberosus*) tubers in response to wounding and chemical treatments. We compared induction of C4H and other P450-catalyzed activities. C4H was moderately induced by chemicals relative to other P450s. Increases in enzyme activity, C4H protein, and transcripts were quantified and compared in tuber tissue 48 h after wounding and chemical treatments. Our data suggest that induction of the enzyme activity results primarily from gene activation. Time-course experiments were performed after wounding and aminopyrine treatment. Compared with wounded tissues, aminopyrine triggered an additional and delayed peak of transcript accumulation. The timing of the induced changes in activity, protein, and transcripts confirms that C4H induction results primarily from an increase in *CYP73A1* mRNA, in both wounded and aminopyrine-treated tissues. However, posttranscriptional mechanisms might also contribute to the regulation of C4H activity.**

P450s are ubiquitous enzymes that catalyze the activation of molecular oxygen and the insertion of one of its atoms into physiological and artificial substrates (Porter and Coon, 1991). In plants P450s perform many oxygenation reactions in secondary metabolism, in sterols and fatty acid derivative synthesis, and in the detoxification of xenobiotics (Bolwell et al., 1994; Durst and O'Keefe, 1995). More than 16 P450-catalyzed reactions have been reported in the pathway leading to the biosynthesis of phenylpropanoids (Werck-Reichhart, 1995). The influx of metabolites into the pathway is controlled by a sequence of three catalytic steps leading from Phe to activated 4-coumaroyl CoA. The second reaction of the sequence is catalyzed by a P450 called C4H. Complete cDNA sequences were recently reported for the enzymes from Jerusalem artichoke (*Helianthus tuberosus;* Teutsch et al., 1993), mung bean (*Vigna radiata;* Mizutani et al., 1993), and alfalfa (*Medicago sativa;* Fahrendorf and Dixon, 1993). In accordance with the proposed nomenclature for P450s (Nebert et al., 1993), the *H. tuberosus* enzyme was termed CYP73A1. Enzymes of the general phenylpropanoid pathway are usually expressed in a coordinate manner, but the regulatory mechanisms underlying coordinated expression are unclear. Both the substrate (cinnamic acid) and the product (*p*-coumaric acid) of C4H have been implicated in the regulation of upstream and downstream enzymes of the phenylpropanoid pathway (Dixon and Paiva, 1995; Werck-Reichhart, 1995). Whether C4H activity plays a role in the process, however, remains to be determined.

A characteristic common to many P450s from all organisms is their inducibility by exogenous chemicals. The induction mechanisms of bacterial and animal P450s by xenobiotics have been extensively studied. In some cases (i.e. induction by polyaromatic hydrocarbons or hypolipidemic drugs) the induction was shown to involve gene activation, and the signal transduction pathways involved have been elucidated (Denison and Whitlock, 1995). It is believed that the transduction pathways activated by these molecules are normally triggered by endogenous signaling molecules the nature and function of which are not yet understood (Nebert and Feyereisen, 1994; Okey et al., 1994). Aside from activated transcription, the regulation of animal P450s has also been shown to involve regulation at several posttranscriptional levels, depending on the effector and particular P450. These include stabilization of mRNA and protein, modification of intranuclear transcript processing, and enhanced translation (Porter and Coon, 1991; Roberts et al., 1994). Little information is available concerning the mechanism of the regulation of plant P450s. C4H, which is easily detected in many plant tissues and catalyzes a reaction important for defense and lignification, has been one of the most extensively studied P450s. C4H activity is induced by a number of stresses, including wounding and chemical effectors (Werck-Reichhart, 1995). In addition, several reports suggested that the enzyme might be glycosylated (Kochs et al., 1992; Fahrendorf and Dixon, 1993; Smith et

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Abbreviations: C4H, *trans*-cinnamate 4-hydroxylase (NADPH: oxygen oxidoreductase [4-hydroxylating], EC 1.14.13.11); DEHP, bis(2-ethylhexyl)-phthalate; ECOD, 7-ethoxycoumarin *O*-deethylase; EROD, 7-ethoxyresorufin (or 7-ethoxyphenoxazone) *O*-deethylase; NA, naphthalic anhydride; P450, Cyt P450.

al., 1993; Werck-Reichhart et al., 1993), thus suggesting a possible regulatory effect of this glycosylation. In previous studies, increases in protein or steady-state mRNA concomitant with enzymatic activity have been reported (Fahrendorf and Dixon, 1993; Teutsch et al., 1993; Werck-Reichhart et al., 1993; Hotze et al., 1995; Logemann et al., 1995; Frank et al., 1996); however, induction of C4H activity has never been correlated with C4H protein and mRNA levels in an attempt to determine if this enzyme is subject to posttranscriptional regulation.

