Cloning and Characterization of a Glutathione S-Transferase That Can Be Photolabeled with 5-Azido-indole-3-acetic Acid

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Previously, we identified a soluble protein from Hyoscyamus muticus that was photolabeled by 5-azido-indole-3-acetic acid. This protein was determined to be a glutathione S-transferase (GST; J. Bilang, H. Macdonald, P.J. King, and A. Sturm [1993] Plant Physiol 102: 29–34). We have examined the effect of auxin on the activity of this H. muticus GST. Auxins reduced enzyme activity only at high concentrations, with 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid being more effective than indole-3-acetic acid (IAA) and naphthylactic acid. IAA was a noncompetitive inhibitor, whereas inhibition by 2,4-D was competitive with respect to 1-chloro-2,4-dinitro-benzene. We also present the sequence of a full-length cDNA clone that codes for a GST and contains all partial amino acid sequences of the purified protein. The auxin-binding GST was found in high amounts in roots and stems and low amounts in leaves and flower buds. The steady-state mRNA level was not regulated by IAA or naphthylactic acid, whereas 2,4-D and 2,3-dichlorophenoxyacetic acid increased mRNA levels. We propose a model in which 2,4-D is a substrate for GST, whereas IAA binds at a second site, known as a ligandin-binding site for the purpose of intracellular transport.

Auxins are phytohormones that influence a wide range of developmental and growth responses in plants (for a review, see Davies, 1987). Many of these responses are well known and characterized at the cellular level. However, the mechanisms leading to these responses are poorly understood. The paradigm that binding of auxin to a soluble or a membrane-bound receptor protein is one of the early steps in signal transduction has led to the investigation of ABPs. Several ABPs have been identified with the help of photoaffinity-labeling techniques or anti-idiotypic antibodies, and a number of them have also been isolated (recently reviewed by Palme, 1993; Jones, 1994). A receptor function, however, has not been demonstrated unambiguously for any of these proteins, and their precise function for the most part remains speculative.

In our laboratory, a 25-kD protein has been identified by photoaffinity labeling with azido-[3H]IAA in the soluble fraction of Hyoscyamus muticus (Macdonald et al., 1991). We have purified this protein, now designated Hmgst-I, by conventional techniques (Bilang et al., 1993). Tryptic peptides obtained from Hmgst-I showed a high similarity to GSTs from maize and tobacco. We have demonstrated that Hmgst-I has GST activity in a standard assay using CDNB as a substrate. In addition, we purified the same protein by affinity chromatography on GSH-agarose, strongly suggesting that Hmgst-1 indeed is a GST. Recently another ABP, Atpm24 from Arabidopsis thaliana, was identified as a GST. In contrast to the soluble Hmgst-I, Atpm24 appears to be associated with the plasma membrane (Zettl et al., 1994).

GSTs are a family of dimeric proteins that catalyze the nucleophilic attack of GSH at electrophilic centers in a wide variety of molecules. They are widely distributed in mammals, insects, and higher plants. GSTs are involved in the detoxification of cytotoxic products and in protection against oxidative damage. In plants, GSTs have been studied mainly in connection with the detoxification of xenobiotic compounds like herbicides (Irzyk and Fuerst, 1993, and refs. cited therein). In mammals, GSTs also catalyze key steps in the synthesis of leukotrienes and prostaglandins (Söderström et al., 1985; Uijhara et al., 1988). In addition to the catalytic binding site, some animal GSTs also possess a noncatalytic binding site. This site may be involved in intracellular transport of hydrophobic and amphipathic molecules (Ketley et al., 1975).

In addition to the two ABPs identified as GSTs, a few auxin-regulated genes have been shown to encode GSTs (van der Zaal et al., 1991; Takahashi et al., 1989; Takahashi and Nagata, 1992a; Droog et al., 1993). In some cases, expression of these genes is also regulated by heavy metals (Hagen et al., 1988) or salicylic acid (Boot et al., 1993).

The identification of GSTs as ABPs raises questions about their possible functions in auxin mode of action. In this context, we have performed experiments to try to answer four questions: (a) Do auxins have a direct effect on GST activity? (b) If so, do auxins compete with the artificial substrate CDNB? (c) In which organs of an H. muticus plant is Hmgst-I expressed? (d) Is the expression of Hmgst-I regulated by auxins? These experiments and the cDNA cloning of Hmgst-1 are described in this present report.

