

# Photoaffinity labeling of *Arabidopsis thaliana* plasma membrane vesicles by 5-azido-[7-<sup>3</sup>H]indole-3-acetic acid: Identification of a glutathione S-transferase

(photoaffinity labeling/auxin-binding protein)

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**ABSTRACT** We used 5-azido-[7-<sup>3</sup>H]indole-3-acetic acid (5-azido-[7-<sup>3</sup>H]IAA), a photoaffinity analogue of the plant hormone indole-3-acetic acid (IAA), to search for auxin-binding proteins in *Arabidopsis thaliana* membranes. We identified an auxin-binding protein with a molecular mass of 24 kDa (Atpm24) in microsomes as well as in plasma membrane vesicles. Atpm24 was solubilized by 1% Triton X-100 and partially purified. A cDNA clone (Atpm24.1) corresponding to Atpm24 was isolated. The amino acid sequence predicted from the Atpm24.1 cDNA contains 212 amino acid residues with a relative molecular mass of 24,128 Da. Data base searches revealed that the predicted protein has homology to glutathione S-transferases (GSTs; EC 2.5.1.18). When Atpm24.1 was expressed in *Escherichia coli*, we found a high level of GST activity in the bacterial extracts. We have analyzed the substrate specificity of this protein and found that cumene hydroperoxide and *trans*-stilbene oxide but not *trans*-cinnamic acid or IAA-CoA were substrates. A role for this GST in physiological processes of plants is discussed.

Auxins are phytohormones that influence a wide range of growth and developmental responses in plants (for review, see ref. 1). In any of the various cellular responses to auxin, the first step will probably be the recognition of the hormone by molecules that may be considered as receptors coupling the auxin signal to an appropriate cellular response. The search for such proteins has led to the identification of soluble and membrane-associated auxin-binding sites. Early studies demonstrated the existence of plasma membrane (PM)-localized auxin-binding sites (2). It has been argued that these proteins could be involved in auxin uptake, auxin efflux, and in control of elongation growth. Initial biochemical characterization using equilibrium auxin-binding assays as a guide for purification of these proteins was met with limited success due to technical difficulties. Photoactivatable auxin analogues, such as 5-azido-[7-<sup>3</sup>H]indole-3-acetic acid (5-azido-[7-<sup>3</sup>H]IAA), turned out to be valuable tools to identify auxin-binding proteins (3–9). Here we report the photoaffinity labeling of a 24-kDa polypeptide in PM vesicles of *Arabidopsis thaliana*. This protein was partially purified and sequenced<sup>†</sup> and its cDNA was found to encode a glutathione S-transferase (GST; EC 2.5.1.18). After expression in *Escherichia coli* we have analyzed its substrate specificity.

## MATERIALS AND METHODS

**Membrane Isolation and Photoaffinity Labeling.** Six-week-old *A. thaliana* plants were harvested without roots and frozen in liquid nitrogen. Two hundred grams of frozen material was homogenized in 500 ml of buffer 1 [500 mM

sucrose/50 mM Hepes-KOH, pH 7.5/5 mM ascorbic acid/3.6 mM cysteine/Trasyol (3.5 μg/liter)/leupeptin (0.1 μg/liter)]. The combined homogenates were centrifuged at 4°C for 15 min at 10,000 × g, and a microsomal pellet was obtained by centrifugation (142,000 × g for 40 min at 4°C). Chlorophyll-free PM vesicles were enriched from the microsomal fraction by two-phase partitioning. PM vesicles were resuspended in buffer 2 (10 mM Mes/Tris, pH 6.5/250 mM sucrose) and frozen in liquid nitrogen. Photoaffinity labeling was as described (10). Aliquots of resuspended PM vesicles containing 100 μg of protein were diluted to 80 μl with buffer 2. The pH was adjusted to 4.5 or 5.5 with 50 mM citric acid or to pH 7.5 with 20 mM Tris base. Optionally, 1 mM IAA or 1 mM 1-naphthylphthalamic acid (NPA) was added for competition analysis. To each assay, 10 μl of a 10 μM solution of 5-azido-[7-<sup>3</sup>H]IAA in buffer 2 was added.