This paper describes the effects of wounding and of different chemical effectors, including those triggering gene activation and important signal transduction pathways in animals or bacteria (e.g. phenobarbital, benzo[*a*]pyrene, and clofibrate), on C4H activity and accumulation of CYP73A1 protein and transcripts. The impact of these effectors on C4H is compared with their effect on other P450 (ECOD and EROD)-catalyzed reactions in the same plant. The effect of wounding and of aminopyrine, the most potent P450 inducer in *H. tuberosus*, was studied in more detail and in a time-dependent manner. Our results suggest that induction of C4H in response to all effectors tested results primarily from transcriptional regulation.

MATERIALS AND METHODS

Chemicals

trans-[3-^{14}C]Cinnamate was from Isotopchim (Ganobie, France). NA and isosafrole were purchased from Aldrich, phenobarbital (5-ethyl-5-phenyl-barbituric acid, sodium salt) from Fluka, 1,8-ethoxyresorufin from Pierce, and DEHP from EGA-Chemie (Steinheim, Germany). All other chemicals were obtained from Sigma.

Plant Material

Jerusalem artichoke (*Helianthus tuberosus* L. var Blanc commun) tubers were grown in an open field, harvested in November, and stored in plastic bags at 4°C in the dark. For aging experiments tubers were sliced (1.5 mm thick), washed, and incubated for 48 h in aerated (4 dm^3 min^{-1}), distilled water containing various chemicals, as described previously (Reichhart et al., 1980). Chemicals tested as C4H effectors were prototype P450 inducers known to trigger different signal transduction pathways in animals and bacteria (phenobarbital, aminopyrine, β-naphthoflavone, isosafrole, 3-methylcholanthrene, benzo[*a*]pyrene, clofibrate, and DEHP) (Bresnick, 1993), plant metabolites (flavone and 8-methoxypsoralen), metals ($MnCl_2$, $HgCl_2$, and $CdCl_2$), and agrochemicals (biphenyl, lindane, and NA), all known to induce animal or plant P450s (Reichhart et al., 1979; Fujita, 1985; Letteron et al., 1986; Fonne-Pfister et al., 1988; Borlakoglu and John, 1989; Siess et al., 1989; McFadden et al., 1990; Batard et al., 1995). The $MnCl_2$ solution was adjusted to pH 7. Water-insoluble compounds (β-naphthoflavone, flavone, biphenyl, NA, 8-methoxypsoralen, and benzo[*a*]pyrene) were first dissolved in 4 mL of DMSO and then added to the aging medium (1.5 L).

Preparation of Microsomal Fractions

Plant tissues were extracted and microsomes prepared as described by Werck-Reichhart et al. (1990).

Enzyme Assays

C4H was assayed, as described previously (Reichhart et al., 1980). ECOD and EROD activities were determined fluorimetrically (Werck-Reichhart et al., 1990). The results are means ± SD of duplicates or triplicates.

Analytical Methods

P450 and microsomal protein contents were determined as described by Gabriac et al. (1991). CYP73A1 content was quantified by differential spectrophotometry (presence versus absence of saturating concentration of substrate) using an absorption coefficient (ϵ) for the A_{389} to A_{423} difference of 128 mM^{-1} (Urban et al., 1994).

SDS-PAGE (10% total monomer, 0.3% cross-linker), electroblotting onto nitrocellulose membranes (Hybond C, Amersham), and immunoblot staining using the rabbit polyclonal antibody C4Hpa2/2 were performed as described by Werck-Reichhart et al. (1993). The immunoreactive protein was quantified using a densitometer (model SC930, Shimadzu, Columbia, MD) and comparison with known amounts of purified protein run on the same gel.

Total RNA was isolated according to the method of Lesot et al. (1990). Denatured RNA was separated in the presence of formaldehyde through a 1.2% agarose gel and transferred to a nylon membrane (Hybond N^+, Amersham) (Sambrook et al., 1989). RNA blot prehybridization and hybridization with a full-length *CYP73A1* nucleotidic probe radiolabeled with [α-^{32}P]dCTP by random priming was carried out at 65°C according to established procedures. Membranes were washed twice for 10 min with 2× SSC, 0.1% SDS, once for 10 min with 0.2× SSC, 0.1% SDS at room temperature, and then twice for 30 min with 0.2× SSC, 0.1 SDS at 55°C. Hybridization signals were quantified using a phosphor imager (model BAS1000, Fuji, Tokyo, Japan). RNA amounts were standardized by hybridization at 55°C to a 300-bp *Capsicum annuum* 25S rRNA probe.

RESULTS

Modulation of C4H by Chemical Treatments

A preliminary experiment was performed to determine the constitutive levels of C4H activity and P450 content and the effect of DMSO on these levels in tuber tissues (Table I). C4H activity, just detectable in dormant tissues, was increased 14-fold after 48 h of aging in aerated water. A slightly greater induction of activity was observed in the presence of 0.25% DMSO (approximately 18-fold). In comparison, P450 content was induced only approximately 4-fold in response to DMSO. Increases in C4H activity were more than twice the corresponding increases in total P450, which is indicative of a selective induction of CYP73A1 relative to other P450 forms.

Table I. *C4H activity in microsomes from dormant and control H. tuberosus tuber tissues*

Microsomes were prepared from dormant tubers or from tissues sliced and aged for 48 h in aerated, distilled water or in water plus 0.25% (v/v) DMSO.