Abbreviations: ABP, auxin-binding protein; azido-[3H]IAA, 5-N3-[7-3H]indole-3-acetic acid; CDNB, 1-chloro-2,4-dinitro-benzene; CRABP, cytoplasmic retinoic acid-binding protein; 2,3-D, 2,3-dichlorophenoxyacetic acid; GST, glutathione S-transferase; Hmgst-I, H. muticus GST isoform 1; Hmgst-1, gene for Hmgst-1; NAA, naphthylactic acid; RA, retinoic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid.

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MATERIALS AND METHODS

Plant Material and Cell Cultures

Plants of Hyoscyamus muticus were grown in soil in a greenhouse with a photoperiod of 14 h (daylight, supplemented with halogen light) at 26°C (day) and 18°C (night). The cell-suspension culture of H. muticus and the culture conditions were as described by Gebhardt et al. (1983), except that auxin was not added unless otherwise noted.

GST Enzyme Assay

GST activity was measured according to the method of Edwards and Owen (1986). Purified Hmgst-1 (2.5 μg) was diluted to 1 mL using 0.17 M potassium phosphate buffer (pH 7.5). The protein solution was then mixed in a UV-transparent acrylic cuvette (Semadeni, Ostermundigen, Switzerland) with 0.5 mL of 3 mM CDNB (Sigma) and the substance to be tested (100 μL of a stock solution in ethanol diluted to 0.5 mL with potassium phosphate buffer). The reaction was started by the addition of 1 mL of 10 mM GSH and the formation of the reaction product was measured at 340 nm. The value for the nonprotein blank was subtracted and the result expressed as a percent of the activity of the control.

Preparation of Polyclonal Antiserum

Hmgst-1 was isolated as previously described (Bilang et al., 1993). An aliquot of 75 μg of protein in 45 μL of 20 mM Tris-HCl (pH 7.4), containing 0.05% (w/v) SDS, was mixed with 355 μL of PBS (10 mM Na2HPO4 and 150 mM NaCl [pH 7.5]) and emulsified with 400 μL of Freund’s complete adjuvant (Difco Laboratories, Detroit, MI) for the first injection and with Freund’s incomplete adjuvant for subsequent injections. The emulsion was injected subcutaneously in five or six portions at intervals of 2 weeks into a female Chinchilla rabbit obtained from a local breeder. Preimmune serum was taken from the ear vein before the first injection, and immune serum was collected 2 weeks after the third antigen injection and subsequently every 2 weeks. For the preparation of serum, blood was allowed to clot for 2 h at room temperature and then overnight at 4°C. The clot was removed by centrifugation at 23,500g for 30 min. The supernatant was collected and recentrifuged.

Western Blotting

For western blotting, total protein was extracted by homogenization of plant tissue in extraction buffer (20 mM Tris-HCl [pH 8.0], 2.5% [w/v] Polyclar AT [Serva, Heidelberg, Germany], 1.8 mg/mL diethyldithiocarbamate, and 0.3% [v/v] β-mercaptoethanol) as described by Macdonald et al. (1991). The homogenate was squeezed through Miracloth (Calbiochem) and centrifuged at 23,300g for 1 h at 4°C. Protein (100 μg) was precipitated with 10% (v/v) TCA containing 1% (w/v) gelatin in Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl). The blot was then washed, decorated with secondary antibodies, and developed as described previously (Bilang et al., 1993).

