J Plant Growth Regul (1997) 16:95–107

Journal of
Plant
Growth
Regulation

Plant Glutathione *S*-Transferases, a Tale of Theta and Tau

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Received August 14, 1996; accepted February 6, 1997

Abstract. Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are ubiquitous enzymes that catalyze the addition of glutathione to a wide variety of substrates. Plant GSTs have been studied mainly in relation to their role in the detoxification of herbicides, mostly in maize. Recently, several *gst* genes have been isolated as genes that respond to the plant hormone auxin with increased transcription. Pathogen infections and other treatments have also been found to lead to increased expression of *gst* genes. It is now apparent that different types of plant *gst* genes and activities exist in most plant species, but their functional relationships and their relationships to the action of auxin are still poorly understood. Here, a historic overview on plant GSTs will be presented. Based on primary sequence differences, a new class of plant GSTs, tau, will be proposed. The possible roles of auxin and oxidative stress in inducing *gst* genes will be discussed. Hopefully this review will help develop new ideas and stimulate new research to study the functions of the ever growing family of plant GSTs.

Key Words. Auxin—Evolution—Gene expression—Glutathione *S*-transferase—Oxidative stress—Signal transduction

Glutathione *S*-transferases (EC 2.5.1.18) are a ubiquitous family of multifunctional proteins that catalyze the nucleophilic addition of the thiol of the reduced form of the tripeptide glutathione to a wide variety of hydrophobic electrophiles (for reviews, see Mannervik and Danielson 1988, Rushmore and Pickett 1993). GSTs have evolved together with glutathione in aerobic organisms and are widely distributed in most forms of life: bacteria, fungi, parasites, yeast, insects, mammals, and higher plants. GST isozymes are involved in the detoxification of cytotoxic products and the protection of tissues against oxidative damage. In addition to their enzymatic activities some GST isoforms also possess a ligand binding capacity and are involved in intracellular transport of hydrophobic and amphiphatic compounds (Listoswky et al. 1988).

In general, GST are cytosolic and exist as homo- or heterodimers with a subunit molecular mass of 25–30 kDa. Most studied are mammalian GSTs, which can be classified into four distinct families, alpha, mu, pi, and theta, based on substrate specificities and primary structures. Typically, the amino acid sequence identity within a class is greater than 50%, and between classes smaller than 30%. A fifth class, sigma, is made up by the *S*-crystallins of squid lenses. Theta class isozymes have the widest distribution among species and are therefore proposed to be the ancestral type, from which the others evolved (Buetler and Eaton 1992). The theta class is very heterogeneous and shows lower amino acid sequence identities than the other classes. It has therefore been considered the default class, containing all GST isozymes that do not belong to any of the other classes (Pemble and Taylor 1992). Although plant GSTs have generally been considered to be members of the theta class, it is clear that at least two major types of plant GSTs can be distinguished (Droog et al. 1995a). Based on overall amino acid sequence identify and conservation of specific residues it is proposed here to separate part of the plant GSTs into a new class, which will be named tau, in line with names used so far. This new classification will hopefully be helpful toward studying and understanding the role and function of plant GSTs.

Abbreviations: 2,3-D, 2,3-dichlorophenoxyacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; ABA, abscisic acid; AOS, activated oxygen species; Ap-1, activating protein 1; as, activating sequence; ARE, antioxidant responsive element; CA, cinnamic acid; CDNB, 1-chloro-2,4-dinitrobenzene; DTT, dithiothreitol; EST, expressed sequence tag; GSH, glutathione; GST, glutathione *S*-transferase; IAA, indoleacetic acid; kDa, kilodalton; NAA, 1-naphthylacetic acid; ocs, octopine synthase; SA, salicylic acid; SAR, systemic acquired resistance.

