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Isolation of a Microsomal Enzyme System Involved in Glucosinolate Biosynthesis from Seedlings of *Tropaeolum majus* L.[1]

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An in vitro system that converts phenylalanine to phenylacetaldoxime in the biosynthesis of the glucosinolate glucotropaeolin has been established in seedlings of *Tropaeolum majus* L. exposed to the combined treatment of jasmonic acid, ethanol, and light. The treatment resulted in a 9-fold induction, compared with untreated, dark-grown seedlings, of de novo biosynthesis measured as incorporation of radioactively labeled phenylalanine into glucotropaeolin. Formation of the inhibitory degradation product benzylisothiocyanate during tissue homogenization was prevented by inactivation of the thioglucosidase myrosinase by addition of 100 mM ascorbic acid to the isolation buffer. This allowed the isolation of a biosynthetically active microsomal preparation from the induced *T. majus* plant material. The enzyme, which catalyzes the conversion of phenylalanine to the corresponding oxime, was sensitive to cytochrome P450 inhibitors, indicating the involvement of a cytochrome P450 in the biosynthetic pathway. It has previously been shown that the oxime-producing enzyme in the biosynthesis of *p*-hydroxybenzylglucosinolate in *Sinapis alba* L. is dependent on cytochrome P450, whereas the oxime-producing enzymes in *Brassica* species have been suggested to be flavin monooxygenases or peroxidase-type enzymes. The result with *T. majus* provides additional experimental documentation for a similarity between the enzymes converting amino acids into the corresponding oximes in the biosynthesis of glucosinolates and cyanogenic glucosides.

Glucosinolates are amino acid-derived, secondary plant products containing a sulfate and a thioglucose moiety (Ettlinger and Kjær, 1968). Glucosinolates are found throughout the order Capparales, which includes the family Brassicaceae containing the agriculturally important crop plants oilseed rape and *Brassica* forages. When tissue is damaged or food is processed, glucosinolates are rapidly hydrolyzed by the thioglucosidase myrosinase (EC 3.2.3.1). The presence of glucosinolates in these plants is of economic importance because of the potentially harmful effects of their breakdown products (Duncan, 1992). Glucosinolates and their degradation products may defend plants against insect and fungal attack (Fenwick et al., 1983; Chew, 1988) or serve as attractants to insects that are specialist feeders on cruciferae (Feeny et al., 1970).

Glucosinolates are grouped into aliphatic, aromatic, and indolyl glucosinolates, depending on whether they are derived from Met, Phe, and Tyr, or Trp. Approximately 100 different glucosinolates have been identified. The parent amino acid often undergoes a series of chain elongations prior to entering the biosynthetic pathway, and the glucosinolate product is often subjected to secondary modifications such as hydroxylations, methylations, oxidations, etc. (Ettlinger and Kjær, 1968). In vivo biosynthetic studies using seedlings or excised tissues have demonstrated that *N*-hydroxy amino acids, nitro compounds, oximes, thiohydroximates, and desulfoglucosinolates are precursors of glucosinolates (Poulton and Møller, 1993; Wallsgrove and Bennett, 1995). *Tropaeolum majus* L. has been used extensively in in vivo studies of glucosinolates. This plant produces only a single glucosinolate (glucotropaeolin) derived from Phe, which, in contrast to the chain-elongated amino acids, is commercially available as a radioactively labeled precursor. Early in vivo trapping experiments demonstrated that labeled phenylacetaldoxime accumulates in shoots of *T. majus* upon administration of labeled Phe and unlabeled oxime, indicating the involvement of oximes as intermediates in the biosynthesis of glucosinolates (Underhill, 1967).

Knowledge about the enzymes catalyzing the biosynthesis of glucosinolates is only slowly emerging. The last two steps in the pathway are catalyzed by two soluble enzymes, the UDPG-thiohydroximate glucosyltransferase (EC 2.4.1.-), which glucosylates the thiohydroximate, and the 3′-phosphoadenosine 5′-phosphosulfate:desulfoglucosinolate sulfotransferase (EC 2.8.2.-), which converts the desulfoglucosinolate into the glucosinolate. These two enzymes have been purified and shown to be nonspecific with respect to the nature of the side chain (Glendening and Poulton, 1988; Jain et al., 1990; Reed et al., 1993; Guo and Poulton, 1994). The sulfur-donating enzyme has not been characterized, but feeding experiments suggest that Cys is the sulfur donor (Matsuo, 1968). The nature of the enzymes catalyzing the conversion of amino acids to oximes has been the subject of many discussions. In Brassicaceae, the involvement of flavin monooxygenases (Ben-

