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2,4-Dichlorophenoxyacetic Acid and Related Chlorinated Compounds Inhibit Two Auxin-Regulated Type-III Tobacco Glutathione *S*-Transferases[1]

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Two auxin-inducible glutathione *S*-transferase (GST, EC 2.5.1.18) isozymes from tobacco (*Nicotiana tabacum*, White Burley) were partially characterized. GST1–1 and GST2–1 are members of a recently identified new type of plant GST isozymes that we will here refer to as type III. Both enzymes were active, with 1-chloro-2,4-dinitrobenzene as a substrate, when expressed in bacteria as fusion proteins. The apparent K_m for 1-chloro-2,4-dinitrobenzene was found to be 0.85 ± 0.25 mM for GST1–1 and 0.20 ± 0.15 mM for GST2–1. The apparent K_m for glutathione was similar for both enzymes, 0.40 ± 0.15 mM. The in vitro activity of both enzymes could be inhibited by the synthetic auxin 2,4-dichlorophenoxyacetic acid, with an apparent K_i of 80 ± 40 μM for GST1–1 and 200 ± 100 μM for GST2–1. The GST1–1 was also inhibited by structurally related substances, such as 2,4-dichlorobenzoic acid, with a roughly similar K_i. The nonchlorinated structures benzoic acid and phenoxyacetic acid did not inhibit. *p*-Chloroisobutyric acid, or clofibric acid, an auxin-transport inhibitor, was found to be an active inhibitor as well. The strongest inhibitor identified, however, was a phenylacetic acid derivative, ethacrynic acid, which showed an apparent K_i of 5 ± 5 μM for both enzymes. This substance is a known inducer as well as a substrate of specific mammalian GSTs. The results presented here indicate that the type III plant GSTs might be involved in the metabolism or transport of chlorinated substances that are structurally related to auxins. The possibility that auxins are endogenous ligands or substrates for GSTs is discussed.

GSTs are a ubiquitous family of proteins that catalyze the nucleophilic attack of the thiol group of GSH (γ-glutamylcysteinylglycine) to the electrophilic site of a large variety of substrates (Mannervik and Danielson, 1988; Pickett and Lu, 1989; Coles and Ketterer, 1990; Daniel, 1993). These enzymes are best known for their role in the detoxification of various exogenous substances, whereby the covalent linkage of GSH to the substrate usually leads to a less toxic GSH conjugate. They are also involved in the detoxification of endogenous reactive products of cellular metabolism. In addition to their enzymatic activities, GSTs also possess a ligand-binding capacity and are involved in intracellular transport of hydrophobic and amphiphatic substances (Litwack et al., 1971; Ketley et al., 1975; Listowski et al., 1988).

So far, the only role clearly established for plant GSTs is the detoxification of several classes of herbicides (Timmerman, 1989). These studies were done primarily in maize, where at least four distinct GST activities have now been identified, GST I, II, III, and IV (Timmerman, 1989; Fuerst et al., 1993; Irzyk and Fuerst, 1993). In maize, both the GST activity and herbicide metabolism are stimulated by safeners, compounds structurally related to herbicides but not active as such (Moore et al., 1986; Wiegand et al., 1986). Most of the other plant GSTs have been isolated as a result of their inducibility by various treatments, including IAA, ethylene, pathogen infection, heavy metals, and heat shock. A *Hyoscyamus muticus* GST was isolated as an auxin-binding protein (MacDonald et al., 1991; Bilang et al., 1993).

For a number of the GSTs that have been identified in a variety of plant species, the complete primary amino acid sequence has been determined. Based on these primary sequences two major groups were distinguished (Droog et al., 1993). The first group includes GSTs from maize (Shah et al., 1986; Grove et al., 1988), wheat (Dudler et al., 1991), tobacco (*Nicotiana tabacum*) (Takahashi and Nagata, 1992a), carnation (Meyer et al., 1991b; Itzhaki and Woodson, 1993), *Silene cucubalus* (Kutchan and Hochberger, 1992), and *Arabidopsis thaliana* (Bartling et al., 1993; Zhou and Goldsbrough, 1993). The second group, originally identified as a group of auxin-regulated proteins, includes GSTs from soybean (Czarnecka et al., 1988; Hagen et al., 1988), potato (Taylor et al., 1990), tobacco (Takahashi et al., 1989, 1992b; Van der Zaal et al., 1991; Droog et al., 1993), and *Nicotiana plumbaginifolia* (Dominov et al., 1992).

The existence of several groups or types of GSTs in plants is similar to the situation in mammals, where at least four distinct types of soluble GSTs are now distinguished, α, μ, π, and θ (Mannervik et al., 1985, 1992; Meyer et al., 1991a), and a fifth type, σ (Buetler and Eaton, 1992), has been proposed. However, in other animals this classification is different. For instance, in *Drosophila* a separate group called the D-type is found that is quite divergent from the types identified in mammals (Toung et al., 1990,

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Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; ethacrynic acid, 2,3-dichloro-4(2-methylene-butyryl)phenoxyacetic acid; GST, glutathione *S*-transferase; NAA, naphthylacetic acid; PAA, phenylacetic acid.

1993). This can be explained by the divergence of the species occurring before the divergence of the different types of GST. According to this hypothesis, the θ type would be the evolutionary most ancient GST (Pemble and Taylor, 1992).

We have previously isolated and characterized an auxin-regulated gene family from tobacco to study the signal transduction pathway of the plant hormone auxin (Van der Zaal et al., 1987, 1991). The gene family was found to consist of three subfamilies, Nt103, Nt107, and Nt114, and encode proteins with a low level of sequence similarity to GSTs. For one of the proteins, NT103, we have shown that it is active as a GST in vitro (Droog et al., 1993).