Microsome	C4H Activity	P450 Content
	pkat mg^{-1} protein	*pmol mg^{-1} protein*
Dormant tuber	0.8 ± 0.07	9 ± 2
Wounding (water)	10.9 ± 0.3	44 ± 7
Water + DMSO	14.1 ± 1	36 ± 9

The modulation of C4H activity in tubers treated with compounds previously documented to induce P450 activities was examined (Table II; Figs. 1 and 2). Addition of some chemicals to the incubation medium resulted in a decrease in C4H activity. Clofibrate and DEHP, inducers of fatty acid ω-hydroxylase in animals and plants (Salaün et al., 1986), did not significantly alter the total P450 content (Table II), but at 5 mM, reduced C4H activity by 80 and 50%, respectively (Fig. 1). 8-Methoxypsoralen (or xanthotoxin), a coumarin accumulated by Apiaceae that was previously shown to induce P450s in animals (Letteron et al., 1986), increased the total P450 content but decreased the C4H activity. Isosafrole, benzo(*a*)pyrene, 3-methylcholanthrene, and β-naphthoflavone, prototype inducers of animal P450s (Bresnick, 1993), did not alter significantly the activity at the concentrations assayed, even though an increase in the total P450 content of the tissues was observed (Table II). $HgCl_2$ and $CdCl_2$, which were previously reported to induce P450 and the accumulation of terpenes in sweet potato discs (Fujita, 1985), were apparently toxic at the concentrations used in our experiments (Fig. 2). All other chemicals, including Mn^{2+}, phenobarbital, aminopyrine, lindane, biphenyl, NA, and flavone, increased both C4H activity and P450 content 1.6- to 3.3-fold. The highest induction was obtained with chemicals ($MnCl_2$, aminopyrine, and phenobarbital) that had been previously tested for dose response (Reichhart et al., 1979; Fonne-Pfister et al., 1988), which were therefore used at optimal concentrations, but several aromatic planar molecules such as NA, biphenyl, and flavone also appeared to be good inducers of C4H.

We previously reported the induction of alkoxycoumarin and alkoxyphenoxazone *O*-dealkylating activities in *H. tuberosus* tuber tissues by the same xenobiotics (Batard et al., 1995). Figure 1 shows that the induction patterns of ECOD and EROD activities measured in the same plant microsomes are clearly different from that of C4H. On the whole, *O*-dealkylating activities are more susceptible to chemical stress than is C4H. They are, in particular, strongly induced by aminopyrine, phenobarbital, benzo(*a*)pyrene, or 8-methoxypsoralen. In contrast, C4H activity is inhibited by 8-methoxypsoralen treatment. However, some planar aromatic molecules (biphenyl, NA, and flavone) seem to induce C4H equally or better than dealkylase activities.

Expressed in yeast microsomes, C4H has been shown to be fully low-spin, i.e. with maximum A_{423} resulting from the presence of one molecule of water as the sixth ligand of the heme iron (Urban et al., 1994). Binding of the substrate releases the water molecule and shifts the maximum of *A* to 389 nm. A differential coefficient of absorption for the A_{389} to A_{423} difference measured in the presence versus the absence of saturating substrate was determined. Assuming that the enzyme is also low-spin in plant microsomes, we made a rough estimation of the contribution of C4H to the total P450 in wounded and xenobiotic-treated tissues. Table III shows that in wounded tuber tissues, C4H is by far the major P450. Its relative abundance, however, is less in tissues treated with phenobarbital, $MnCl_2$, and, in particular, aminopyrine. This is in good agreement with the data shown in Figure 1 and Table II, which indicate that other P450s are more strongly induced by these treatments than is C4H.

CYP73A1 Protein and Transcript Levels in Treated Tissues

Figure 2 shows the level of C4H protein immunodetected in the control and treated microsomes, and the correlation between the amount of C4H protein present in the tissues and the C4H activity. The correlation is imperfect, probably resulting from imprecise immunoblot quantification of the protein, or it may reflect some sort of posttranslational regulation. In a few cases, discrepancies between activity and protein content can be explained. Clofibrate and DEHP are peroxisome proliferators and induce the production of activated oxygen species and lipid peroxidation in both animal and plant tissues (Palma et al., 1991). These molecules were described as inducers of fatty acid hydroxylases, and sometimes of C4H, in plants (Salaün et al., 1986).

Table II. *Increases in P450 content in microsomes from H. tuberosus tuber tissues after chemical treatments*

Dormant tissues were sliced and aged for 48 h in aerated, distilled water containing the indicated concentrations of chemicals. P450 induction is given as a ratio to the activity or P450 content measured in tissues aged in distilled water or in water plus DMSO (Table I).