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Abbreviations: Tricine, *N*-tris(hydroxymethyl)-methylglycine; solvent system 1, 2-propanol:ethyl acetate:H_2O (7:1:2, v/v); solvent system 2, ethyl acetate:toluene (1:5, v/v).

nett et al., 1993; Dawson et al., 1993), peroxidase types of enzymes (Ludwig-Muller et al., 1990), and Cyt P450 enzymes has been suggested. Recently, we demonstrated that the conversion of Tyr to *p*-hydroxyphenylacetaldoxime in the biosynthesis of *p*-hydroxybenzylglucosinolate in *Sinapis alba* L. involves a Cyt P450-dependent monooxygenase as evidenced by photoreversible carbon monoxide inhibition (Du et al., 1995). Taxonomically, *S. alba* belongs to the Brassicaceae family, and *T. majus* belongs to the Tropaeolaceae family. Therefore, characterization of the oxime-producing enzyme system in *T. majus* would be of interest in providing evidence in support of one of the suggested enzyme types in the Brassicaceae family for taxonomic reasons. Earlier attempts to isolate a biosynthetically active in vitro enzyme system from *T. majus* were hampered by low biosynthetic activity and by the inhibitory effect of benzylisothiocyanate generated by degradation of glucotropaeolin by myrosinases during tissue homogenization (Lykkesfeldt and Møller, 1993).

In the present paper, we report the isolation of a biosynthetically active microsomal enzyme system catalyzing the conversion of Phe into phenylacetaldoxime in seedlings of *T. majus*. The enzyme activity was induced 9-fold by exposure of the seedlings to the combined treatment of jasmonic acid, ethanol, and light, compared with untreated, dark-grown seedlings. Furthermore, we show that the enzyme system responsible for oxime production in *T. majus* resembles the system involved in biosynthesis of *p*-hydroxybenzylglucosinolate in *S. alba* in that they are both dependent on Cyt P450.

MATERIALS AND METHODS

Chemicals

Phenylacetaldoxime was chemically synthesized from phenylacetaldehyde using hydroxylamine hydrochloride in an alkaline medium as described previously (Sekiya et al., 1961). L-[U-^{14}C]Phe (464 mCi/mmol) was purchased from Amersham. [U-^{14}C]Phenylacetaldoxime was enzymatically synthesized by administration of L-[U-^{14}C]Phe to microsomal preparations from *Tropaeolum majus*. The reaction mixtures were extracted with ethyl acetate, and phenylacetaldoxime was isolated from the extracts by TLC as described below. Benzylglucosinolate was a generous gift from Professor Anders Kjær (Department of Organic Chemistry, The Technical University, Lyngby, Denmark). 2-Amino-indan-2-phosphonic acid was a generous gift from Professor Nikolaus Amrhein. BAS 110, BAS 111, LAB 150 978, and Tetcyclasis were gifts from BASF (Limburgerhof, Germany). SK&F 525-A was a gift from Smith, Kline and French (King of Prussia, PA). Ancymidol was a gift from Lilly Research Laboratories (Greenfield, IN). Phenobarbital was purchased from Nomeco (Copenhagen, Denmark). All other chemicals were purchased from Sigma.

Plant Material

Seeds of *T. majus* L. cv Empress of India were obtained from Dansk Havefrøforsyning (Kolding, Denmark). The seeds were germinated in complete darkness for 1 week in moist vermiculite at 24°C, after which the etiolated seedlings reached a height of approximately 4 cm. At this stage, the seedlings were transferred to continuous light (60 μmol photons m^{-2} s^{-1}, 400–700 nm of light provided by fluorescent lights). After 3 d in the light, the seedlings were treated with various chemical reagents for 24 h and then subjected to in vivo labeling experiments.