**Fractionation of PM Proteins.** After illumination, the membranes were pelleted at 100,000 × g (11 min, 2°C). The membrane pellets were resuspended in 100 μl of buffer 2/1% (vol/vol) Triton X-100 for 30 min at 4°C. The pelleted PM vesicles (100,000 × g, 11 min, 2°C) were resuspended in 20 μl of SDS-containing loading buffer. Solubilized proteins were precipitated from the supernatant by 12% trichloroacetic acid for 2 h on ice and dissolved in 20 μl of SDS loading buffer by heating to 70°C for 5 min. The proteins were separated by SDS/PAGE (12.5%).

**Purification of Atpm24.** PM vesicles (containing 3 mg of protein) were suspended in 2 ml of buffer 2. Twenty microliters of a 10 μM solution of 5-azido-[7-<sup>3</sup>H]IAA was added. After photoaffinity labeling, 200 μl of a 10% solution of Triton X-100 in buffer 2 was added, and the sample was incubated for 30 min at 4°C. After centrifugation (15 min at 100,000 × g), the pellet was resuspended in 1 ml of buffer 2 containing 1% Triton X-100. After solubilization and centrifugation, the supernatants were combined and diluted with 10 mM Mes/Tris (pH 6.5) to a final volume of 30 ml. This solution was loaded onto a Q-Sepharose column (Pharmacia; 600-μl bed volume). After washing with buffer 2 containing 0.06% Triton X-100, proteins were eluted stepwise by buffer 2/0.06% Triton X-100 containing 100 mM, 250 mM, 500 mM, and 1 M sodium chloride. Fractions were analyzed by SDS/PAGE. Coomassie-stained gels were used subsequently for fluorography. Electrophoretic transfer of proteins from polyacrylamide gels to poly(vinylidene difluoride) (PVDF) was performed in 50 mM Tris/50 mM boric acid, pH 8.3, for 8 h at 30 V.

**Sequence Analysis.** *In situ* proteolytic digestion of PVDF-bound proteins was carried out using trypsin or endoprotein-

Abbreviations: GST, glutathione S-transferase; IAA, indole-3-acetic acid; NPA, 1-naphthylphthalamic acid; PM, plasma membrane; GSH, glutathione; PVDF, poly(vinylidene difluoride).

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<sup>†</sup>The sequence reported in this paper has been deposited in the GenBank data base (accession no. X75303).

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ase Asp-N. Sequence analysis was performed using an Applied Biosystems 477 gas-phase sequencer equipped with an on-line phenylthiohydantoin amino acid analyzer (model 120A).

**Recombinant DNA Techniques.** These were performed, with minor variations, according to ref. 11.

**Design of Oligonucleotide Primers.** Two degenerate oligonucleotides were designed: oligo A (5'-CARCCYTTYGGW-CARGTBCCNG-3') and oligo B (5'-CVCACCAWGCIIYTVACACGNGG-3') (where R = A or G; Y = T or C; W = A or T; B = T, C, or G; V = C, G, or A; N = A, T, C, or G).

**PCR and Cloning.** The reaction mixture for PCR contained 1  $\mu$ l of amplified phage lysate of an *A. thaliana* *agt10* cDNA library ( $10^6$ – $10^8$  plaque-forming units per  $\mu$ l), *Taq* polymerase buffer [50 mM KCl/10 mM Tris·HCl, pH 9.5/1.5 mM MgCl<sub>2</sub>/0.01% (wt/vol) gelatin], oligonucleotides A and B each at 50 pM, and water. DNA was denatured for 10 min at 100°C. After cooling on ice, 200  $\mu$ M dNTPs (final concentration) and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega) were added to the reaction mixture. PCR was performed in a total volume of 100  $\mu$ l in a thermocycler for 30 cycles: 1 min at 92°C (denaturation), 1 min at 50°C (annealing), and 1 min at 65°C (extension). After amplification, 1 unit of Klenow polymerase (Boehringer Mannheim) was added, and the mixture was incubated at room temperature for 30 min. A 440-bp DNA fragment of expected size was isolated, and its identity was confirmed by DNA sequence analysis. Three hundred thousand recombinants of an *A. thaliana* *agt10* library were screened. Six out of 15 positive recombinants were selected for further analysis. Double-stranded DNA sequences were determined using fluorescent primers and an automated DNA sequencer (A.L.F.; Pharmacia).