Treatment	Abbreviated Name	Concentration	P450 Relative Induction
		mM	
$MnCl_2$		25	2.5 ± 0.2
$CdCl_2$		2.5	n.d.[a]
$HgCl_2$		2.5	n.d.
Aminopyrine	AP	20	3.1 ± 0.3
Phenobarbital	PB	8	2.6 ± 0.2
β-Naphthoflavone[b]	βNF	1.7	2.0 ± 0.1
Clofibrate	Clo	5	0.8 ± 0.3
Diethylhexyphthalate	DEHP	5	0.9 ± 0.09
Benzo(*a*)pyrene[b]	B(a)P	0.26	1.5 ± 0.3
3-Methylcholanthrene[b]	3-MC	0.24	1.4 ± 0.2
Lindane[b]	Lin	1	1.8 ± 0.2
Biphenyl[b]	Bip	6.5	2.3 ± 0.3
Naphthalic anhydride[b]	NA	0.1	2.6 ± 0.1
		0.2	1.9 ± 0.4
Flavone[b]	Flav	1.5	2.3 ± 0.3
8-Methoxypsoralen[b]	8-MP	0.05	1.4 ± 0.2
Isosafrole	Iso	0.05	0.8 ± 0.1
		0.1	0.9 ± 0.3

[a] n.d., Not detectable. [b] Chemicals solubilized in DMSO (0.25% of the total volume of the aging medium).

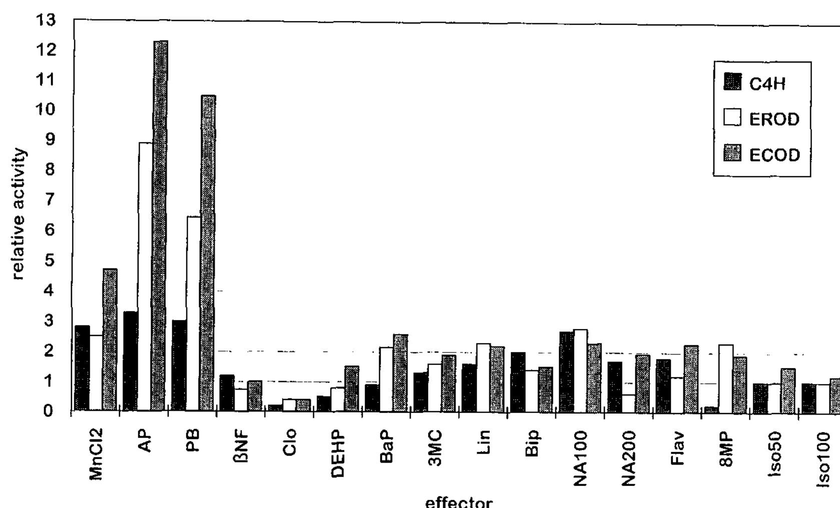

Figure 1. Comparison of the relative increases in C4H, ECOD, and EROD activities in microsomes from *H. tuberosus* tuber tissues after different chemical treatments. Tuber tissues were sliced and aged for 48 h in aerated, distilled water containing the different chemicals before extraction and preparation of the microsomes. Abbreviated names and concentrations of the chemical effectors are defined in Table II. C4H, ECOD, and EROD were measured using radiochemical or fluorimetric assays, as described in "Materials and Methods." Relative activities are given as ratios of the activities measured in tissues aged in distilled water or in water plus DMSO. C4H activities are given in Table I. ECOD and EROD activities measured in microsomes from tissues aged in water were 0.012 ± 0.002 and 0.028 ± 0.004 pkat mg^{-1} protein, respectively, and in water plus DMSO were 0.061 ± 0.005 and 0.035 ± 0.005 pkat mg^{-1} protein, respectively.

In our experiments, we used concentrations more than twice those previously reported to induce P450. Such concentrations still promote an increase in immunodetectable C4H protein, but the oxidative stress seems to result in protein inactivation. If the data concerning clofibrate and DEHP are omitted from Figure 2B, the coefficient of correlation between C4H activity and protein is shifted from 0.58 to 0.81.

CYP73A1 mRNA abundance in some of the same treated tuber tissues is shown in Figure 3. Both wounding and chemical treatments clearly resulted in an accumulation of *CYP73A1* transcripts (Fig. 3A). A good correlation between the amounts of immunodetected C4H protein and the abundance of the corresponding mRNA was also observed (Fig. 3B). C4H induction by wounding and chemical treatments thus seems to result primarily from increases in the steady-state levels of *CYP73A1* mRNA.

Time-Course Induction of CYP73A1 after Wounding and Aminopyrine Treatment

To further test the hypothesis of transcriptional control of *CYP73A1* gene expression, two separate experiments were performed to monitor C4H enzyme activity, the level of the C4H protein, and steady-state levels of C4H mRNA after wounding and aminopyrine treatment.

After wounding, two successive waves of induction were observed (Fig. 4). Transcript accumulation proceeded without a detectable lag phase. A first peak of C4H transcript accumulation was observed at approximately 10 h following wounding. A second maximum occurred after another 10 h. The steady-state content of C4H messenger then decreased until 50 h after wounding, and stayed relatively constant (transcripts still being detectable) for the next 2 d. C4H protein and enzyme activity grossly followed the same pattern of induction, but the maxima were shifted 10 to 20 h later than that observed for the mRNA. Activity appeared slightly delayed compared with protein accumulation.