In Vivo Biosynthesis of Glucotropaeolin

Excised seedlings of *T. majus* were inserted into a test tube containing 1 μCi of L-[U-^{14}C]Phe or trace amounts of [U-^{14}C]phenylacetaldoxime in 20 μL of water and incubated for 24 h in continuous light. After incubation, the plant material was extracted with 90% (v/v) methanol (Lykkesfeldt and Møller, 1993). The extracts were concentrated in vacuo and the residue was dissolved in water. Qualitative analysis of the glucosinolate content was carried out using TLC (Kieselgel 60 F254; Merck, Darmstadt, Germany) in solvent system 1. Radioactive bands on TLC plates were visualized using autoradiography. Quantitative determinations of glucotropaeolin were carried out on an HPLC system equipped with UV (model LKB-2141, LKB, Bromma, Sweden) and radioactivity (model LB-506 C1, Berthold, Wildbad, Germany) monitors. Aliquots (50 μL) of extracts were applied to the column (Nucleosil 100–10C$_{18}$ 250 × 4.6 mm; Macherey-Nagel, Düsen, Germany) and eluted isocratically with 10% (v/v) solvent B (70% [v/v] methanol) in solvent A (0.1 M ammonium acetate) at a flow rate of 1.0 mL/min (Du et al., 1995). The UV absorption of the effluent was recorded at 254 nm, and the radioactivity was simultaneously monitored by the Berthold monitor fed liquid scintillator (Monoflow 2; National Diagnostics, Manville, NJ) at a rate of 3.0 mL/min. The retention time of glucotropaeolin was verified with an authentic sample. The biosynthetic activity was calculated from the radioactivity incorporated into glucotropaeolin with correction for the loss of one carbon atom by decarboxylation. All assays were carried out in duplicate.

Preparation of Microsomal Enzyme System

The microsomes were prepared as described earlier by Du et al. (1995). Typically, 100 g of chemically treated seedlings of *T. majus* were ground in a prechilled mortar with 10 g of polyvinylpolypyrrolidone and 30 g of acid-washed sea sand in 200 mL of isolation buffer (250 mM Tricine, 250 mM Suc, 100 mM ascorbic acid, 50 mM of $NaHSO_3$, 2 mM DTT, 2 mM EDTA, 1 mM PMSF, and 5 mg/mL BSA, pH 8.2), which was degassed and argon flushed three times prior to use. After the sample was filtered and centrifuged for 10 min at 15,000*g*, the microsomal pellet was obtained by centrifugation of the 15,000*g* supernatant for 30 min at 200,000*g*. The microsomal pellet was resuspended in isolation buffer and dialyzed sequentially under a nitrogen atmosphere against the isolation buffer for 1 h; against 50 mM Tricine, pH 7.9, 100 mM ascorbic acid, 2 mM DTT for 1 h; and twice against 50 mM Tricine, pH 7.9, 2 mM DTT for 1 h each. After dialysis, the

microsomal preparation was adjusted to a final protein concentration of 10 mg/mL. The isolation procedure was carried out at 4°C.

In Vitro Biosynthesis of Phenylacetaldoxime

The in vitro biosynthetic activity of the microsomal preparation from *T. majus* was determined as production of phenylacetaldoxime using Phe as substrate. A standard reaction mixture contained 0.4 mg of microsomal protein, 1 μCi (2.2 nmol) of L-[U-^{14}C]Phe (464 mCi/mmol) diluted with varying amounts (0–200 nmol) of unlabeled Phe, and 0.6 μmol of NADPH in a total volume of 280 μL of 50 mM Tricine, pH 7.9, 2 mM DTT. The reaction was started by the addition of NADPH. After incubation at 35°C for 30 min, the reaction was stopped by extraction with 750 μL of ethyl acetate. After the sample was centrifuged at 15,000*g* for 5 min, the ethyl acetate phase was carefully removed without interference with the protein-rich interphase. The reaction mixture was re-extracted once. The combined extracts were concentrated in vacuo, streaked onto TLC plates, and eluted with system 1. Radioactive bands on the TLC plates were visualized using autoradiography. The area corresponding to *p*-coumaric acid was scraped off and eluted with ethyl acetate. The eluate was concentrated and rerun on a TLC plate eluted with solvent system 2. This allowed the separation of phenylacetaldoxime from *p*-coumaric acid in extracts from green plant material as verified with authentic standards. The ^{14}C-labeled oxime was eluted by ethyl acetate and quantified by scintillation counting (LKB-RackBeta, Bromma, Sweden). The production of oxime was calculated from the radioactivity incorporated into oxime with correction for the loss of the C-1 atom by decarboxylation.