Here we report on the partial characterization of two representatives of the auxin-regulated group of GSTs, a new type of plant GST isozyme (Droog et al., 1993). Because they are the most recent to be discovered, we will refer to this group as type III plant GSTs. That three types of GST isozymes can be recognized in plants is shown by an evolutionary tree, created by using the complete primary sequences of plant proteins established as GSTs and their homologs. We extend our previous observations concerning the GST activity of the NT103 (or GST1–1) protein by showing that the NT107 (or GST2–1) protein is also an active GST. Both enzymes were characterized to establish their relationship to previously identified GSTs. The observed inhibition of in vitro GST activity by auxin-related compounds suggests a possible connection between the function of the proteins and their induction by auxins.

MATERIALS AND METHODS

Cloning of Nt107 into an Expression Vector

The cDNA of clone pCNT107 (Van der Zaal et al., 1987) was isolated as an *Eco*RI fragment from the pUN121 vector (Nilsson et al., 1983) and ligated into pUC21 (Vieira and Messing, 1991) cut with *Eco*RI. The resulting plasmid, plac107, expresses the NT107 protein as a fusion protein, with an extra 29 amino acids derived from β-galactosidase on the N-terminal end, under control of the *plac* promoter. As a control the same *Eco*RI fragment was cloned in a reverse orientation, leading to the construct placanti107. Plasmids were transformed into *Escherichia coli* host bacteria, strain XL1-blue (Stratagene, La Jolla, CA). Constructs made to express the cDNA of clone pCNT103 (Van der Zaal et al., 1987), i.e. plac103 and placanti103, were described by Droog et al. (1993).

GST Enzyme Assay

Bacteria containing the expression plasmids were grown overnight in the presence of 1 mM isopropylthiogalactoside and collected by centrifugation. The bacteria were resuspended in 100 mM Tris-HCl, pH 7.5, 1 mM DTT (one-eighth of their original volume) and lysed by sonication. Enzyme activity for the NT107 protein was determined with a 200-μL extract using CDNB as a substrate as described previously (Droog et al., 1993), employing a standard procedure (Mannervik and Guthenberg, 1981). Enzyme activity for the NT103 protein was determined with a 2-μL extract of bacteria expressing the NT103 protein, isolated in a similar manner (Droog et al., 1993). Activity toward ethacrynic acid was determined using 0.2 mM substrate and 0.25 mM GSH, as described (Habig et al., 1974).

Kinetic Analysis

The apparent K_m value for GSH was determined using a range from 0.1 to 1 mM GSH and a fixed concentration of 1 mM CDNB. The apparent K_m value for CDNB was determined using a range from 0.2 to 0.8 mM CDNB and a fixed concentration of 1 mM GSH. Data were plotted as a double-reciprocal Lineweaver-Burk plot to determine the apparent K_m.

To determine the apparent K_i for 2,4-D and ethacrynic acid the GST assay was performed in the presence of a fixed concentration of inhibitor, 50 or 100 μM for 2,4-D and 5 or 10 μM for ethacrynic acid, and a range of CDNB concentrations, from 0.2 to 0.8 mM. The data were represented as a double-reciprocal Lineweaver-Burk plot from which the K_m and K_i values were calculated.

The relative inhibitory capacity of the other substances tested was obtained by determining the concentration giving 50% inhibition in a standard GST assay using 1 mM CDNB. These "apparent K_i values" will therefore be underestimates of the real apparent K_i values. For comparison, the concentration of 2,4-D leading to 50% inhibition was also determined in this manner. The data presented in Table II are from a representative experiment in which all inhibitors were tested simultaneously. Actual values varied slightly from experiment to experiment but the relative order in which the inhibitors were active was always the same.

Phylogenetic Analysis

The amino acid sequences available for proteins, shown to be active GSTs or homologous to these, were aligned using the Wisconsin Genetics Computer Group package (Devereux et al., 1984) and used to construct a phylogenetic tree. The evolutionary probabilistic ancestor matrix distances were determined and used to graphically represent the relations between the sequences using the option PhyloTree of the AllAll program, which was run at the Computational Biochemistry Research Group at the Eidgenössische Technische Hochschule at Zurich.

RESULTS

GST Activity of Protein Encoded by pCNT107

To test the activity of the NT107 protein, or GST2–1 (Droog et al., 1993), the pCNT107 cDNA (Van der Zaal et al., 1991), which encodes a full-length protein, was fused to the *lacZ* gene in the expression vector pUC21. The resulting fusion protein was used in a standard GST activity assay, using CDNB as the substrate (Mannervik and Guttenberg, 1981). As a control for the activity of the bacterial extracts, a construct was also made with the cDNA in the reverse orientation.

Table I. *GST activity of NT103 and NT107 against CDNB*

Construct	Activity
	$\Delta A_{340}\ mg^{-1}\ min^{-1}$
plac107	0.10± 0.05
placanti107	≤ 0.02
plac103	8.70± 1.20
placanti103	≤ 0.02

The GST activity measured for the NT107 (GST2–1) was low but distinct (Table I). The activity was about 100-fold lower than that observed for NT103 (GST1–1; Droog et al., 1993), but it was well above the negative controls. The activities of GST2–1 and GST1–1, however, are not directly comparable, since the values are expressed per total bacterial protein, and the percentage of the respective proteins might very well be different, for example, due to differences in the expression plasmids used. The activity detected for GST2–1 corroborates the identification of the group of auxin-regulated proteins reported on earlier (Droog et al., 1993) as plant GSTs with activity toward CDNB.