**Heterologous Expression of the *A. thaliana* GST in *E. coli*.** The coding region of the *A. thaliana* GST was amplified by PCR using oligonucleotide primers C (27-mer, 5'-CCG-GATCCCATATGGCAGGTATCAAAG-3') and D (26-mer, 5'-ACGGATCCCTCACTGAACCTTCTCGG-3'). The GST coding region was inserted into pET 3a resulting in pET-GSTex. The *E. coli* host BL21 (DE3) (12) was transformed with pET-GSTex, and after isopropyl  $\beta$ -D-thiogalactoside induction Atpm24 was expressed as a soluble protein ( $\approx$ 90% of the total soluble *E. coli* protein). Reaction products were analyzed according to ref. 13 or by HPLC on a C<sub>4</sub> reversed-phase column (4.6  $\times$  250 mm, Vydac; Separations Group, U.S.A.). After equilibration of the column with 0.06% trifluoroacetic acid (solvent A), compounds were eluted at a flow rate of 0.4 ml/min with a linear gradient of 0–100% solvent B in 61 min. Solvent B was 80% acetonitrile (HPLC grade; Baker)/0.056% trifluoroacetic acid. Eluting compounds were detected by UV absorbance at 214 nm and 280 nm.

## RESULTS

**In *A. thaliana* Membrane Vesicles Two Polypeptides Are Labeled with 5-Azido-[7-<sup>3</sup>H]IAA.** Photoaffinity labeling of crude microsomal membranes by 5-azido-[7-<sup>3</sup>H]IAA at  $-196^\circ\text{C}$  resulted in identification of a polypeptide with an apparent molecular mass of 23 kDa (Atpm23) (Fig. 1, lane 1). In PM vesicles a polypeptide with a similar molecular mass is radioactively labeled by 5-azido-[7-<sup>3</sup>H]IAA at  $-196^\circ\text{C}$  (Fig. 1, lane 2). In addition, a major polypeptide with an apparent molecular mass of 24 kDa (Atpm24) was detected in PM vesicles (Fig. 1, lane 2). A signal in this molecular mass range was detected in crude microsomes only after exposure of fluorograms for more than 28 days (data not shown).

To analyze the strength of association of Atpm23 and Atpm24 to membrane vesicles, photoaffinity labeled proteins were extracted according to the scheme shown in Fig. 2A. Most of the Atpm24 was detected in the Triton X-100 extract

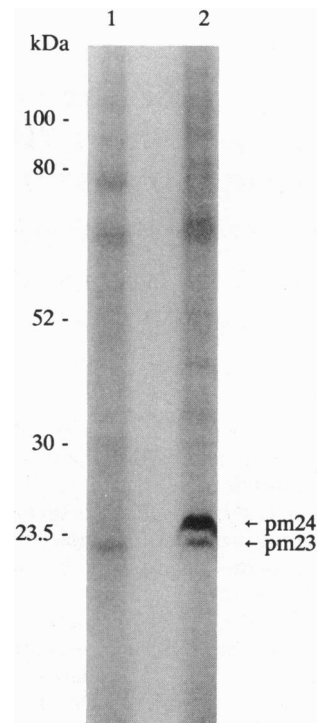


FIG. 1. Photoaffinity labeling of *A. thaliana* membrane proteins using 5-azido-[7-<sup>3</sup>H]IAA. Crude microsomal membranes (lane 1) and PM vesicles (lane 2; each containing 100  $\mu$ g of proteins) were subjected to photoaffinity labeling as described in *Materials and Methods*. Radioactively labeled proteins were separated by SDS/12.5% PAGE and visualized by fluorography. Sizes of molecular mass standard proteins are indicated.

(Fig. 2B, lane 4), suggesting a weak association of Atpm24 to membranes. This conclusion is also supported by our observation that  $\approx$ 20% of Atpm24 could be removed from PM vesicles by repeated mechanical homogenization and subsequent ultracentrifugation (Fig. 2B, lane 1). Due to its weak labeling by 5-azido-[7-<sup>3</sup>H]IAA, Atpm23 is difficult to detect in either the Triton X-100 extract or in the PM pellet (Fig. 2B, lanes 3 and 4). Atpm23 was partially solubilized by Triton X-100 from PM vesicles; it was bound to Q-Sepharose from which it could be eluted with 250 mM NaCl (see Fig. 4, lane 5).