Effects of Cyt P450 Inhibitors on Phenylacetaldoxime Production

Microsomal reaction mixtures containing 1.0 μCi of L-[U-^{14}C]Phe were incubated in the presence of 0.1 mM of Cyt P450 inhibitors as described previously (Halkier and Møller, 1991). After 30 min of incubation at 35°C, the reaction mixtures were extracted with ethyl acetate and analyzed by TLC eluted with solvent system 1. The area corresponding to *p*-coumaric acid was extracted with ethyl acetate, rerun on a TLC plate, and eluted with solvent system 2. The relative intensity of the oxime band on autoradiography was quantified using a visual imager.

GC-MS Analysis

Phenylacetaldoxime for GC-MS was obtained by incubating standard microsomal reaction mixtures with 0.7 mM Phe, followed by purification on TLC as described above. The isolated phenylacetaldoxime was dissolved in ethyl acetate and analyzed on a mass spectrometer (JMS AX505W, JEOL) directly interfaced to a gas chromatograph (HP5890 Series II, Hewlett-Packard). Splitless injection at 180°C was applied. The capillary column (HP-1, Hewlett-Packard) was 25 m, 0.2 mm i.d., 0.33-μm film thickness. The head pressure was 70 kPa, and the oven temperature was programed as follows: 40°C for 1 min, 10°C/min to 250°C, and 250°C for 5 min. Electron impact mass spectra (70 eV, 200–250°C) were obtained at a repetition rate of 1 scan/s.

RESULTS

Induction of de Novo Biosynthesis of Glucotropaeolin

Seedlings of *T. majus* were treated with chemical reagents known to induce either phytoalexin biosynthesis or Cyt P450 enzymes to define whether these reagents would also induce de novo biosynthesis of glucosinolates. The induction was measured as incorporation of radioactivity into glucotropaeolin upon administration of ^{14}C-labeled Phe to the seedlings. Exposure of the seedlings to light for 3 d increased incorporation of radioactivity into ^{14}C-labeled glucotropaeolin approximately 2-fold compared with dark-grown plants (Fig. 1). Exposure to light combined with treatment with 50% ethanol resulted in an approximately 4-fold induction. A 9-fold induction, compared with untreated, dark-grown seedlings of de novo biosynthesis of glucotropaeolin, was obtained by the combined treatment of seedlings with light and 50 μM jasmonic acid dissolved in 50% ethanol.

As much as 40% of the radioactivity of the fed [^{14}C]Phe was incorporated into glucotropaeolin, when the seedlings were exposed to light and jasmonic acid dissolved in ethanol. The other chemical reagents showed a lower level of induction, varying from 2- to 4-fold (Fig. 1). The combined effect of light, jasmonic acid, and ethanol on the de novo biosynthesis of glucotropaeolin was transient, with an increase in the first 2 d after treatment followed by a decrease to normal level within the next 2 d (Fig. 2). The total content of glucotropaeolin in *T. majus* seedlings was not affected by the treatment (data not shown).

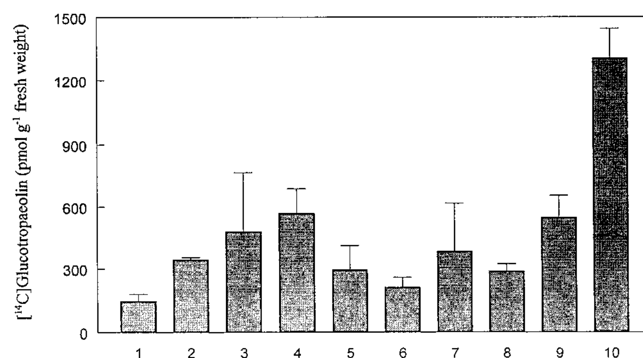

Figure 1. Induction of de novo biosynthesis of glucotropaeolin in seedlings of *T. majus* upon treatment with various chemical reagents. One-week-old etiolated seedlings were exposed to light for 3 d, followed by treatment (10 mL/100 g fresh weight) with water (bar 2), 2 mM salicylic acid (bar 3), 2 mM methylcholanthrene (bar 4), 2 mM phenobarbital (bar 5), 2 mM $MnCl_2$ (bar 6), 2 mM Phe (bar 7), 2% (v/v) ethanol (bar 8), 50% (v/v) ethanol (bar 9), and 50 μM jasmonic acid in 50% (v/v) ethanol (bar 10). Control seedlings (bar 1) were kept in the dark and treated with water. After 24 h of treatment, the biosynthetic activity was measured as incorporation of radioactivity into glucotropaeolin upon administration of 1 μCi (2.2 nmol) of L-[U-^{14}C]Phe to excised seedlings. Results are means ± SE.