Determination of K_m for GSH and CDNB of GST1–1 and GST2–1

The activity of the GST1–1 and GST2–1 enzymes as a function of various GSH and CDNB concentrations was determined and results for GST1–1 are shown in Figure 1. Michaelis-Menten kinetics were observed for both changes in GSH concentration at a fixed CDNB concentration and changes in the CDNB concentration at a fixed GSH concentration. The apparent K_m value for GSH was calculated from the Lineweaver-Burk plot to be 0.40 ± 0.15 mM for GST1–1 (Fig. 1A). A similar value was obtained for the GST2–1 enzyme (data not shown). The apparent K_m for the model substrate CDNB was calculated to be 0.85 ± 0.25 mM for GST1–1 (Fig. 1B). For GST2–1 a value of 0.20 ± 0.15 mM was found (data not shown).

Both kinetic parameters determined here fall well within the range observed for other GSTs of either plant or animal origin. Therefore, in these features GST1–1 or GST2–1 do not differ from other plant GSTs characterized to date.

Inhibition of Activity of GST1–1 and GST2–1 by 2,4-D

To evaluate the relationship between the auxin-induced expression of the proteins and their activity as GST, we tested whether the synthetic auxin 2,4-D, the strongest inducer of the GST1–1- and GST2–1-encoding genes (Droog, 1995), had any direct effect on the in vitro GST activity. The results of experiments, including a fixed 2,4-D concentration in a GST assay with GST1–1, using varying concentrations of CDNB present are shown in Figure 2A. It is clear from the data presented that 2,4-D inhibits the activity of GST1–1 toward CDNB. This inhibition occurs in a competitive manner. The apparent K_i calculated from the plotted data for 2,4-D was 80 ± 40 μM. A similar assay with the GST2–1 enzyme showed an apparent K_i value of 200 ± 100 μM (data not shown).

These results indicate that 2,4-D is not only an inducer of the GST1–1 and GST2–1 proteins but also can be bound by them, albeit with a rather low affinity. When other auxins, including IAA, indole-3-butyric acid, and NAA, were tested in similar experiments, no or only a slight inhibitory action could be observed. However, IAA concentrations above 1 mM were found to interfere with the assay due to the absorption at 340 nm by IAA itself. Therefore, a lower-affinity interaction between the GSTs and IAA cannot be ruled out completely.

Inhibition by Other Chlorinated Substances

The results obtained using different auxins pointed to structural characteristics of 2,4-D, not necessarily identical to those determining its auxin activity, as important for the inhibitory action on the GST activity of GST1–1 and GST2–1. Therefore, several structural analogs were tested. Shown in Figure 2B are the results obtained for ethacrynic acid, the strongest inhibitor detected for both GST1–1 and GST2–1. This PAA derivative is known as both an inducer and a substrate of specific mammalian GSTs. The apparent

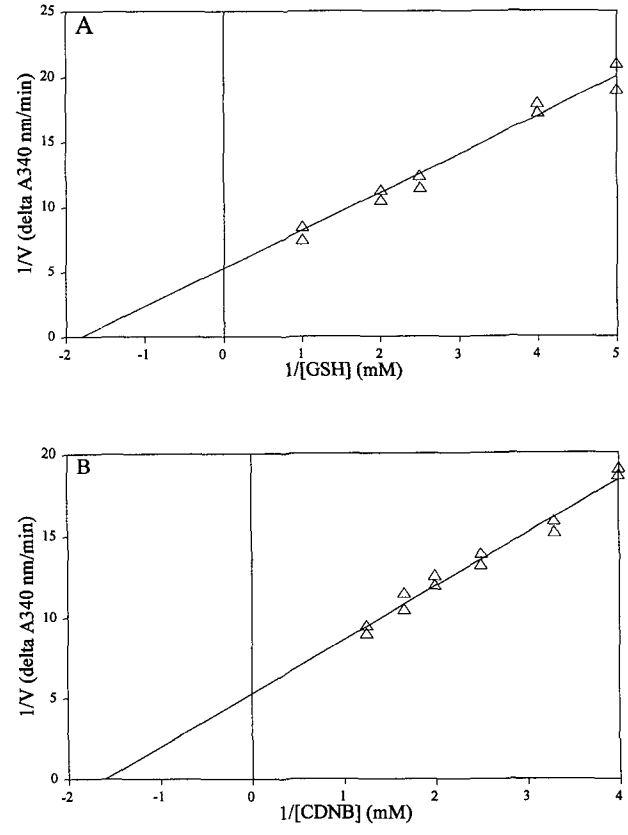

Figure 1. Kinetic analysis of GST1–1 activity. A, Enzyme activity as a function of GSH concentration was measured in the presence of 1 mM CDNB. B, Enzyme activity as a function of CDNB concentration was measured in the presence of 1 mM GSH. Data are represented as double-reciprocal plots. Plotted values represent duplicates within one experiment. Apparent K_m values were calculated from four separate experiments.