**Photoaffinity Labeling of Atpm24 with 5-Azido-[7-<sup>3</sup>H]IAA Is pH Dependent and Competable by IAA.** Labeling of Atpm24 was influenced by pH. We observed optimal labeling at pH 7.5 for Atpm24, whereas labeling of Atpm23 was pH independent (Fig. 3, lanes 1–3). When 1 mM IAA was added to the photoaffinity labeling assay prior to illumination, the labeling of Atpm24 but not Atpm23 was strongly reduced (Fig. 3, lane 4). The addition of an inhibitor of polar auxin transport, NPA, at 1 mM resulted in a slight reduction of the labeling efficiency of Atpm24. The photoaffinity labeling of Atpm23 was not significantly influenced by pH or by addition of unlabeled IAA or NPA to the labeling assays.

**Partial Purification of Atpm24 from *A. thaliana* PM Vesicles.** PM vesicles from *A. thaliana* were enriched from crude microsomal membranes by aqueous two-phase partitioning; PM vesicles containing 5–10 mg of protein were obtained from 200 g of *A. thaliana* tissue. A purification protocol for Atpm24 was established starting with PM vesicles containing 3 mg of protein (see Fig. 4). Radioactively labeled Atpm24 was found in the 100 mM NaCl eluate from a Q-Sepharose column (see Fig. 4, lane 4). To get a sufficient amount of material for proteolytic digestion and subsequent peptide sequencing, we scaled up the isolation procedure but omitted

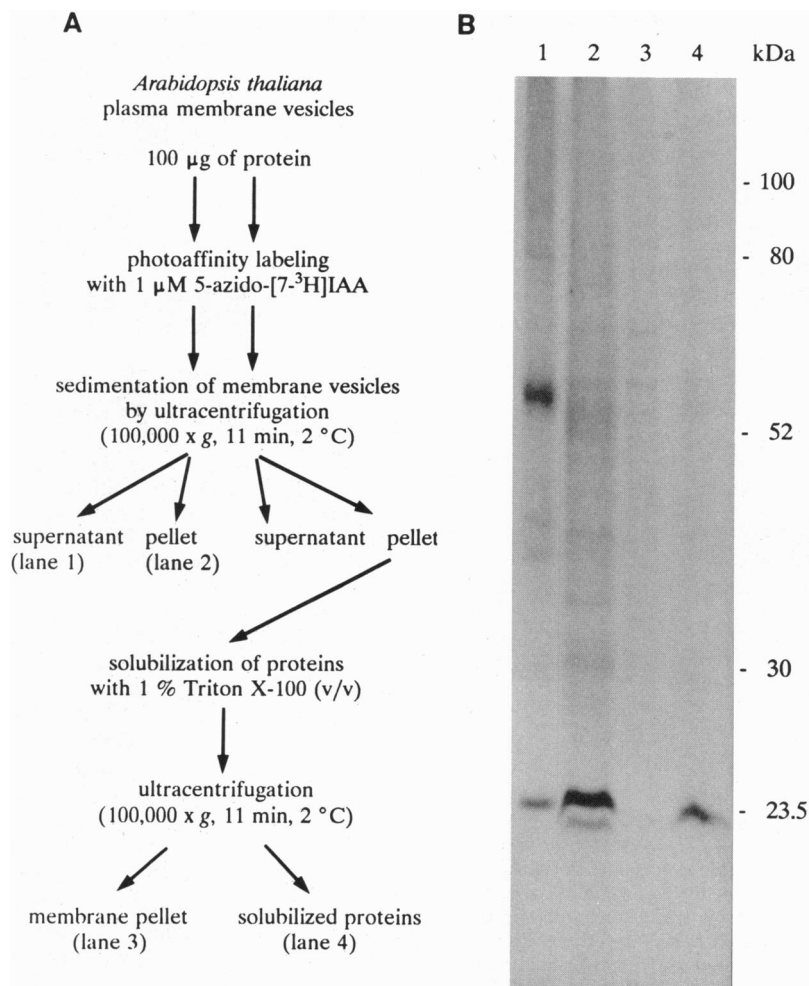


FIG. 2. Solubilization of photoaffinity labeled proteins with Triton X-100. PM vesicles (100  $\mu$ g of protein) were used for photoaffinity labeling with 1  $\mu$ M 5-azido-[ $^3$ H]IAA. After labeling, PM proteins were fractionated as indicated in A and described in *Materials and Methods*. (B) Labeled polypeptides were analyzed by SDS/12.5% PAGE and fluorography. Sizes of molecular mass standard proteins are indicated.