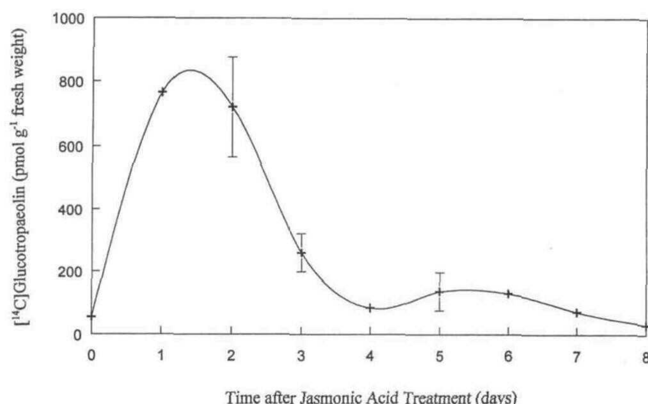

Figure 2. Time course of de novo biosynthesis of glucotropaeolin in *T. majus* seedlings exposed for 24 h to the combined treatment of light and 50 μM jasmonic acid dissolved in 50% ethanol. The biosynthesis was measured as incorporation of [^{14}C]Phe into [^{14}C]glucotropaeolin. Results are means ± SE. SE values not shown were smaller than the symbol.

In Vitro Biosynthesis of Phenylacetaldoxime

A biosynthetically active microsomal preparation was isolated from *T. majus* using jasmonic acid-induced, light-grown seedlings. Production of ^{14}C-labeled phenylacetaldoxime from ^{14}C-labeled Phe by *T. majus* microsomal reaction mixtures was demonstrated by co-migration of a labeled component with authentic phenylacetaldoxime on TLC (Fig. 3). The production of oxime by the microsomal system was strictly NADPH dependent. Phe is also a precursor for the ubiquitous phenylpropanoid pathway. This results in a concomitant accumulation of cinnamic acid and *p*-coumaric acid in the microsomal reaction mixtures, as seen on the TLC chromatogram (Fig. 3). Solvent systems 1 and 2 allow the separation of standards of phenylacetaldoxime and *p*-coumaric acid on the TLC plate. This is not the case when phenylacetaldoxime and *p*-coumaric acid accumulated in microsomal reaction mixtures are extracted with ethyl acetate and applied to the TLC plates (Fig. 3A). Under these conditions, phenylacetaldoxime and *p*-coumaric acid do not separate in either system, presumably because of co-extraction of sticky, hydrophobic material from microsomal membranes. However, when the area corresponding to *p*-coumaric acid on the TLC plate eluted in solvent system 1 is extracted with ethyl acetate and applied to a second TLC plate, phenylacetaldoxime and *p*-coumaric acid separate in either solvent system (Fig. 3B). The production of oxime has been confirmed by GC-MS (Fig. 4). On GC, the isolated phenylacetaldoxime gave two peaks at retention times 13.14 min (*E* isomer) and 13.49 min (Z isomer), as did the authentic phenylacetaldoxime (data not shown). The MS profile of the two peaks were identical, with a molecular ion of *m/z* 135, a $[M-H_2O]^+$ ion of *m/z* 117, and a tropylium ion of *m/z* 91 (Fig. 4).

In vivo administration of enzymatically produced ^{14}C-labeled phenylacetaldoxime to seedlings of *T. majus* resulted in the production of ^{14}C-labeled glucotropaeolin as evidenced by co-migration of authentic glucotropaeolin on TLC (Fig. 5). This supports the identification of oximes as intermediates in the biosynthetic pathway of glucosinolates.