Purification of Anti-Hmgst IgG

Anti-Hmgst-1 IgG was purified by affinity chromatography as described by the manufacturer’s protocol (Pharmacia). Briefly, purified Hmgst-1 (1 mg) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) as described in the manufacturer’s protocol. The affinity gel was tumbled overnight with anti-Hmgst-1 antiserum at 4°C and washed successively with 5 column volumes of PBS, PBS with 0.1% Tween 20, borate buffer (0.1 M Na2B4O7, 0.1% Tween 20, and 1 M NaCl [pH 8.5]), and PBS again. The column was then eluted with 4 column volumes of Gly/NaCl buffer (0.2 M Gly and 0.5 M NaCl [pH 2.2]) and the eluate was immediately neutralized with 1 M Tris-HCl (pH 9). Proteins were precipitated with (NH4)2SO4 (25 g/100 mL eluate), harvested by centrifugation, and redissolved in 0.5 mL of PBS containing 0.1% (v/v) sodium azide.

cDNA Synthesis and Screening Procedures

Total RNA from cells 4 d after subculture was prepared by the method of Prescott and Martin (1987) modified by adding 50 mg of Polyclar AT/g cells before grinding in liquid nitrogen. Poly(A)+ RNA was isolated by oligo(dT)-cellulose chromatography (Sambrook et al., 1989) and was used for cDNA synthesis (cDNA Synthesis System Plus, Amersham). After EcoRI linkers were added, the cDNA was ligated into the λ ZAP II vector (Stratagene) and packaged into phages [5 X 104 plaque-forming units/5 μg of poly(A)+ RNA]. The nonamplified library was screened according to the method of Young and Davis (1983) with the anti-Hmgst-1 IgG. One positive clone was isolated and both strands were sequenced by the dideoxynucleotide chain-termination reaction (Messing, 1983).

Analysis of RNA and DNA

For RNA gel blot analyses, 10 μg of total RNA were separated on 1.2% agarose gels containing 7.4% formaldehyde (Sambrook et al., 1989). Equal loading of the gels was judged by comparing the amount of ethidium bromide-stained rRNA in each lane. Genomic DNA was prepared from leaves of H. muticus (Mettler, 1987), digested with AvaII, BamHI, or EcoRI restriction enzymes, and separated on 0.7% agarose gels (5 pg/lane) (Sambrook et al., 1989). RNA and DNA gel blot analyses were performed on nylon membranes (Hybond N, Amersham) with probes that had been labeled with 32P by random priming (Sambrook et al., 1989). The complete Hmgst-1 cDNA was used as a probe. Prehybridizations were done at 65°C in hybridization buffer (6× SSC, 5× Denhardt’s solution, 100 μg/mL denatured calf thymus DNA, and 0.5% SDS) (Sambrook et al., 1989) for 5 h. Hybridizations were carried out in the same buffer overnight at 65°C. Unless otherwise noted, the blots were washed twice in 2× SSC at 65°C for 30 min.
Analysis of DNA and Protein Sequences

Computer-assisted analyses of DNA and protein sequences were carried out with the Beta version of the Genetics Computer Group Sequence Analysis software package (version 7.3-Unix, June 1993; University of Wisconsin, Madison, WI).

RESULTS

Effect of Auxins on GST Activity in Vitro

The Hmgst-1 protein was identified by photoaffinity labeling with azido-[3H]IAA (Bilang et al., 1993). To test whether the binding of auxin to GST has an effect on the GST activity, we measured the GST activity in the presence of different concentrations of IAA, NAA, 2,4-D, 2,4,5-T, and 2,3-D. As a control we used ethanol at the same final concentration as in the other samples (3%, v/v). At intermediate concentrations (greater than $4 \times 10^{-6}$ M), 2,4,5-T, 2,4-D, and, to a lesser extent, 2,3-D caused a reduction of GST activity, with 50% inhibition at approximately $2 \times 10^{-3}$, $3 \times 10^{-3}$, and $4 \times 10^{-3}$ M, respectively (Fig. 1). IAA and NAA reduced the GST activity at least 10 times less efficiently than 2,4-D, and inhibitions of only 20 and 30%, respectively, were reached at a hormone concentration of $4 \times 10^{-3}$ M.

We determined the type of competition of IAA and 2,4-D toward CDNB. In three independent experiments, IAA at $4 \times 10^{-5}$ or $10 \times 10^{-3}$ M showed a noncompetitive inhibition (Fig. 2A), with the apparent $K_m$ value for CDNB at $4 \times 10^{-3}$ M IAA not being significantly different from the control ($1.18 \pm 0.47$ mM, $n = 6$, and $1.66 \pm 0.63$ mM, $n = 5$, respectively), whereas 2,4-D at $4 \times 10^{-3}$ M (Fig. 2B) turned out to be a competitive inhibitor toward CDNB with an apparent $K_m$ of $3.94 \pm 1.15$ mM ($n = 6$).