the initial labeling step. We started with PM vesicles containing  $\approx$ 20 mg of protein and observed similar patterns when proteins of the different fractions were analyzed by SDS/PAGE followed by Coomassie staining (data not shown).

**Atpm24 Is a GST.** After electrophoretic transfer of Atpm24 to PVDF membranes, *in situ* proteolytic digestion using

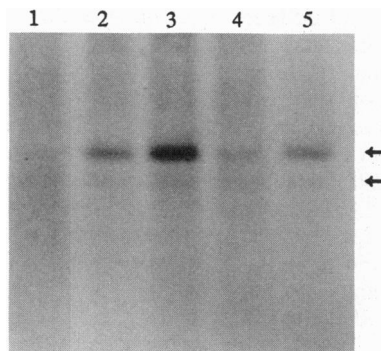


FIG. 3. Influence of pH and competitors on the efficiency of photoaffinity labeling. PM vesicles (100  $\mu$ g of protein) were incubated for 10 min at pH 4.5 (lane 1), pH 6.0 (lane 2), or pH 7.5 (lane 3). In addition, incubation at pH 7.5 was performed in the presence of 1 mM IAA (lane 4) or 1 mM NPA (lane 5). After photoaffinity labeling with 1  $\mu$ M 5-azido-[ $^3$ H]IAA, membrane proteins were analyzed by SDS/12.5% PAGE and fluorography. Arrows indicate Atpm24 and Atpm23.

trypsin and Asp-N was performed. After sequencing of obtained peptides, homology searches identified Atpm24 as a GST. The best sequence homology was found to a GST from tobacco encoded by the auxin-induced *parB* gene (13). The DNA sequence of Atpm24.1 was determined (GenBank accession number X75303). The predicted protein has 212 amino acid residues and a molecular mass of 24,128 Da. Significant homologies were found to other GSTs from maize (14) and wheat (15) (Fig. 5).

**Atpm24 Substrates.** After expression of Atpm24 in *E. coli* BL21, GST activity was assayed using 1-chloro-2,4-dinitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane as substrates (see Table 1). In addition *trans*-cinnamic acid, cumene hydroperoxide, IAA-CoA, and *trans*-stilbene oxide were tested. Whereas cinnamic acid, a substrate for a microsomal GST of pea (16), was not a substrate, cumene hydroperoxide and *trans*-stilbene oxide were substrates for Atpm24.

## DISCUSSION

Photoaffinity labeling techniques have proven to be useful for identification of hormone receptors and ligand binding proteins. Using 5-azido-[ $^3$ H]IAA we report here that two polypeptides with molecular masses of  $\approx$ 23 kDa and  $\approx$ 24 kDa are photoaffinity labeled in *A. thaliana* membrane vesicles. We found that Atpm24 was enriched during isolation of the