The Effect of Cyt P450 Inhibitors on Phenylacetaldoxime Production

The production of phenylacetaldoxime by *T. majus* microsomes was carried out in the presence of several known inhibitors of Cyt P450 enzymes (0.1 mM; Table I). All of the inhibitors reduced the amount of oxime produced by the microsomes to varying degrees. This indicates that a Cyt P450 is involved in the oxime production. Tetcyclasis had the strongest effect by inhibiting 86%.

DISCUSSION

Ixn this paper we report the isolation of an in vitro enzyme system that actively converts Phe into phenylacetaldoxime in the biosynthesis of glucotropaeolin in seedlings of *T. majus*. Low biosynthetic activity was overcome by the combined treatment of seedlings with light and 50 μM jasmonic acid dissolved in 50% ethanol. We found that de novo biosynthesis

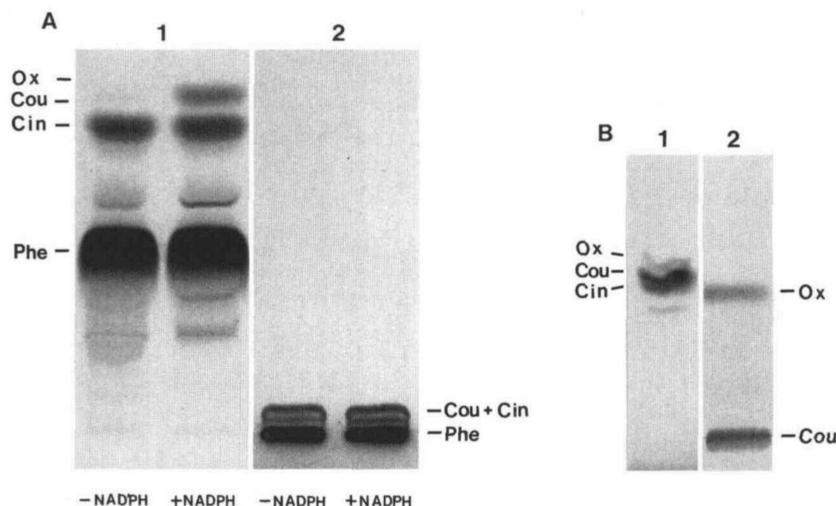

Figure 3. Production of phenylacetaldoxime from Phe by *T. majus* microsomes. Standard reaction mixtures were incubated with 1 μCi of L-[U-^{14}C]Phe for 30 min at 35°C. A, TLC chromatogram of ethyl acetate extracts of microsomal reaction mixtures eluted with solvent system 1 (panel 1) and solvent system 2 (panel 2). B, TLC chromatogram of the ethyl acetate eluate of the area corresponding to *p*-coumaric acid in the TLC chromatogram in A, which was eluted in solvent system 1. The eluate was rerun in solvent system 1 and 2. Ox, Phenylacetaldoxime; Cou, *p*-coumaric acid; Cin, cinnamic acid.

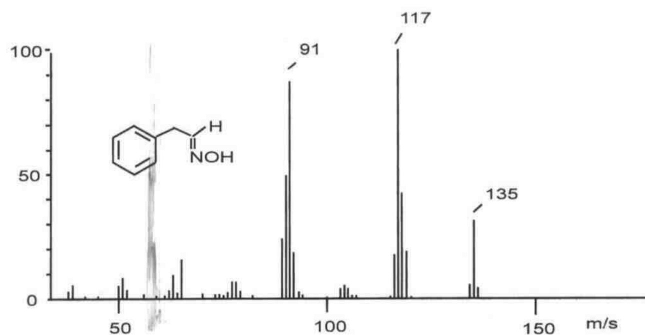

Figure 4. Identification of phenylacetaldoxime by GC-MS analysis. Microsomes from *T. majus* were incubated with Phe (200 nmol) in standard reaction mixtures. The oxime produced was extracted with ethyl acetate, purified by TLC (for details, see legend to Fig. 3), and analyzed by GC-MS. The mass spectrum contains the molecular ion of *m/z* 135, the $[M\text{-}H_2O]^+$ ion of *m/z* 117, and the tropylium ion of *m/z* 91.