Plant Glutathione *S*-Transferase Sequences

The first GST activity to be described in plants was a maize enzyme responsible for the detoxification of the herbicide atrazine (Frear and Swanson 1970). Subsequently, numerous GST activities have been characterized which are involved in detoxification of several classes of herbicides in different plant species. These various GST activities show differences in their regulation and display distinct but sometimes overlapping substrate specificities. Notably, conspicuous species-specific differences in metabolism and susceptibility toward certain herbicides have been observed. The variation in regulation and substrates also illustrates the versatility of the GST isozymes and indicates that studies on the regulation and activity of individual GST isozymes in one species need to be interpreted with caution. In maize, the most studied system, four isoforms have been characterized, and four cDNAs or genes encoding the subunits have been isolated. GSTI, III, and IV are homodimers of GST29, GST26, and GST27 subunits respectively, whereas GSTII is a heterodimer of GST29 and GST27 (Grove et al. 1988, Irzyk et al. 1995, Jepson et al. 1994, Shah et al. 1986).

In completely unrelated research, aimed at studying auxin-regulated gene expression, several genes were isolated which were found to have a very limited yet functionally significant homology to the previously identified plant GST sequences (Droog et al. 1993). These genes were detected in several species and, in tobacco, three closely related genes were isolated named *Nt103, Nt107/parC,* and *Nt114/parA* (Droog 1995, Takahashi and Nagata 1992a, Takahashi et al. 1989, van der Zaal et al. 1987, 1991). Interestingly, a fourth tobacco auxin-regulated gene, called *parB,* was also isolated which was much more similar to the maize sequences involved in herbicide detoxification than to the other auxin-regulated tobacco genes (Takahashi and Nagata 1992b). In related research on auxin-binding proteins two *gst* genes were isolated from *Hyoscyamus muticus* and *Arabidopsis* which also were more similar to the maize detoxifying sequences than to the tobacco auxin-regulated sequences (Bilang and Sturm 1995, MacDonald et al. 1991, Zettl et al. 1994). The same *Arabidopsis* sequence was isolated independently as an ethylene-responsive gene (Zhou and Goldsbrough 1993).

Several other lines of research also led to isolation of similar sequences. A pathogen-inducible wheat gene and two dehydration-induced *Arabidopsis* sequences were found to be closely related to the herbicide-detoxifying GSTs (Dudler et al. 1991, Kiyosue et al. 1993). On the other hand, a pathogen-inducible potato gene and a multiple stimulus response curled leaved tobacco gene were closely related to the auxin-regulated GSTs (Dominov et al. 1992, Hahn and Strittmatter 1994). It soon became apparent that plants have numerous GST genes and numerous ways of regulating their expression. Based on the primary sequences it seemed that there were two major groups, but referring to these as *herbicide-detoxifying* or *auxin-regulated* is clearly inaccurate.

The number of plant GST sequences is growing rapidly, and at least 35 different genes or cDNAs encoding GST isozymes from 13 different plant species have now been fully or partially sequenced. They are listed in Table 1 and include sequences from *Arabidopsis* (10), tobacco (9), maize (5), wheat (2), soybean (2), mung bean, potato, curled leaved tobacco, broccoli, rice, *Silene cucubalus, H. muticus,* and *Eucalyptus globulus.* Most data so far strongly suggest that most plant species will have multiple *gst* genes and point to the existence of up to ten or more genes per species, indicating the importance of the activities of GSTs for the well-being of modern day plants. The data also indicate that the two major types of plant GSTs which have been recognized previously (Droog et al. 1995a) exist in most plant species.

Classification of Plant GST Isozymes

In mammals five classes of species-independent GST subunits are recognized, mainly based on percentage NH_2- terminal amino acid identity and cross-reactivity with antibodies to human GST subunits. They are referred to as alpha, mu, pi (Mannervik and Danielson 1988), theta (Meyer et al. 1991a), and sigma (Buetler and Eaton 1992). Since the last count (Buetler and Eaton 1992) the number of GST isozyme sequences has more than doubled, and more than 150 are now known, with all newly discovered sequences fitting into the proposed classes.