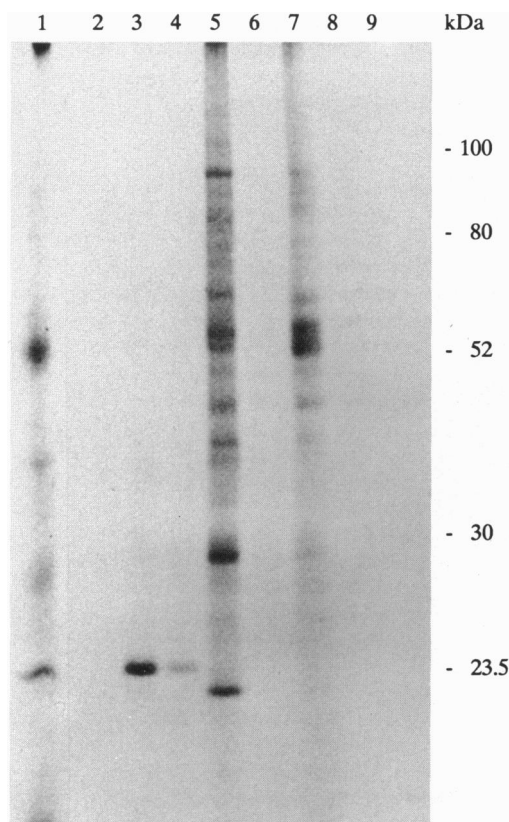


FIG. 4. Purification of Atpm24. Atpm24 was partially purified as described in *Materials and Methods*. After photoaffinity labeling of PM vesicles (3 mg of total protein) with 1 mM 5-azido-[7-<sup>3</sup>H]IAA, proteins were solubilized by 1% Triton X-100, and the extract was fractionated by anion-exchange chromatography (Q-Sepharose). Elution was performed stepwise with 100 mM, 250 mM, 500 mM, and 1 M NaCl. Between the main elution peaks (indicated by I and representing  $\approx 90\%$  of the peak area) minor peaks (indicated by II) were collected to avoid loss of radioactivity. The Triton X-100 extract (lane 1) and all eluates [flow through, lane 2; 100 mM NaCl (I), lane 3; 100 mM NaCl (II), lane 4; 250 mM NaCl (I), lane 5; 250 mM NaCl (II), lane 6; 500 mM NaCl (I), lane 7; 500 mM NaCl (II), lane 8; 1 M NaCl, lane 9] were analyzed by SDS/12.5% PAGE and subsequent fluorography. Sizes of molecular mass standard proteins are given at right.

PM vesicles ( $\approx 10$ -fold), suggesting an association of this protein with PMs.

To elucidate the identity of Atpm24, we partially purified this protein for microsequencing. *A. thaliana* PMs were labeled with 5-azido-[7-<sup>3</sup>H]IAA, which enabled us to easily monitor the purification. Amino acid sequence analysis of peptides obtained after proteolytic cleavage of Atpm24 allowed the deduction of oligonucleotides and isolation of a corresponding cDNA. The protein sequence predicted from the open reading frame of this clone contained all amino acid residues that were identified during microsequencing. Database searches revealed that the Atpm24.1 cDNA encodes a GST. Recently Macdonald and coworkers (9) reported several proteins that were labeled with 5-azido-[7-<sup>3</sup>H]IAA in a soluble fraction of *Hyoscyamus muticans* (6); one of these proteins was meanwhile shown to be a GST.

GSTs are dimeric multifunctional proteins that catalyze the conjugation of the tripeptide glutathione to a large variety of electrophilic and hydrophobic compounds. GSTs have been shown to be involved in the reduction of organic hydroperoxides and certain glutathione (GSH)-dependent isomerizations, and they have been shown to be binding proteins for a large number of hydrophilic chemicals (17, 18). While the

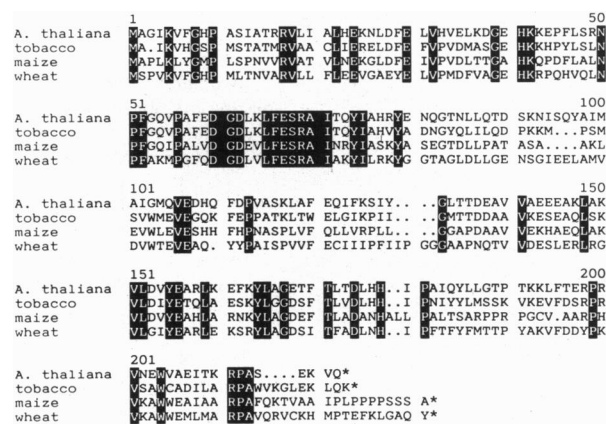


FIG. 5. Comparison of the deduced amino acid sequence of Atpm24.1 and GSTs from different plant species. Amino acid sequence of Atpm24.1 is compared with those of tobacco GST (*parB*; ref. 13), maize GST (MGST III; ref. 14), and wheat GST (*WIR5*; ref. 15). Identical amino acids are shown in white type with a black background.

physiological role of plant GSTs in these processes is not yet fully understood, it has been suggested that they detoxify, for example, lipid peroxides formed after pathogen attack of plant tissues and thereby protect the tissue from further damage (15); a role for GSTs in herbicide detoxification has meanwhile been well documented (19).