of the aromatic glucotropaeolin in *T. majus* was transiently induced 9-fold upon this treatment. Jasmonates are known to elicit the accumulation of numerous phytoalexins (e.g. terpenoids, flavonoids, anthraquinones) (Blechert et al., 1995). It has been reported that the total content of indolylglucosinolates, but not aromatic and aliphatic glucosinolates, can be induced by jasmonic acid in several *Brassica* spp. (Bodnaryk, 1994; Doughty et al., 1995). In our experiments, the total content of glucotropaeolin was not affected, which is in contrast to an up to 9-fold increase in the total content of specific indolylglucosinolates in several *Brassica* spp. upon jasmonic acid treatment (Bodnaryk, 1994). This suggests that the pool of free Phe in *T. majus* might be limiting, or that competing pathways, e.g. the phenylpropanoid pathway, are induced simultaneously. The induction pattern for glucotropaeolin biosynthesis upon jasmonic acid treatment in *T. majus* resembles the pattern for induction of *p*-hydroxybenzylglucosinolate synthesis in *S. alba* (Du et al., 1995). Both plants show

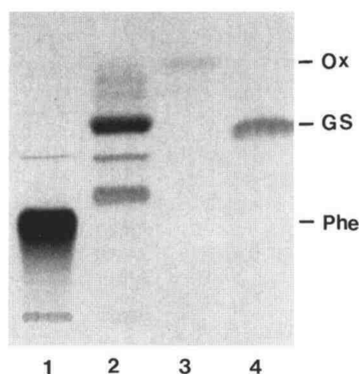

Figure 5. Production of glucotropaeolin from [U-^{14}C]phenylacetaldoxime in seedlings of *T. majus* exposed to the combined treatment of light, jasmonic acid, and ethanol. The induced seedlings were fed 1 μCi of L-[U-^{14}C]Phe or trace amounts of [U-^{14}C]phenylacetaldoxime for 24 h. Extracts of the seedlings were analyzed by TLC in solvent system 1. Lane 1, Phe tracer; lane 2, glucotropaeolin produced in vivo from Phe; lane 3, phenylacetaldoxime tracer; and lane 4, glucotropaeolin produced in vivo from phenylacetaldoxime. Ox, Phenylacetaldoxime; GS, glucotropaeolin.

transient inductions in de novo biosynthesis of glucosinolates, without a concomitant increase in the total content of glucosinolates.

During homogenization of plant material from *T. majus*, glucotropaeolin is hydrolyzed by myrosinase to benzylisothiocyanate. Glucosinolate isothiocyanates are known to react with the hydroxyl groups of the Ser and Thr residues in proteins (Björkman, 1973). Typically, microsomes from *Sorghum bicolor* L. Moench are highly active in catalyzing the biosynthesis of the cyanogenic glucoside dhurrin (Conn, 1980). However, when sorghum microsomes are prepared in the presence of leaves from *T. majus*, the biosynthetic enzymes are inactivated, and the inhibitory component has been shown to be benzylisothiocyanate (Lykkesfeldt and Møller, 1993). In the present study, the formation of benzylisothiocyanate was prevented by using buffers containing 100 mM ascorbic acid, which is known to inhibit myrosinase activity (Björkman and Lönnerdal, 1973). The buffer has previously been used to isolate successfully biosynthetically active microsomes from the glucosinolate-producing *S. alba* (Du et al., 1995). Under these conditions, the presence of equal amounts of *T. majus* plant material during homogenization resulted in the recovery of more than 50% of the biosynthetic activity in sorghum microsomes (data not shown), indicating that the formation of benzylisothiocyanate was significantly reduced.

The detection of phenylacetaldoxime accumulating in the reaction mixtures was hampered because of interference from simultaneously produced intermediates from the relatively more active phenylpropanoid pathway. Furthermore, the enzymes in the phenylpropanoid pathway were also induced by jasmonic acid, as evidenced by an increased accumulation of cinnamic acid and *p*-coumaric acid upon jasmonic acid treatment (data not shown). When ethyl acetate extracts of microsomal reaction mixtures were analyzed by TLC, phenylacetaldoxime and *p*-coumaric acid co-migrated on the TLC plates. Re-extraction from the TLC plate of the area correspond-

Table I. *Effects of Cyt P450 inhibitors on the production of phenylacetaldoxime*

T. majus microsomes were incubated with 1.0 μCi of ^{14}C-labeled Phe in the presence of 0.1 mM Cyt P450 inhibitors in a standard reaction mixture. After incubation, the microsomal reaction mixture was extracted with ethyl acetate and analyzed by TLC eluted with solvent system 1. The area corresponding to *p*-coumarate was extracted with ethyl acetate and rechromatographed on a TLC plate eluted with solvent system 2. The relative intensity of the oxime band on autoradiography was quantified using a visual imager.