In a second experiment, tuber slices were aged in water or in water containing 20 mM aminopyrine (Fig. 5). The two waves of induction elicited by wounding were less apparent in this experiment since (a) the tubers were not used at exactly the same stage of dormancy release and dormancy stage influences induction (amplitude and time course), and (b) a smaller set of samples was analyzed. In aminopyrine-treated tissues, the initial induction consistent with the wounding response was slightly delayed and reduced, but still obvious. It was followed by another increase in transcripts and protein. C4H mRNA peaked after 45 h in the presence of aminopyrine, then decreased steadily. C4H protein increased until 60 h and remained so until 84 h. Transcripts and protein levels during this second phase of induction never exceeded 140% of the maxima observed after wounding.

DISCUSSION

P450s in animals and microorganisms are highly inducible by exogenous molecules. This inducibility is usually related to the detoxifying function of these enzymes, and P450 forms very active in the metabolism of xenobiotics are usually more susceptible to exogenous inducers than are isozymes involved in essential physiological pathways (Porter and Coon, 1991). Our findings suggest that this may also be the case in plants. We have shown that C4H, a central enzyme in the phenylpropanoid pathway, is induced by a wide range of chemicals, but is induced less

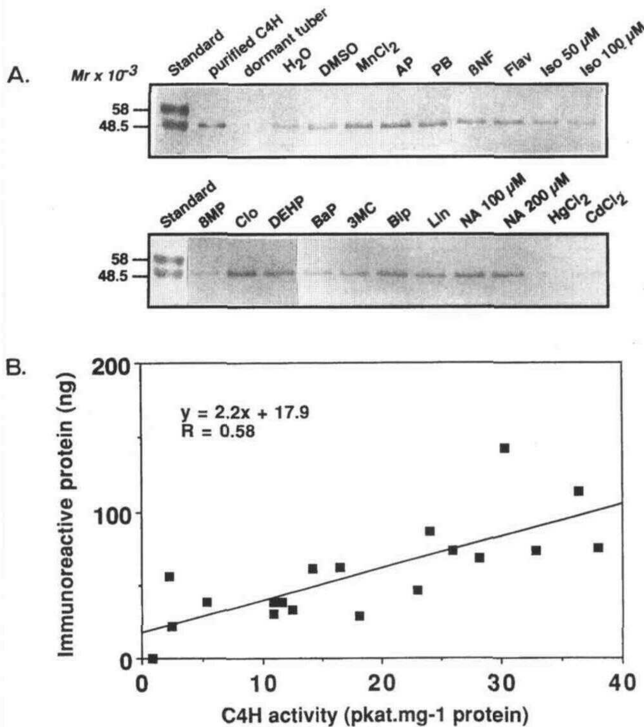

Figure 2. Immunoquantification of the CYP73A1 protein in microsomes from tuber tissues treated with different chemicals. A, Ten micrograms of microsomal protein from dormant *H. tuberosus* tuber tissue or tissue that had been aged for 48 h in water, in solutions of chemicals (full names and concentrations given in Table II), or 100 ng of immunopurified C4H were analyzed by SDS-PAGE, transferred onto nitrocellulose membrane, and immunostained for C4H. Staining was performed using rabbit polyclonal anti-C4Hpa2/2 serum diluted 1:10,000 (Werck-Reichhart et al., 1993). Standard indicates prestained molecular mass markers. B, Immunoreactive C4H was then quantified by densitometry with reference to the immunopurified enzyme. Data were plotted against C4H activity measured in the same microsomes.

than other P450s in the same plant. Several of the potent inducers of animal P450s (phenobarbital, aminopyrine, flavone, biphenyl, and DMSO) also trigger an increase in C4H activity in plants. Other chemical agents, in particular those capable of inducing CYP1A1 (i.e. benzo[*a*]pyrene, 3-methylcholanthrene, and β-naphthoflavone), do not affect C4H, but do increase P450 content and P450-dependent dealkylase activities. Common induction mechanisms, receptors, or signal transduction pathways are possibly conserved in animals and plants.

NA was one of the best inducers of C4H in *H. tuberosus*. This was unexpected, since this compound, which was the first commercial herbicide safener, is usually thought to exert a selective action on monocots (Hatzios, 1991). In mung bean, NA has no effect on C4H activity and increases lauric acid or herbicide metabolism only when applied in combination with other inducers (Moreland et al., 1995). In grass crops NA induces the metabolism of various herbicides but represses C4H activity (Zimmerlin et al., 1992; Persan and Schuler, 1995).

The absence of a response to some of the chemicals tested in our experiments does not exclude the possibility of a response at other concentrations, since many concentrations used in this work were chosen from studies on animals or other plant materials and were not optimized for *H. tuberosus*. Clofibrate, for example, may induce C4H at lower concentrations (Salaün et al., 1986).