Less well understood is the role of GSTs in phytohormone action. It was found recently that in tobacco an auxin-regulated gene encodes a GST (13). After completion of review of this manuscript, a paper by Zhou and Goldsberg (20) was published in which these authors reported the cloning of an ethylene-induced *Arabidopsis* GST that is identical to Atpm24. These authors suggested that this GST might use auxin as a substrate for xenobiotic detoxification. Our finding that a GST from *A. thaliana* is labeled by 5-azido-[7-<sup>3</sup>H]IAA supports the view that auxin could be a substrate. Chemically activated derivatives of IAA such as IAA-thioesters (i.e., IAA-CoA) might be suitable endogenous substrates for GSTs (21). The putative conjugate resulting from transfer of glutathione to IAA-CoA (i.e., IAA-GSH) could then be an intermediate either for degradation of IAA or, alternatively, represent a compound allowing transport of this phytohormone to cellular target sites. Yet another speculative role for IAA-GSH was recently suggested (9) describing a possible involvement in regulation of GSH levels. Changes of GSH levels could alter the cellular redox potential and in consequence influence cellular differentiation by influencing the switch from somatic embryogenesis to cell proliferation in carrot cells (22).

To investigate whether Atpm24 can catalyze the formation of IAA-GSH *in vitro*, we used IAA-CoA as a substrate, but preliminary HPLC analysis of the reaction products did not indicate that IAA-CoA is a substrate under the reaction conditions tested. Alternatively, it is equally possible that auxin could bind to Atpm24; in fact, for some animal GSTs a second binding site for steroid and porphyrin derivatives, distinct from the active center of the enzyme, has been demonstrated (23, 24). Whereas the active site, consisting of a highly specific GSH-binding region and a less specific hydrophobic binding region, was precisely oriented in the N-terminal half of the GST protein as seen by x-ray crystallography (25, 26), this nonsubstrate-ligand-binding site is not yet precisely identified. Nevertheless biochemical data indicate that this site is located in the C-terminal half of the protein. For Atpm24 it will be of interest to test whether IAA binds to the substrate or nonsubstrate-binding site. This

Table 1. Substrate specificity of Atpm24

Substrate	Assay	Activity	Specific activity, nmol·min <sup>-1</sup> ·mg <sup>-1</sup>
1-Chloro-2,4-dinitrobenzene	A	+	82
1,2-Epoxy-3-( <i>p</i> -nitrophenoxy)propane	A	+	44
Cumene hydroperoxide	B	+	nd
<i>trans</i> -Stilbene oxide	B	+	nd
<i>trans</i> -Cinnamic acid	B	-	nd
IAA-CoA	B	-	nd

Assays were performed as described in *Materials and Methods*, and products were analyzed either spectrophotometrically at 340 nm (assay A) or after HPLC separation simultaneously at 214 nm and 280 nm (assay B).

should be possible by cocrystallization of Atpm24 with auxin followed by analysis of its crystal structure.

Another interesting feature of Atpm24 was its copurification with PMs. GSTs are mostly soluble enzymes localized to the cytosol, and a soluble auxin-binding GST was indeed found (9), but membrane-associated GSTs have been also found in a variety of organisms including plants (16, 27–29). Although these membrane-associated GSTs apparently do not contain membrane targeting or spanning domains and share similarity with other GSTs, they do not seem to associate artifactually with membranes (30). It has been suggested that such membrane-associated GSTs could be involved in the formation of leukotriene C, which acts as an important inter- and intracellular signaling molecule (31). Whereas arachidonic acid is a key precursor for leukotrienes in animals, linolenic acid in higher plants is a precursor for growth regulators such as jasmonic acid (32). Intermediates of this pathway, particularly linolenic acid epoxides and hydroperoxides, could be potential substrates for plant GSTs like Atpm24 thereby modulating the level of these growth regulators. This hypothesis is supported by the observation that cumene hydroperoxide, a model substrate for linoleate hydroperoxide and arachidonate hydroperoxide, was used as a substrate by Atpm24.

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