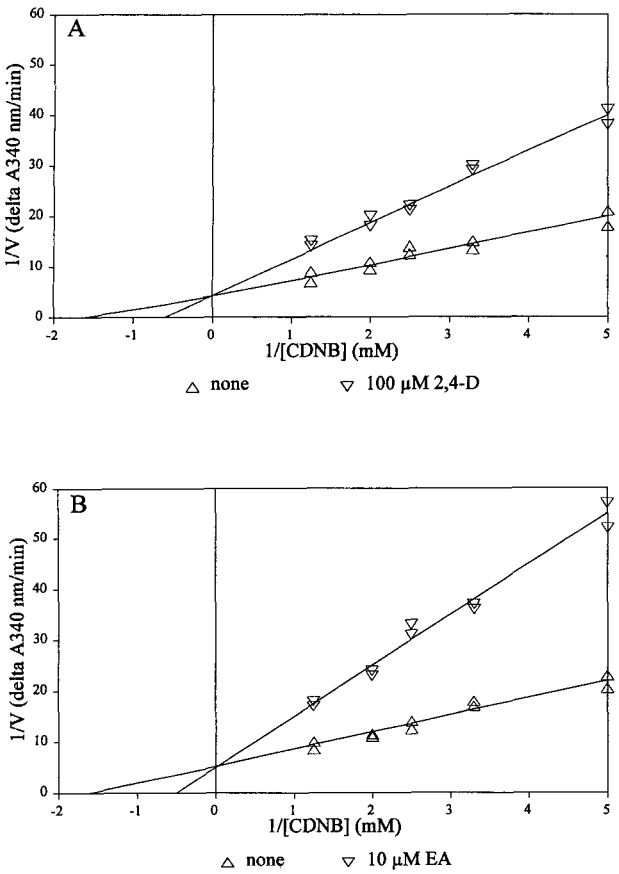

Figure 2. Inhibition of GST1–1 activity. Enzyme activity as a function of CDNB concentration was measured in the absence or presence of a fixed concentration of 2,4-D (A) or ethacrynic acid (B). Data are represented as a double-reciprocal plot. Plotted values are duplicate values from a single experiment. Apparent K_i values were calculated as an average of four separate experiments.

K_i, calculated from the data plotted in Figure 2B, was found to be 5 ± 5 μM for GST1–1. Approximately the same value was obtained when GST2–1 was used in similar assays.

Several other substances were selected to determine both the effect of the type of ring structure and the effect of the substituent present on the apparent K_i, using GST1–1 (Table II). The represented values were determined by varying the concentration of inhibitor in a standard assay, using a fixed concentration of CDNB, and are therefore slightly higher than the actual apparent K_i values. For comparison, the value determined for 2,4-D in this way is also shown. Values shown are from a representative experiment. All substances active as inhibitors demonstrated concentrations giving 50% inhibition that are slightly higher than that for 2,4-D. The first conclusion that can be drawn from the data presented is that at least one chlorine atom is needed for inhibition to occur. The nonsubstituted parent structures benzoic acid, PAA, and phenoxyacetic acid were found to be inactive. The presence of a second chlorine atom increased the effectiveness as an inhibitor only slightly. Second, it is clear that a benzoic acid backbone is sufficient and just as effective as a PAA or phenoxyacetic acid backbone. It is thus clear that the structural requirements for the inhibitory action on the activity of GST1–1 and GST2–1 are different from those necessary for auxin activity.

Classification of Plant GSTs into Three Types

In view of the findings described recently (Droog et al., 1993) and corroborated here that a group of auxin-regulated proteins possess GST activity, we decided to analyze the evolutionary relationship between these proteins and previously recognized plant GSTs. We determined the evolutionary distances between 16 plant GST protein sequences available and used these values to construct a phylogenetic tree. The graphic representation of this phylogenetic tree is shown in Figure 3, where the evolutionary distances are indicated by the length of the lines connecting the sequences. What is most obvious is that three separate groups can be distinguished. On a historical basis we would like to refer to the group containing the first identified plant GSTs as type I. This group so far includes the maize GSTI and GSTIII, the wheat GSTA1, the tobacco PAR-B, a *Silene cucubalus* GST, and two *Arabidopsis* GSTs. The type II GST enzymes are so far represented only by the GST from carnation. The third type includes the auxin-regulated proteins: the tobacco GST1–1 (NT103), GST2–1 (NT107), and GST3–1 (NT114), the soybean GmHSP26A, and the *Nicotiana plumbaginifolia* LS216, and also the tobacco C7, the potato PRP1, and the soybean GmGXI.

Given the situation observed in mammals, where now five types of soluble GSTs are recognized, and the relatively recent discovery of the plant GSTs, it seems very likely that more types exist and will be discovered in the near future. This will undoubtedly shed more light on the relationships between plant GSTs and their endogenous functions.

DISCUSSION

In this paper we have shown that the tobacco protein encoded by the cDNA pCNT107 is a functional GST en-

Table II. *Inhibition of GST1–1 activity against CDNB*

The concentration leading to 50% inhibition in a standard GST assay using 1 mM CDNB was determined.

Inhibitor	50% Inhibition[a]
Benzoic acid	>1
4-Chlorobenzoic acid	0.5
2,4-Dichlorobenzoic acid	0.4
Phenylacetic acid	>1
4-Chlorophenylacetic acid	0.4
2,4-Dichlorophenylacetic acid	0.4
Phenoxyacetic acid	>1
4-Chlorophenoxyacetic acid	0.3
2,4-Dichlorophenoxyacetic acid	0.2
3,5-Dichlorophenoxyacetic acid	0.4
2,4,5-Trichlorophenoxyacetic acid	0.2
2(4-Chlorophenoxy)propionic acid	0.4
2(4-Chlorophenoxy)methylpropionic acid	0.4
2(2,4-Dichlorophenoxy)propionic acid	0.4

[a] Concentration in mM.