Table III. *C4H to P450 ratio in H. tuberosus tuber tissues after wounding and chemical treatments*

Microsomes were prepared from tuber tissues aged for 24 h in water, for 48 h in 8 mM phenobarbital, or 20 mM aminopyrine, or for 78 h in 25 mM $MnCl_2$ (aging times previously reported to be optimal for induction in each case). P450 and C4H contents were determined using spectrophotometrical methods, as described in the text.

Microsome	P450 Content	C4H Content	C4H/P450
	pmol mg⁻¹ protein		*%*
Water	85 ± 2	48 ± 2	56
PB	240 ± 16	102 ± 2	42
$MnCl_2$	347 ± 43	106 ± 5	31
AP	427 ± 49	125 ± 5	29

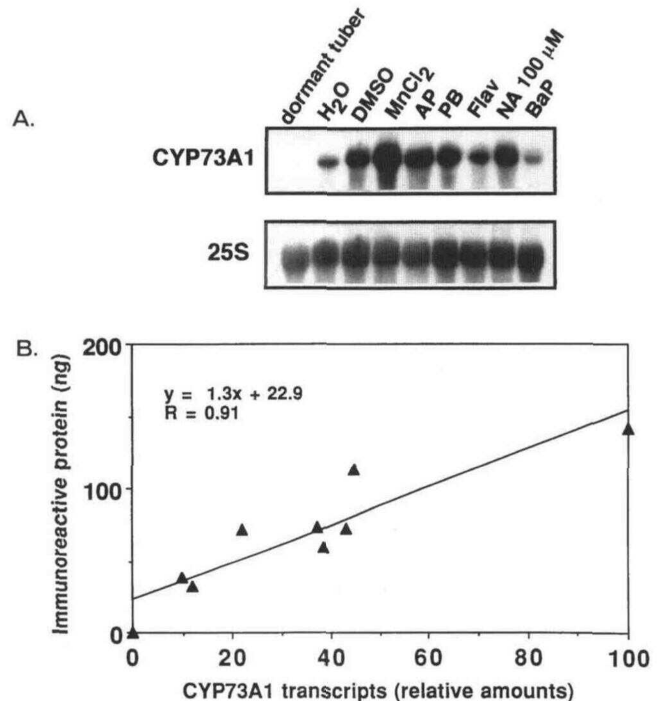

Figure 3. Accumulation of *CYP73A1* transcripts in tuber tissues treated with different chemicals. A, Twenty micrograms of total RNA extracted from dormant *H. tuberosus* tuber tissue or tissue that had been aged for 48 h in water or in solutions of chemicals (full names and concentrations given in Table II) was electrophoresed through denaturing formaldehyde gels and transferred by capillarity onto a nylon membrane. The RNA blot was successively hybridized with a full-length *CYP73A1* DNA probe at high stringency and with a 300-bp pepper probe coding for a 25S rRNA at low stringency, as described in "Materials and Methods." B, *CYP73A1* transcripts were quantified using a phosphor imager. Transcript abundance was calculated by comparison with the $MnCl_2$-treated sample, which was arbitrarily assigned a value of 100.