Identification of a cDNA Clone Coding for a GST

Hmgst-1 was purified and partially sequenced (a total of 76 amino acids) as described by Bilang et al. (1993). Poly(A)+ RNA was isolated from a cell-suspension culture of H. muticus 4 d after subculture. cDNA was synthesized and a λ ZAP II expression library was prepared. This library was screened with affinity-purified anti-Hmgst-1 IgG.

One positive clone with an insert of 872 bp was isolated, and both strands were sequenced (Fig. 3). The clone contained the coding sequence for a polypeptide of 212 amino acid residues and a molecular mass of 23.7 kD. The encoded protein contained all previously identified peptides (underlined in Fig. 3). The cDNA clone also contained 13...
between positions 48 and 76 was 100% conserved (Fig. 4).

A 215-bp 3' untranslated region. A 215-bp 3' untranslated region was followed by a poly(A) tail (not shown in Fig. 3).

Figure 3. Nucleotide sequence of the cDNA for Hmgst-1. Nucleotides are numbered from the first base of the cDNA clone, excluding EcoRl linkers. The deduced amino acid sequence (one-letter code) is indicated below the nucleotide sequence. Underlined sequences indicate previously identified tryptic peptide sequences (Bilang et al., 1993).

Figure 4. Comparison of the amino acid sequences of Hmgst-1 (H. muticus, H., upper sequence) and parB (tobacco, N., lower sequence). Vertical bars indicate identical amino acids; colons indicate conservative amino acid replacements.

Genomic DNA Gel Blot

DNA gel blot analyses (Southern, 1975) were performed to determine the number of genes that hybridize to the isolated cDNA clone. DNA from leaves of adult H. muticus plants was digested with AvaII, BamHI, or EcoRI. A DNA gel blot was probed with the \( ^{32} \)P-labeled cDNA for Hmgst-1. Under high-stringency conditions (the blot was washed twice with 2X SSC and once with 0.1X SSC containing 0.5% [w/v] SDS at 65°C for 30 min), the cDNA hybridized to only a few restriction fragments, indicating the presence of a single- or low-copy-number gene (Fig. 5). Under low-stringency conditions (the blot was washed once in 2X SSC at 65°C for 30 min), however, a number of faint additional bands were visible, which may correspond to different isoenzymes of GST (data not shown). The fragments were separated by agarose gel electrophoresis and blotted before hybridization with the \( ^{32} \)P-labeled cDNA for Hmgst-1.

Figure 5. DNA gel blot analysis of the sequence of Hmgst-1 in the genome of H. muticus. Genomic DNA (5 \( \mu \)g/lane) was digested with Avall (lane A), BamHI (lane B), or EcoRI (lane E). The fragments were separated by agarose gel electrophoresis and blotted before hybridization with the \( ^{32} \)P-labeled cDNA for Hmgst-1.

bp of a 5' untranslated region. A 215-bp 3' untranslated region was followed by a poly(A) tail (not shown in Fig. 3). A consensus sequence for polyadenylation has not been identified.

The cDNA-deduced polypeptide sequence of Hmgst-1 has no glycosylation consensus sequences and no other particular features such as putative membrane-spanning domains or a signal peptide. It has a net charge of -4 and a calculated pI of 6.25. The Hmgst-1 protein shares the greatest sequence identity with the parB protein from tobacco (Takahashi and Nagata, 1992a) (Fig. 4). parB was isolated as an auxin-regulated gene and its product was identified as a GST. The sequences of Hmgst-1 and parB are 83% similar (identical amino acids and conservative exchanges) and 70% identical. In addition to a high identity throughout the whole protein, a stretch of 29 amino acids between positions 48 and 76 was 100% conserved (Fig. 4). This confirmed the finding of Droog et al. (1993) that the N-terminal part of the protein between amino acid residues 60 and 80 are highly conserved in both plant and animal GSTs.
isolated cDNA clone did not contain an internal EcoRI restriction site, and the pattern observed in Figure 5, lane E, therefore, indicates the presence of at least one intron.