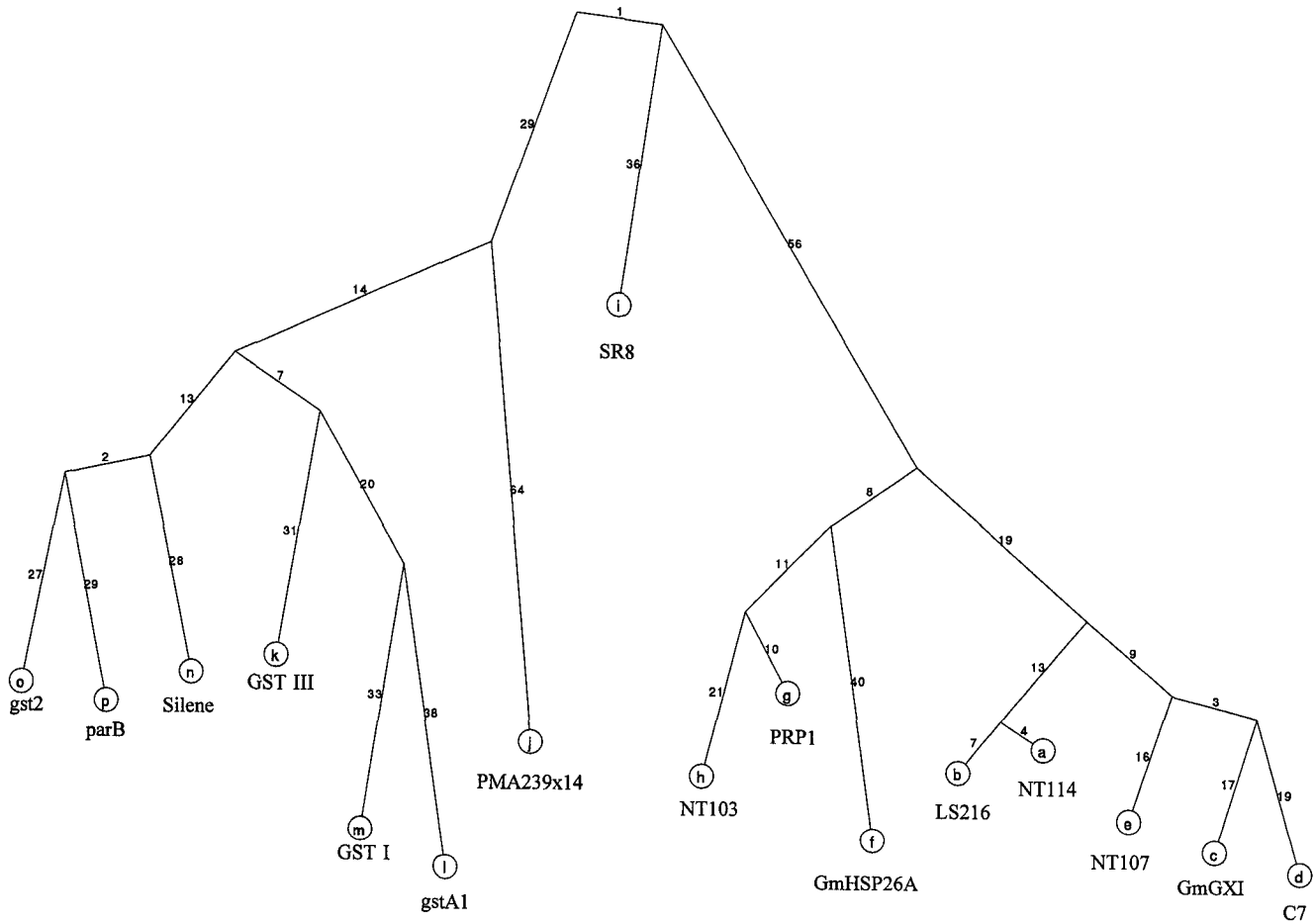

Figure 3. Phylogenetic tree of plant GST isozymes. The tree was constructed using the option PhyloTree of the AllAll program (run at the Computational Biochemistry Research Group at the Eidgenössische Technische Hochschule in Zurich). The peptides derived from the following sequences have been used. a, Tobacco *Nt114–4* (Droog, 1995; identical to tobacco parA, Takahashi et al., 1989); b, *N. plumbaginifolia* LS216 (Dominov et al., 1992); c, soybean GmGXI (H.-J. Jacobsen, personal communication.); d, tobacco C7 (Takahashi et al., 1992); e, tobacco pCNT107 (van der Zaal et al., 1991; nearly identical to par C, Takahashi et al., 1992b); f, soybean GmHSP26A (Czarnecka et al., 1988); g, potato PRP1 (Taylor et al., 1990); h, tobacco pCNT103 (van der Zaal et al., 1991); i, carnation SR8 (Meyer et al., 1991); j, *A. thaliana* PMA239x14 (Bartling et al., 1993); k, maize GST III (Grove et al., 1988); l, wheat *gstA1* (Dudler et al., 1991); m, maize GST I (Shah et al., 1986); n, *S. cucubalus* gst (Kutchan and Hochberger, 1992); o, *A. thaliana gst2* (Zhou and Goldsbrough, 1993); and p, tobacco parB (Takahashi and Nagata, 1992a).

zyme. We will name this enzyme tobacco GST2–1, as was previously proposed when a similar activity was described for the related pCNT103-encoded tobacco GST1–1 (Droog et al., 1993). The GST1–1 and GST2–1 enzymes share a 46% amino acid identity (Van der Zaal et al., 1991). Both enzymes were characterized for several kinetic parameters, which were found to be comparable. Both enzymes had an equal affinity for GSH, with apparent K_m values of 0.40 ± 0.15 mM. These values are similar to those observed for other GSTs, either from plants or animals. This probably reflects the conservation of the GSH-binding site (Reinemer et al., 1991; Rushmore and Pickett, 1993). The affinity for the model substrate CDNB varied slightly between the two enzymes, with an apparent K_m for GST1–1 of 0.85 ± 0.25 mM and for GST2–1 of 0.20 ± 0.15 mM. These values are well within the range found for other plant GSTs that show activity toward this substrate.

The tobacco GST1–1 and GST2–1, together with their homologs from other plant species, form a group of auxin-regulated proteins that have only a limited homology to the previously identified plant GSTs (Droog et al., 1993). They behave, however, similarly in an in vitro assay using CDNB as a substrate. This has recently been confirmed in our laboratory for a protein encoded by an *A. thaliana* cDNA clone, which was isolated as hybridizing to the tobacco pCNT103 (D. Van der Kop, unpublished data). For the homologous proteins isolated from potato, PRP1, and soybean, GmHSP26A, the GST activity against CDNB has now been confirmed (Guilfoyle et al., 1993; Hahn et al., 1994). In tobacco the gene family encoding these GSTs consists of at least three subfamilies, the Nt103, Nt107, and Nt114 families (Van der Zaal et al., 1987, 1991; F.N.J. Droog, unpublished data). The Nt103 family contains at least three expressed genes, whereas the Nt107 and Nt114 families

have two expressed members each. Identical or nearly identical genes have been isolated independently and are called *parC* (Takahashi and Nagata, 1992b), which is nearly identical to Nt107 genes, and *parA* (Takahashi et al., 1989), which is identical to the Nt114 genes.