The theta class is the most diverse and includes members in the widest range of species, including bacteria, insects, plants, fish, and mammals. It is also the largest class of GST isozymes with more than 50 members. Although it has been regarded as a default class, several strict sequence conservations can be used to distinguish it from the other classes. In the region considered crucial for glutathione binding a conserved glutamine residue is present in all GSTs. This residue has been shown to interact directly with the γ-glutamic acid moiety of GSH (Reinemer et al. 1991, Wilce and Parker 1994). In the theta class at this same position a conserved glutamic acid is present instead, and it has been shown to perform a similar function (Reinemer et al. 1996, Wilce et al. 1995). A second essential difference between the theta class and the other four classes is a conserved tyrosine residue near the NH_2-terminal end which, in the expressed protein, is part of the active site and is situated within hydrogen bonding distance of the thiol group of bound glutathione. The structurally

Table 1. Plant glutathione *S*-transferase sequences.

Code	Name	Species	Reference
a	C7	Tobacco	Takahashi and Nagata 1992b
b	Nt107/GST2-1	Tobacco	van der Zaal et al. 1991, Droog 1995
	parC	Tobacco	Takahashi and Nagata 1992b
d	Egpar	*Eucalyptus globulus*	Martin et al. 1996
e	Igul/GmGxI	Soybean	Accession no. P46417
f	parA/Nt114/GST3-1	Tobacco	Takahashi et al. 1989, Droog 1995
	msr	Curled leaved tobacco	Dominov et al. 1992
g	MII-4	Mung bean	Accession no. U20809
h	pCE54/Gmhsp26-A	Soybean	Czarnecka et al. 1988, Hagen et al. 1988
i	prp1-1/gst1	Potato	Hahn and Strittmatter 1994
	pGNT35/GST1-3	Tobacco	van der Zaal et al. 1991, Droog et al. 1993
	pGNT1/GST1-2	Tobacco	van der Zaal et al. 1991, Droog et al. 1993
j	Nt103/GST1-1	Tobacco	van der Zaal et al. 1991, Droog et al. 1993
l	gst5/At103-1a	*Arabidopsis*	Watahiki et al. 1995, van der Kop et al. 1996
m	At103-1b	*Arabidopsis*	van der Kop 1996
n	pBC591/CIP	Rice	Binh and Oono 1992
o	bronze-2	Maize	Nash et al. 1990, Schmitz and Theres 1992
q	gst1	Carnation	Meyer et al. 1991b, Itzhaki and Woodson 1993
s	PM239 × 14	*Arabidopsis*	Bartling et al. 1993
t	ERD13	*Arabidopsis*	Kiyosue et al. 1993
u	''GSTI''/GST29	Maize	Shah et al. 1986, Grove et al. 1988
v	''GSTIV''/GST27	Maize	Jepson et al. 1994, Irzyk et al. 1995
w	gstA1	Wheat	Dudler et al. 1991
	gstA2	Wheat	Dudler et al. 1991
x	''GSTIII''/GST26	Maize	Grove et al. 1988
z	Sc gst	*Silene cucubalus*	Kutchan and Hochberger 1992
	apiC	Tobacco	Ezaki et al. 1995
A	parB	Tobacco	Takahashi and Nagata 1992a
B	Hmgst-1	*Hyoscyamus muticus*	Bilang and Sturm 1995
C	gst2/Atpm24	*Arabidopsis*	Zhou and Goldsbrough 1993, Zettl et al. 1994
D	ERD11	*Arabidopsis*	Kiyosue et al. 1993
c	est3	*Arabidopsis*	Accession no. Z33955
k	est4	*Arabidopsis*	Accession no. Z34012
p	est6	*Arabidopsis*	Accession no. Z26546
r	Bo gst	Broccolli	Lopez et al. 1994
y	est1	*Arabidopsis*	Accession no. Z18158

Note. Duplicate names exist for several of the sequences because of different names for the cDNA, genes, or encoded proteins or because they have been isolated independently by several groups. Code refers to the lettercode used for the phylogenesis in Fig. 1.

corresponding residue in theta class isozymes is a conserved glycine that unambiguously is not part of the enzyme's active site (Reinemer et al. 1996). The residue that seems to take over the role of tyrosine in the catalytic side is a conserved serine, positioned several residues further toward the COOH terminus (Reinemer et al. 1996, Wilce et al. 1995).