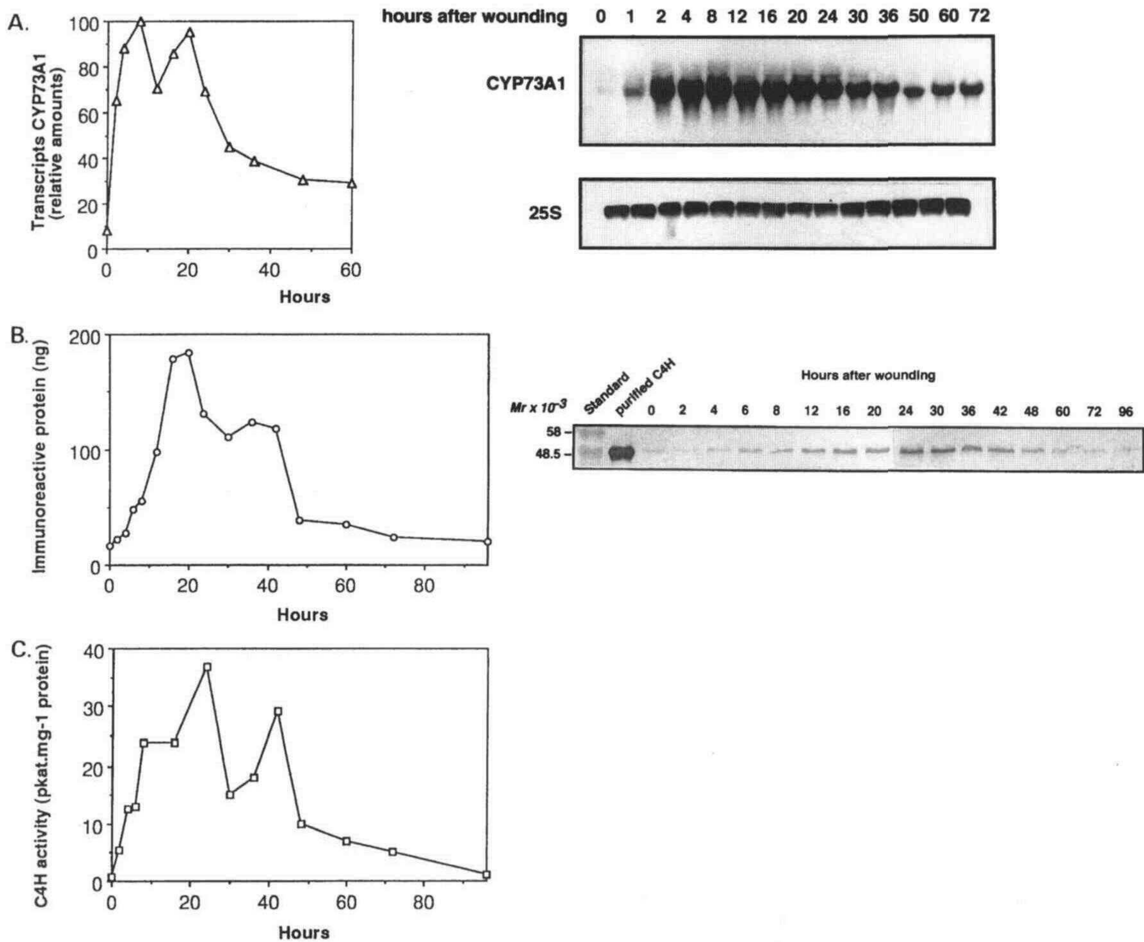

Figure 4. Time courses of induction of C4H activity, CYP73A1 protein, and *CYP73A1* transcripts in wounded *H. tuberosus* tuber tissues. Tuber tissues were peeled, sliced, washed, and aged in aerated, distilled water. A, Accumulation of *CYP73A1* transcripts. Twenty micrograms of total RNA extracted from the tissues was fractionated, transferred to membrane, and hybridized successively with CYP73A1 and 25S rRNA nucleotidic probes, as described above. *CYP73A1* transcripts were quantified using a phosphor imager. Transcript abundance was calculated by comparison with the sample prepared after 8 h of aging, which was arbitrarily assigned a value of 100. B, Immunoquantification of the CYP73A1 protein. Ten micrograms of microsomal protein or immunopurified C4H was submitted to immunoblot analysis, as described above. Standard indicates prestained molecular mass markers. Immunoreactive C4H was then quantified by densitometry with reference to the immunopurified enzyme. C, C4H activity detected in microsomal preparations.

In animals changes in P450 levels resulting from exposure to xenobiotics are primarily the result of gene activation. Depending on the P450 form and on the effector, however, all types of posttranscriptional regulation have been observed (Porter and Coon, 1991; Denison and Whitlock, 1995). In plants, the only data available are indicative of transcriptional activation. Light-, wounding-, or infection-promoted increases in C4H activity occur after a lag period of 1 to 3 h and are prevented by treatment with cycloheximide, puromycin, cordycepin, or actinomycin D, which indicates de novo synthesis of the enzyme resulting from gene activation (Hyodo and Yang, 1971; Tanaka et al., 1974; Durst, 1976; Rhodes et al., 1976; Benveniste et al., 1977; Lamb, 1977; Oba and Conn, 1988). Results recently obtained by RNA blot hybridization confirmed increases in steady-state levels of C4H mRNA after wounding, treatment with xenobiotics, or fungal elicitation of cell suspension cultures (Fahrendorf and Dixon, 1993; Teutsch et al., 1993; Hotze et al., 1995; Logemann et al., 1995). Our results corroborate these data and are consistent with the suggestion that enhanced transcription is primarily responsible for the induction of C4H in response to wounding and chemical inducers. Wounding is followed by two peaks in transient mRNA accumulation. These two peaks are also observed at the level of the C4H protein and enzyme activity in the microsomes. Such a biphasic response to wounding has already been observed (Morelli et al., 1994; Logemann et al., 1995) and seems to be correlated with two distinct phases of stimulation of translational activity synchronized with the expression of two classes of wound-induced genes involved in wound healing and prevention of pathogen invasion. In tubers, an important function of these genes is the synthesis of suberin for the wound periderm. It has been proposed