In view of the limited homology to previously identified plant GSTs and the considerable size of the auxin-regulated gene family in tobacco, we were interested in the evolutionary relationships that exist between the different GSTs identified so far. Therefore, we constructed a phylogenetic tree that showed that these groups of plant GSTs can be distinguished. To avoid future confusion on which proteins are being discussed, we propose to start a classification of plant GSTs based on the phylogenetic tree derived here. In mammals a division is based on similar grounds, and given the number of plant sequences now available it seems appropriate to start a nomenclature for plant GSTs. So we propose to use the group names suggested here: type I, II, and III, as a starting point. Whether the division made here is representative for different functions or the use of different substrates remains to be determined.

For the two type III plant GST isozymes described here, GST1–1 and GST2–1, it was found that 2,4-D, the synthetic auxin that is a very strong inducer of the genes encoding these enzymes, is capable of inhibiting the in vitro activity. The apparent K_i for GST1–1 was 80 ± 40 μM and for GST2–1 it was 200 ± 100 μM. However, other tested auxins, IAA, indole-3-butyric acid, NAA, and PAA, were not found to be inhibitory at the concentrations tested. Besides being active as an auxin, 2,4-D is also active as an herbicide at higher concentrations. Plant GSTs are known to be involved in the detoxification of several classes of pesticides and herbicides (Wiegand et al., 1986; Timmerman, 1989). That phenoxyacetic acids like 2,4-D can inhibit GST activity has also been demonstrated for specific mammalian isozymes (Vessey and Boyer, 1984).

Several structural analogs of 2,4-D were tested, and it was found that only those substances that contained at least one chlorine atom were inhibitory in the in vitro GST assay. The parent structures benzoic acid, PAA, and phenoxyacetic acid were all inactive. When a chlorine atom was introduced at position four, inhibition followed. When two chlorine atoms were introduced at positions two and four, only a slightly higher inhibition was measured. Although the apparent K_i values of all active substances differed only marginally, 2,4-D was found to be the strongest inhibitor of this series. Positioning the two chlorine atoms at positions three and five, as in the inactive auxin-analog 3,5-D, led to an only slight decrease in the inhibitory activity. Adding a third chlorine atom at position five, leading to the active auxin-analog 2,4,5-T, did not change the inhibitory activity. These data clearly suggest that the structural requirements for the inhibitory action of 2,4-D on the GST activity of GST1–1 and GST2–1 are different from those determining its auxin activity.

That substances that are not active auxins can inhibit the in vitro activity of GST1–1 and GST2–1 was further corroborated by using several other related substances. An interesting one is *p*-chloroisobutyric acid [2(4-chlorophenoxy)propionic acid], a substance known in plant hormone research as an anti-auxin. In animal research it is known as clofibric acid, a so-called peroxisome proliferator. It induces several genes, most notably acyl-CoA oxidase, by activating the peroxisome proliferator-activated receptor to bind to the peroxisome proliferator response element present in the promoter (Tugwood et al., 1992; Boie et al., 1993).

Ethacrynic acid, a PAA derivative, is an inducer as well as a substrate of specific mammalian GSTs (Yamada and Kaplowitz, 1980; Ahokas et al., 1985). Surprisingly, ethacrynic acid was found to be the most potent inhibitor of the GST1–1 and GST2–1 activity, with an apparent K_i of only 5 ± 5 μM. This might mean that ethacrynic acid is more strongly binding both enzymes because it more closely resembles the possible endogenous substrate(s) for the GST1–1 and GST2–1 isozymes. However, no GST activity toward ethacrynic acid could be observed (data not shown).

The affinity of GST1–1 and GST2–1 toward 2,4-D could indicate that these GST isozymes are involved in the detoxification, metabolism, or transport of auxins and auxin-like substances. Although IAA only slightly inhibited the in vitro activity at the highest concentrations tested, a low-affinity interaction could still be envisaged. Several mammalian GSTs function as intracellular transporters of hydrophobic and amphiphatic molecules (Litwack et al., 1971; Ketley et al., 1975; Listowski et al., 1988), and the same might be true in plants. This possibility is also suggested by the recent isolation of *H. muticus* and *A. thaliana* GSTs as auxin-binding proteins (Bilang et al., 1993; Zettl et al., 1994). Interestingly, the *H. muticus* protein showed a higher affinity toward IAA than toward 2,4-D (Bilang et al., 1993), with an apparent displacement constant for IAA of 100 μM (MacDonald et al., 1993), which is similar to the value we observed for 2,4-D. However, IAA was not inhibitory toward the GST activity of this protein, indicating that an interaction between a GST and IAA does not necessarily lead to inhibition of the in vitro activity toward CDNB. Alternatively, the data presented here might indicate that the inhibitory effect of 2,4-D is not directly related to its activity as an auxin but a consequence of its chlorinated structure.

The data presented here clearly establish the existence of an extensive superfamily of GST isozymes in plants. Several of these are induced by auxins and several show some form of interaction with auxins. Whether there is any direct connection between the induction of GST genes by auxins and the interaction of GST proteins with auxins remains an open question. Further investigation of the relationships between auxins, auxin effects, and GSTs will be of great interest, both for the elucidation of auxin signal transduction pathways and for defining the functional role of plant GSTs.