To prevent further confusion about the relationships among different plant GSTs and whether they are herbicide-detoxifying or auxin-regulated enzymes, a classification as used for mammalian GSTs would be very helpful. Recently, a first proposal for such a classification of plant GST subunits was made, based on a phylogenetic analysis from 16 full-length GST subunit sequences (Droog et al. 1995a). Three classes of plant GSTs were recognized, and these were referred to as types I, II, and III. Here, the phylogenesis is extended to 30 sequences by including several new full-length sequences as well as several partial ones. (Although the calculated evolutionary distances for the partial sequences will not be exactly correct, as the conservation of amino acids is not equal for all positions in the protein, their incorporation in the tree does allow a more extended discussion of the merit of the classification as more sequences are included.) The results obtained are shown in Fig. 1 and essentially confirm the previous conclusions. It is clear that there are two major groups of plant GST subunits, but it is equally clear that some sequences cannot be easily grouped. The first major group, previously named type I, contains the maize GST26, 27, and 29, the wheat *gstA1*, the tobacco *parB*, the *S. cucubalus gst*, the *H. muticus gst*, the broccoli *gst*, and the *Arabidopsis gst2*, ERD11, ERD13, and EST1. The second major group, previously named type III, is formed by the tobacco C7, Nt107-5, Nt114-4,

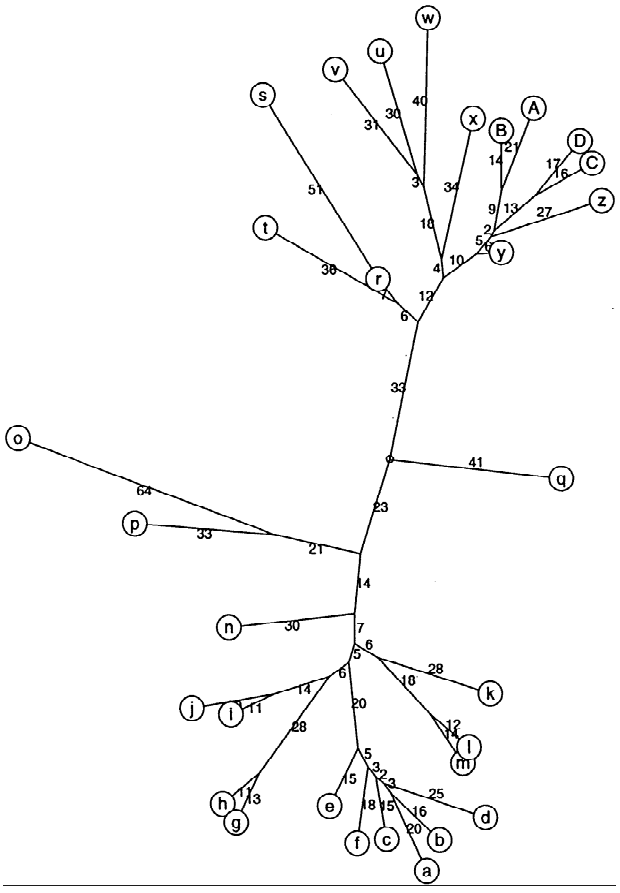

Fig. 1. Phylogenetic analysis of plant glutathione *S*-transferase sequences. Sequences were obtained from literature and from databases using the University of Wisconsin GCG software package (Devereux et al. 1984) and the Blast program (Altschul et al. 1990). Phylogenesis was done by running the RootedTree option of the AllAll program at the ETH in Zürich. *a,* C7; *b,* Nt107; *c,* AtEST3; *d,* Egpar; *e,* GmGxI; *f,* Nt114; *g,* MII-4; *h,* GmHsp26-A; *i,* PRP1-1; *j,* Nt103; *k,* AtEST4; *l,* At103-1a; *m,* At103-1b; *n,* CIP; *o, Bz2; p,* AtEST6; *q,* Dc gst; *r,* Bo gst; *s,* Pm239x14; *t,* ERD13; *u,* ZmGST29; *v,* ZmGST27; *w,* Ta gstA1; *x,* ZmGST26; *y,* AtEST1; *z,* Sc; *A,* parB; *B,* Hmgst-1; *C,* Atgst2; *D,* ERD11. For references see Table I, where the lettercode used for the phylogenesis is indicated. ESTs are numbered according to their submission date.