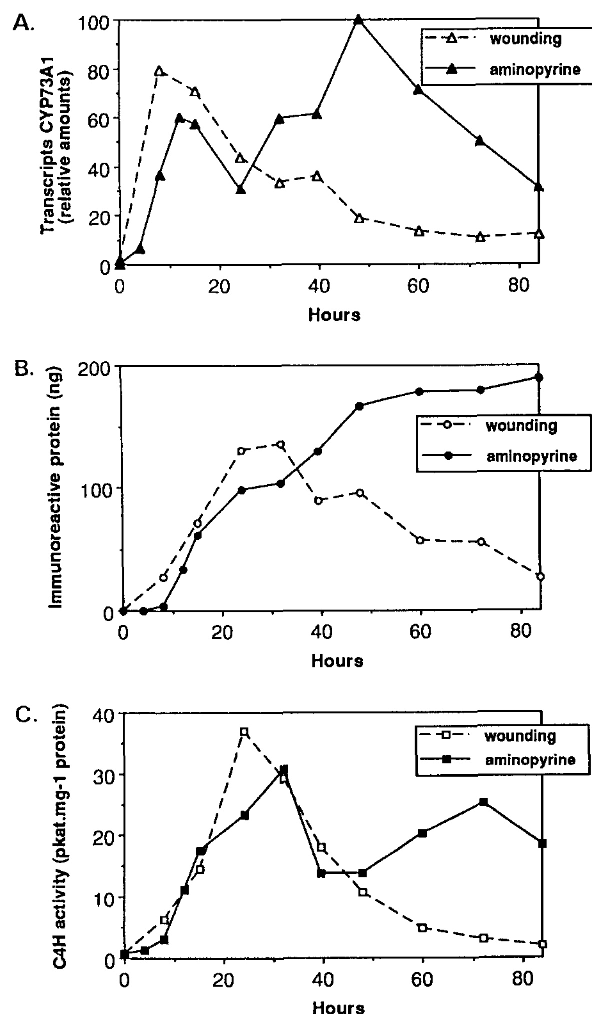

Figure 5. Time course of induction of C4H activity, CYP73A1 protein, and *CYP73A1* transcripts in tuber tissues aged in water or in 20 mM aminopyrine. Tubers were peeled, sliced, washed, and aged in aerated, distilled water alone or containing 20 mM aminopyrine. A, Accumulation of *CYP73A1* transcripts. Twenty micrograms of total RNA extracted from the tissues was fractionated, transferred to membrane, and hybridized with CYP73A1 and 25S rRNA nucleotidic probes, as described above. *CYP73A1* transcripts were quantified using a phosphor imager. Transcript abundance was calculated by comparison with the sample prepared after 48 h of aging in the presence of aminopyrine, which was arbitrarily assigned a value of 100. B, Immunoquantification of the CYP73A1 protein. Ten micrograms of microsomal protein or immunopurified C4H was submitted to immunoblot analysis, as described above. Immunoreactive C4H was quantified by densitometric scanning by comparison with the reference immunopurified enzyme. C, C4H activity detected in microsomal preparations.

that these two gene classes are triggered by either immediate injury and cell division (Morelli et al., 1994). Aminopyrine treatment triggers an additional phase of transcript accumulation, delayed by about 38 h compared with the wound response. This response to chemical treatment is apparently stronger than, and independent from, the wound-induced gene activation. It occurs a long time after the beginning of the treatment and follows a slight inhibition of wound induction. Aminopyrine induction does not seem to result from gene activation directly triggered by the exogenous molecule, but rather from the onset of a cascade of events and signals that apparently promote *CYP73A1* gene transcription 30 h after the beginning of the treatment.

Considering the successive waves of increases in steady-state levels of *CYP73A1* mRNA elicited by wounding and aminopyrine treatment, it is tempting to speculate that there is an activation of two or three independent *CYP73A1* genes in *H. tuberosus*. The presence of at least two variant cDNAs has been detected (Teutsch et al., 1993). These two variants, however, showed 98.25 and 100% identity in nucleic acids and amino acids, respectively. Noncoding sequences were not available for comparison.

A delay of about 15 to 20 h is observed between maximal increases in *CYP73A1* mRNA and in the accumulation of the C4H protein or enzyme activity. This is a surprisingly long delay compared with the lag observed in cell cultures (Hotze et al., 1995; Logemann et al., 1995) and is difficult to understand at this time. It is unlikely to reflect the time necessary for protein maturation and correct membrane targeting of the C4H inserted into the ER. Transcript processing or availability of the components of the translational apparatus in wounded tuber tissues might slow the expression of the enzyme (Morelli et al., 1994).

Although some correlation between accumulation of *CYP73A1* transcripts and increases in C4H protein and activity was observed in our experiments, detailed analysis of our results reveals several discrepancies. For example, Mn^{2+}-, clofibrate-, or DEHP-treated tissues show a high C4H protein content relative to enzymic activity. Such data could be indicative of a posttranslational regulation. It may be pointed out, however, that all three chemicals are likely to favor an oxidative degradation of the protein. The time-course experiments also indicate that the regulation of C4H activity in tuber tissues brings into play mechanisms more complex than simple activation of the *CYP73A1* gene(s) expression. For example, the elevated levels of C4H protein recorded after long aging on aminopyrine (Fig. 5) and the progressive increase in the delay between message and protein accumulation observed in all experiments suggest that transcript maturation and translation, but also protein stabilization or inactivation, possibly have an impact on C4H regulation.

Direct measurements of the rates of *CYP73A1* transcription, determination of the number of *CYP73A1* copies in the *H. tuberosus* genome, and evaluation of C4H stability after different tuber treatments will be needed to obtain a more accurate picture of the mechanisms regulating C4H expression.

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