**Expression of \textit{Hmgst-1} in Vivo**

Knowledge of the organ-specific expression of \textit{Hmgst-1} in \textit{H. muticus} plants may provide hints about the function of the enzyme in vivo. Therefore, adult plants were separated into roots, leaves, stems, and flower buds. The stems were divided into four sections ranging from young tissue (stems a; approximately 1 cm from the apical end) to old woody tissue (stems d; approximately 2 cm above the ground). Total RNA was isolated and probed on RNA gel blots with the $^{32}$P-labeled cDNA. Total protein extracted from these tissues was used directly for western blot analysis.

Whereas in flower buds almost no mRNA of \textit{Hmgst-1} was detectable, we observed a strong signal in stems and a somewhat weaker signal in leaves and roots (Fig. 6A). In the analysis of proteins on western blots (Fig. 6B), the greatest difference from the mRNA analysis was noted in the roots, where the highest amount of immunoreactive protein was found. The stem sections contained slightly less immunoreactive protein than roots, with little difference between apical and basal parts. Lower amounts were detectable in leaves and flower buds. In the lanes containing proteins of leaves and buds an additional, weak band could be observed at a higher molecular mass (approximately 26 kD). The 24- and the 26-kD polypeptides were the only proteins with which the antibodies cross-reacted on the blots. This may indicate that the antiserum used for these blots is not absolutely specific for \textit{Hmgst-1} (24 kD) and may detect other GST isoenzymes in the same molecular mass range.

**Effect of Auxins and Herbicides on the Expression of \textit{Hmgst-1}**

Because some GSTs are encoded by auxin-regulated genes (Takahashi and Nagata, 1992a; Droog et al., 1993), we analyzed the effect of some auxins and structural analogs on the steady-state mRNA levels of \textit{Hmgst-1}. Two herbicides were included in these experiments because some herbicides are known to be inducers of GST (Mauch and Dudler, 1993).

Suspension cells were grown for 24 h in the presence of 2 mg/L IAA, 1-NA, 2,4-D, 2,4,5-T, or 2,3-D or 10 μM atrazine or metolachlor. The results of an RNA gel blot analysis are summarized in Figure 7. Subculturing led to an increase in steady-state mRNA levels. To compare the effects of the different treatments, the data were normalized to the control (grown for 24 h in medium containing 0.01% [v/v] ethanol, the same final concentration as in the other samples). The auxins IAA and 1-NA did not induce \textit{Hmgst-1} over control levels. In contrast, 2,4-D and its structural homologs 2,4,5-T (an active auxin) and 2,3-D (inactive as an auxin) caused an up to 3-fold higher induction of \textit{Hmgst-1} expression within 24 h. The two herbicides atrazine and metolachlor had no significant effect when compared to the control, indicating that \textit{Hmgst-1} may not be involved in their detoxication.

**DISCUSSION**

\textit{Hmgst-1} was identified and isolated as a protein that can be photoaffinity labeled with azido-$[^3]H$IAA (Bilang et al., 1993). Previously, we showed that IAA and NAA could compete with azido-$[^3]H$IAA for binding, whereas 2,4-D only competed weakly (Macdonald et al., 1991; Bilang et al., 1993). This suggests that \textit{Hmgst-1} may be involved in the detoxification of auxins, atrazine, and metolachlor.
acterization of Hmgst-1 as well as a study of the expression of its gene.

To understand the function of the binding of auxins and auxin analogs to Hmgst-1, the effects of these molecules on GST activity were analyzed. All of the substances reduced enzyme activity but at high concentrations (2.4 × 10^{-4} M). In the case of 2,4-D, this inhibition was due to competition with CDNB. Whether 2,4-D is actually used as a substrate and conjugated with GSH was not studied. IAA, on the other hand, showed only a weak effect on GST activity even at nonphysiological concentrations (4 × 10^{-3} M IAA reduced the GST activity by 20%), and this reduction was due to noncompetitive inhibition of GST. These differences between IAA and 2,4-D suggest that Hmgst-1 does not bind 2,4-D and structurally related compounds as auxins but rather as xenobiotic compounds. The inability of Hmgst-1 to conjugate IAA with GSH under standard GST assay conditions (data not shown) suggests that IAA is also not used as a substrate in a conjugation reaction.