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LITERATURE CITED

Ahokas JT, Nicholls FA, Ravenscroft PJ, Emmerson BT (1985) Inhibition of purified rat liver glutathione S-transferase isozymes by diuretic drugs. Biochem Pharmacol **34:** 2157–2161

Bartling D, Radzio R, Steiner U, Weiler EW (1993) A glutathione S-transferase with glutathione-peroxidase activity from *Arabidopsis thaliana*. Eur J Biochem **216:** 579–586

Bilang J, MacDonald H, King PJ, Sturm A (1993) A soluble auxin-binding protein from *Hyoscyamus muticus* is a glutathione *S*-transferase. Plant Physiol **102:** 29–34

Boie Y, Adam M, Rushmore TH, Kennedy BP (1993) Enantioselective activation of the peroxisome proliferator-activated receptor. J Biol Chem **268:** 5530–5534

Buetler TM, Eaton DL (1992) Glutathione S-transferases: amino acid sequence comparison, classification and phylogenetic relationship. J Environ Sci Health Part C Environ Carcinog Ecotoxicol Rev **10:** 181–203

Coles B, Ketterer B (1990) The role of glutathione and glutathione transferases in chemical carcinogenesis. CRC Crit Rev Biochem Mol Biol **25:** 47–70

Czarnecka E, Nagao RT, Key JL (1988) Characterization of Gmhsp26-A, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. Mol Cell Biol **8:** 1113–1122

Daniel V (1993) Glutathione S-transferases: gene structure and regulation of expression. Crit Rev Biochem Mol Biol **28:** 173–207

Devereux I, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res **12:** 387–395

Dominov JA, Stenzier L, Lee S, Schwarz JJ, Leisner S, Howell SH (1992) Cytokinins and auxins control the expression of a gene in *Nicotiana plumbaginifolia* cells by feedback regulation. Plant Cell **4:** 451–461

Droog FNJ (1995) Promoter and functional analysis of an auxin-regulated tobacco gene family. PhD thesis, State University Leiden, The Netherlands

Droog FNJ, Hooykaas PJJ, Libbenga KR, Van der Zaal EJ (1993) Proteins encoded by an auxin-regulated gene family of tobacco share limited but significant homology with glutathione S-transferases and one member indeed shows in vitro GST activity. Plant Mol Biol **21:** 965–972

Dudler R, Hertig C, Rebman G, Bull J, Mauch F (1991) A pathogen-induced wheat gene encodes a protein homologous to glutathione S-transferases. Mol Plant Microbe Interact **4:** 14–18

Fuerst EP, Irzyk GP, Miller KD (1993) Partial characterization of glutathione S-transferase isozymes induced by the herbicide safener benoxacor in maize. Plant Physiol **102:** 795–802

Grove G, Zarlengo RP, Timmerman KP, Li N, Tam MF, Tu C (1988) Characterization and heterospecific expression of cDNA clones of genes in the maize GSH S-transferase multigene family. Nucleic Acids Res **16:** 425–438

Guilfoyle TJ, Hagen G, Li Y, Ulmasov T, Liu Z, Strabala T, Gee M (1993) Auxin-regulated transcription. Aust J Plant Physiol **20:** 489–502

Habig WH, Pabst MH, Jakoby WB (1974) Glutathione S-transferases: the first enzymatic step in mercapturic acid production. J Biol Chem **249:** 7130–7139

Hagen G, Uhrhammer N, Guilfoyle TJ (1988) Regulation of expression of an auxin-induced soybean sequence by cadmium. J Biol Chem **263:** 6442–6446

Hahn K, Herget C, Strittmatter G (1994) PR-gene *prp1-1* from potato: regulation of its expression and functional analysis of the gene product. *In* Abstracts 4th International Congress of Plant Molecular Biology. Kluwer Academic Publishers, Dordrecht, The Netherlands, abstract No. 1764

Irzyk GP, Fuerst EP (1993) Purification and characterization of a glutathione S-transferase from benoxacor-treated maize (*Zea mays*). Plant Physiol **102:** 803–810

Itzhaki H, Woodson WR (1993) Characterization of a ethylene-responsive glutathione S-transferase gene cluster in carnation. Plant Mol Biol **22:** 43–58

Ketley JN, Habig WH, Jakoby WB (1975) Binding of nonsubstrate ligands to the glutathione S-transferases. J Biol Chem **250:** 8670–8673

Kutchan TM, Hochberger A (1992) Nucleotide sequence of a cDNA encoding a constitutively expressed glutathione S-transferase from cell suspension cultures of *Silene cucubalus*. Plant Physiol **99:** 789–790

Listowski L, Abramovitz M, Homma H, Niitsu Y (1988) Intracellular binding and transport of hormones and xenobiotics by glutathione S-transferases. Drug Metab Rev **19:** 305–318

Litwack G, Ketterer B, Arias IM (1971) Ligandin: a hepatitic protein which binds steroids, bilirubin, carcinogens and a number of exogenous organic anions. Nature **234:** 466–467

MacDonald H, Jones AM, King PJ (1991) Photoaffinity labeling of soluble auxin-binding proteins. J Biol Chem **266:** 7393–7399

Mannervik B, Alin P, Guthenberg C, Jenson H, Tahir MK, Warholm M, Jornvall H (1985) Identification of three classes of cytosolic glutathione common to several mammalian species: correlation between structural data and enzymatic properties. Proc Natl Acad Sci USA **82:** 7202–7206

Mannervik B, Awasthi YC, Board PG, Hayes JD, Di Ilio C, Ketterer B, Listowsky I, Morgenstern R, Muramatsu M, Pearson WR, Pickett CB, Sato K, Widerstern M, Wolf CR (1992) Nomenclature for human glutathione transferases. Biochem J **282:** 305–306

Mannervik B, Danielson UH (1988) Glutathione transferases. Structure and catalytic activity. CRC Crit Rev Biochem **23:** 283–337