Inhibitor	Relative Oxime Production
	%
None	100
SK&F 525-A	68
Ancymidol	47
BAS 110	41
LAB 150 978	61
Tetcyclasis	16
BAS 111	51

ing to phenylacetaldoxime and *p*-coumaric acid, followed by a rerun on a second TLC plate, allowed the proper separation of the two compounds. This opens the possibility that earlier attempts to obtain in vitro biosynthetic activity from *T. majus* microsomes might have been unsuccessful because of the lack of a proper detection method for phenylacetaldoxime. Attempts to inhibit specifically Phe ammonia-lyase, the first enzyme in the phenylpropanoid pathway, with 2-aminoindan-2-phosphonic acid, a known Phe ammonia-lyase inhibitor (Zon and Amrhein, 1992), were unsuccessful. This was presumably because of a similar inhibition of the enzyme converting Phe into phenylacetaldoxime.

The enzyme activity catalyzing the conversion of Phe to phenylacetaldoxime in *T. majus* microsomes was shown to be inhibited by several Cyt P450 inhibitors. This indicates that the enzyme activity is dependent on Cyt P450. Tetcyclasis, which showed the highest level of inhibition of oxime production, has also been shown to be a strong inhibitor of the Cyt P450 enzyme involved in the biosynthesis of *p*-hydroxybenzylglucosinolate in *S. alba* (Du et al., 1995). Demonstration of Cyt P450 involvement by photoreversible carbon monoxide inhibition of enzyme activity was not possible because of an approximately 40% inactivation of enzymic activity upon irradiation of the reaction mixtures. The inactivation was probably due to chlorophyll radicals generated in the green microsomes by the light. Cyt P450 involvement in the biosynthesis of glucotropaeolin in *T. majus* and of *p*-hydroxybenzylglucosinolate in *S. alba* suggests that homologous enzyme systems are catalyzing the conversion of amino acids to oximes in at least two different families within the Capparales order, namely in the Brassicaceae and Tropaeolaceae.

Wallsgrove and coworkers have demonstrated NADPH-dependent CO_2 release and oxime production from homophenylalanine by microsomes isolated from young leaves of oilseed rape (*Brassica napus*) (Dawson et al., 1993). The ability to measure NADPH-dependent release of CO_2 was extended to several aliphatic and aromatic amino acid precursors of glucosinolates in oilseed rape and other *Brassica* spp. (Bennett et al., 1993, 1995a). The decarboxylation reactions were shown not to be sensitive to the presence of carbon monoxide or the Cyt P450 inhibitor 1-aminobenzotriazole, indicating that Cyt P450 was not involved in these reactions (Bennett et al., 1995a). Wallsgrove and coworkers suggested that the conversion of aromatic and aliphatic amino acids to oximes in *Brassica* spp. was catalyzed by flavin monooxygenases (Bennett et al., 1993). Ludwig-Muller et al. (1990) and Bennett et al. (1995b) found indications that the oximes in the biosynthesis of indolylglucosinolates in *Brassica* spp. are synthesized by a plasma membrane-bound peroxidase type of enzyme. If all of these sets of observations are correct, this would imply that the enzyme system catalyzing the conversion of amino acids to oximes within the Brassicaceae family has evolved independently three times.

Cyanogenic glucosides are a related group of secondary plant products that also have amino acids as precursors and oximes as intermediates (Conn, 1980). In the biosynthesis of dhurrin, the Tyr-derived cyanogenic glucoside in *S. bicolor* L. Moench, a single, multifunctional Cyt P450 has been shown to catalyze the conversion of Tyr to *p*-hydroxyphenylacetaldoxime (Sibbesen et al., 1995). Our results on Cyt P450 involvement in the oxime production by microsomes from *T. majus* and *S. alba* provide experimental documentation that homologous enzyme systems catalyze the conversion of amino acids into the corresponding oximes in the biosynthesis of cyanogenic glucosides and glucosinolates.

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