The competition studies of IAA and 2,4-D with azido-[3H]IAA (Macdonald et al., 1991; Bilang et al., 1993) and the effect of these two auxins on GST activity in vitro (Figs. 1 and 2) strongly indicate that IAA and 2,4-D do not bind to the same binding site on the Hmgst-1 protein. Based on the competition of 2,4-D with CDNB, we propose that 2,4-D, like CDNB, binds to the catalytic binding site of Hmgst-1. Probably the same is true for 2,4,5-T and 2,3-D. Because of the noncompetitive inhibition of Hmgst-1 by IAA and the high concentration of IAA required to measure an effect on GST activity, we suggest that IAA binds to a noncatalytic binding site.

The biochemical characterization of Hmgst-1 strongly suggests that photoaffinity labeling is not an artifact but, instead, that IAA is a genuine ligand. The presence of photolysis is very short lived (Macdonald et al., 1991), and its mobility in solution is reduced because of the labeling conditions (-196°C). It is, therefore, very likely that azido-[3H]IAA binds Hmgst-1 before irradiation and, therefore, before the generation of the nitrene intermediate. Scavengers of nonbound, photoactivated azide did not affect labeling of Hmgst-1 (Macdonald et al., 1991), which also refutes the possibility of the photoactivated nitrene being a substrate.

To gain further insight into the function of Hmgst-1, the spatial expression pattern of its gene (Hmgst-1) in mature plants was studied. For this purpose, we identified and isolated a cDNA clone for Hmgst-1. The cDNA-deduced amino acid sequence showed a high similarity to the sequence of parB, a GST from tobacco (Takahashi and Nagata, 1992a). A comparison of the Hmgst-1 with several plant GSTs, the human GSTs α, π, and μ, and GST θ from rat is shown as a phylogenetic tree (Fig. 8). The plant GSTs seem to be divided into at least three groups, with only limited similarity to human GSTs. Hmgst-1 is in the same group as parB (tobacco, Takahashi and Nagata, 1992a), Silene cucubalus GST (Kutchan and Hochberger, 1992), and Atpm24 (Arabidopsis, Zettl et al., 1994). Although Atpm24 is highly similar to GSTs from the parB group, which appears to consist of soluble proteins, Atpm24 was isolated as a membrane-associated protein that can also be labeled with azido-[3H]IAA. Many of the GSTs isolated as auxin-regulated genes are closely related to each other and constitute the second group (parA [Takahashi et al., 1989], parC [Takahashi and Nagata, 1992b], CNT107, CNT103, CNT110, and CNT111 [van der Zaal et al., 1991]). Two plant GSTs, AtPM239X14 from Arabidopsis (Bartling et al., 1993) and a GST from carnation (Meyer et al., 1991), do not fit into the groups described above and form a third group of related GST polypeptides. The levels of Hmgst-1 in different tissues of adult plants was determined on a western blot. High amounts of immunoreactive polypeptides were found in roots, slightly less in stems, and lower amounts in leaves and flower

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**Figure 8.** Phylogenetic tree of GSTs from plants and animals. The sequences were derived from the following cDNAs or genes: human π pGP12 (Kano et al., 1987), μ GSTM1 (Campbell et al., 1990), and α GTH2 (Rhoads et al., 1987); tobacco pCNT 103, pCNT 107, pCNT 110, pCNT 111 (Van der Zaal et al., 1991), parB (Takahashi et al., 1989), parA (Takahashi and Nagata, 1992a), parC (Takahashi and Nagata, 1992b); wheat GSTA1 (Dudler et al., 1991); maize I pGTC2 and maize III pGTC27 (Grove et al., 1988); H. muticus Hmgst-1 (this report); a Silene cucubalus cDNA (Kutchan and Hochberger, 1992); Arabidopsis Atpm24 (Zettl et al., 1994) and AtPM239X14 (Bartling et al., 1993); rat θ pGST15 (Pemble and Taylor, 1992); and carnation GST1 (Dianthus caryophyllus, Meyer et al., 1991).
buds. A comparison of the western blot signals with the steady-state mRNA levels revealed some discrepancies. The deviations found may originate from the possibility that the antiserum is not absolutely specific for Hmgst-1 but also recognizes additional GST isoenzymes. Furthermore, expression of Hmgst-1 may be regulated at a post-transcriptional level. An interpretation of the expression in vivo with respect to a function of Hmgst-1 is discussed below.