Mannervik B, Guthenberg C (1981) Glutathione transferase (human placenta). Methods Enzymol **77:** 231–235

Meyer DJ, Coles B, Pemble SE, Gilmore KS, Fraser GM (1991a) Theta, a new class of glutathione transferases purified from rat and man. Biochem J **274:** 409–414

Meyer RC Jr, Goldsbrough PB, Woodson WR (1991b) An ethylene-responsive gene from carnation encodes a protein homologous to glutathione S-transferases. Plant Mol Biol **17:** 277–281

Moore RE, Davies MS, O'Connell KM, Harding EI, Wiegand RC, Tiemeier DC (1986) Cloning and expression of a cDNA encoding a maize glutathione S-transferase in *E. coli*. Nucleic Acids Res **14:** 7227–7235

Nilsson B, Uhlen M, Josephson S, Gatenbeck S, Philipson L (1983) An improved positive selection plasmid vector constructed by oligo-nucleotide mediated mutagenesis. Nucleic Acids Res **11:** 8019–8030

Pemble SE, Taylor JB (1992) An evolutionary perspective on glutathione transferases inferred from class-theta glutathione transferase cDNA sequences. Biochem J **287:** 957–963

Pickett CB, Lu AYH (1989) Glutathione S-transferases: gene structure, regulation, and biological function. Annu Rev Biochem **58:** 743–764

Reinemer P, Dirr HW, Ladenstein R, Schäffer J, Gallay O, Huber R (1991) The three-dimensional structure of a class π glutathione S-transferase in complex with glutathione sulfonate at 2.3 Å resolution. EMBO J **10:** 1997–2005

Rushmore TH, Pickett CB (1993) Glutathione S-transferases, structure, regulation, and therapeutic implications. J Biol Chem **268:** 11475–11478

Shah DM, Hironaka CM, Wiegand RC, Harding EI, Krivi GG, Tiemeier DC (1986) Structural analysis of a maize gene coding for glutathione S-transferase involved in herbicide detoxification. Plant Mol Biol **6:** 203–211

Takahashi Y, Kuroda H, Tanaka T, Machida Y, Takaebe I, Nagata T (1989) Isolation of an auxin-regulated gene cDNA expressed during the transition from G0 to S phase in tobacco mesophyll protoplasts. Proc Natl Acad Sci USA **86:** 9279–9283

Takahashi Y, Nagata T (1992a) *parB*: an auxin-regulated gene encoding glutathione S-transferase. Proc Natl Acad Sci USA **89:** 56–59

Takahashi Y, Nagata T (1992b) Differential expression of an auxin-regulated gene, *parC*, and a novel related gene, C7, from tobacco mesophyll protoplasts in response to external stimuli in plant tissues. Plant Cell Physiol **33:** 779–787

Taylor TL, Fritzemeyer K-H, Hauser I, Kombrink E, Rohwer F, Schroder M, Strittmatter G, Hahlbrock K (1990) Structural analysis and activation of a gene encoding a pathogenesis-related protein in potato. Mol Plant Microbe Interact **3:** 72–77

Timmerman KP (1989) Molecular characterization of corn glutathione S-transferase isozymes involved in herbicide detoxication. Physiol Plant **77:** 465–471

Toung Y-PS, Hsieh T-S, Tu C-PD (1990) *Drosophila* glutathione S-transferase 1–1 shares a region of sequence homology with the maize glutathione S-transferase III. Proc Natl Acad Sci USA **87:** 31–35

Toung Y-PS, Hsieh T-S, Tu C-PD (1993) The glutathione S-transferase D genes. A divergently organized, intronless gene family in *Drosophila melanogaster*. J Biol Chem **268:** 9737–9746

Tugwood JD, Isseman I, Anderson RG, Bundell KR, McPheat WL, Green S (1992) The mouse peroxisome proliferator activated receptor recognizes a response element in the 5′ flanking sequence of the rat acyl CoA oxidase gene. EMBO J **11:** 433–439

Van der Zaal EJ, Droog FNJ, Boot CJM, Hensgens LAM, Hoge JHC, Schilperoort RA, Libbenga KR (1991) Promoters of auxin-inducible genes can lead to auxin-inducible and root tip-specific expression. Plant Mol Biol **16:** 983–998

Van der Zaal EJ, Memelink J, Mennes AM, Quint A, Libbenga KR (1987) Auxin-induced mRNA species in tobacco cell cultures. Plant Mol Biol **10:** 145–157

Vessey DA, Boyer TD (1984) Differential activation and inhibition of different forms of rat liver glutathione S-transferase by the herbicides 2,4-dichlorophenoxyacetate (2,4-D) and 2,4,5-trichlorophenoxyacetate (2,4,5-T). Toxicol Appl Pharmacol **73:** 492–499

Vieira J, Messing J (1991) New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene **100:** 189–194

Wiegand RC, Shah DM, Mozer TJ, Harding EI, Diaz-Collier J, Saunders C, Jaworski EG, Tiemeier DC (1986) Messenger RNA encoding a glutathione S-transferase responsible for herbicide tolerance in maize is induced in response to safener treatment. Plant Mol Biol **7:** 235–243

Yamada T, Kaplowitz N (1980) Binding of ethacrynic acid to hepatic glutathione S-transferase in vivo in the rat. Biochem Pharmacol **29:** 1205–1208

Zettl R, Schell J, Palme K (1994) Photoaffinity labeling of *Arabidopsis thaliana* plasma membrane vesicles by 5-azido-[7-^{3}H]indole-3-acetic acid: identification of a glutathione S-transferase. Proc Natl Acad Sci USA **91:** 2507–2511

Zhou J, Goldsbrough PB (1993) An *Arabidopsis* gene with homology to glutathione S-transferases is regulated by ethylene. Plant Mol Biol **22:** 517–523