The expression of some plant GSTs is regulated by plant hormones. The par cDNAs, for example, were isolated as auxin-responsive genes during induction of cell division (Takahashi et al., 1989). Despite the high similarity between parB and Hmgst-1, the two genes seem to be regulated differently. In cells of H. muticus, the induction of Hmgst-1 seems to be related to the transfer of cells into fresh medium rather than to externally added auxin. In suspension cultures without 2,4-D, mRNA and protein levels are even slightly higher than in cultures with 2,4-D (data not shown). Whereas IAA and NAA did not induce Hmgst-1 above control levels (Fig. 7), 2,4-D and its structural analogs 2,4,5-T and 2,3-D led to a marked increase of Hmgst-1 transcripts. This finding is in contrast with the results of Takahashi and Nagata (1992a), who reported an induction of parB by 2,4-D, IAA, and NAA. There are several possibilities to explain these differences. First, it is not known whether parB and Hmgst-1 have the same functions. Second, Takahashi and Nagata (1992a) studied tobacco mesophyll protoplasts. The effect of auxin on these protoplasts may differ from its effect on the H. muticus suspension-cultured cells that grow without additional external auxin.

Several possible functions for the binding of IAA to Hmgst-1 emerge from our experiments. A noncatalytic binding site has already been described for some animal GSTs (ligandin-binding site, Ketley et al., 1975; Reinemer et al., 1991). This site is thought to be involved in intracellular transport of hydrophobic and amphipathic molecules such as steroid hormones, heme, and bilirubin. In analogy to the ligandin hypothesis, Hmgst-1 may be involved in the intracellular transport of IAA. Binding of the hormone to Hmgst-1 may also protect it from being metabolized by unspecific oxidases.

Alternatively, in analogy to the function of CRABPs, binding of IAA to a noncatalytic site of Hmgst-1 may decrease cellular auxin concentrations and, thereby, have a negative effect on auxin activity. The animal morphogen RA is known to bind to CRABP as well as to the receptors in the nucleus (Leid et al., 1992). Binding of RA to CRABP is thought to regulate the concentration of free RA, enabling differential regulation of gene transcription (Maden et al., 1988).

Although no modulation of Hmgst-1 activity toward the synthetic substrate CDNB by physiological concentrations of IAA was found, binding of IAA to Hmgst-1 may possibly change its activity toward its natural, but still unknown, substrate. As a consequence, such an alteration of enzyme activity may modulate the cellular redox potential. This speculation is interesting, because a link between the redox state of a cell and physiological processes such as proliferation and differentiation has been shown (Earnshaw and Johnson, 1985). A related process supporting this hypothesis was described by Chen et al. (1993). The authors showed that the plant signal molecule salicylic acid binds and, thereby, inhibits catalase without being used as a substrate. The inhibition of the catalase activity increases H$_2$O$_2$ concentrations and finally leads to a modulation of specific gene expression.

The high expression of Hmgst-1 in stems, where basipetal IAA transport takes place, supports a function of Hmgst-1 in auxin transport. For the evaluation of such a function, the low affinity between Hmgst-1 and free IAA has to be taken into consideration (apparent $K_i$ of about 100 $\mu$m, Macdonald et al., 1991); in the range of only 1% to a few percent of the free IAA will be bound to the protein. If the affinity between IAA and its final target is high, such a low affinity for the putative transport protein to the hormone may be sufficient. In addition, a loose association of IAA with Hmgst-1 may assure the easy availability of free IAA in the case of increasing demand. The low binding affinity of Hmgst-1 for IAA and the inability of physiological concentrations of the hormone to modulate GST activity in vitro make it less likely that Hmgst-1 participates in modulation of IAA concentration or cellular redox potential.

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LITERATURE CITED