Nt103-1, the potato *gst1,* the soybean hsp26a and GxI, the mung bean MII-4, the maize *Bz2,* the rice CIP, and the *Arabidopsis* At103-1a, At103-1b, EST3, EST4, and EST6. To bring the names for the plant GST groups more in line with class names used so far for GSTs in general, new names for the two major groups of plant GSTs are proposed, and these will be discussed below. An example of a *gst* that does not fall easily into either of these two major groups is the carnation *gst.*

A comparison of the plant GSTs with nonplant theta class isozymes shows that the type I plant isozymes more closely resemble nonplant theta class isozymes than they do plant type III isozymes. This indicates that the type I plant isozymes represent the most archaic GST isozyme in plants and belong to the theta type class. The plant type III isozymes, on the other hand, do not seem to have any closely related isozymes in other species. They thus seem to be unique to plants, comparable to the sigma class, which seems to be unique to mollusks. Their clear evolutionary distinction from the theta class warrants their classification as a new class, which in line with names used so far will be named tau. The validity of the existence of the class tau as a separate class of plant GSTs is underscored by the presence of several uniquely conserved residues in this class as well as a different gene structure, both of which will be discussed below. Plant GSTs that do not belong to the new class tau will, by default, belong to the class theta. It is not unlikely that as more sequences become available more classes will be distinguished. A possible example of such a new class might be the carnation *gst1* gene, which is clearly quite diverged from both the class tau and the class theta plant sequences and has a unique number of introns. The rice CIP, which has several unique insertions throughout its sequence, might also be the first of a new class or subclass (see also below). Definite assignment to possible new classes awaits further sequence information. It is also possible that evolution of *gst* sequences and GST activity has lead to unique genes and activities only occurring in a limited number of plant species. The occurrence of both class tau and class theta sequences simultaneously in most if not all plant species suggests that the evolutionary event leading to their existence has happened early in the development of plants.

At first glance it may seem that there is a bias toward tau class members in tobacco and toward theta class members in maize. This is however probably at least partly due to the different research interests that have led to the identification of *gst* sequences. In maize this was herbicide detoxification, and in tobacco it was auxin-regulated gene expression. For instance, although the maize *Bz2* is currently the only available maize sequence that belongs to the tau class, it is very likely that the maize clone CT24 is also in this class. It reportedly has a 41% identity and a 61% similarity with *Bz2* (Schmitz and Theres 1992), similar to the other tau class members. It hybridizes to several fragments on a genomic blot and seems to be part of a family of sequences showing close similarity to each other and less to *Bz2,* indicating that maize, as tobacco, has several tau class isozymes. (Unfortunately, the CT24 sequence has not yet been made available.) Assays on GST activity in maize indicated at least 11 separable activities (Dean et al. 1991), leaving enough room for more than the six sequences identified to date. This does not exclude the possible occurrence of different numbers of genes of the different classes in different plant species due to specific evolutionary pres-

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                A.                                                  B.

                50                                        79        155          166
                .* === *     ===      **===   *     .              .= *      =   *.
Zm GST27        NPF.GQVPA.LQDGD.LYLFESRAICKYAARKN                   YLAG.DFL.SLADL
Zm GST29        ---.-K--V.-E---.-T-------ARHVL--H                   ----.---.-----
Ta gstA1        ---.AKM-G.FQ---.-V-------AK-IL--Y                   ----.-SI.TF---
Zm GST26        ---.--I--.-V---.EV-------NR-IAS-Y                   ----.-EF.T---A
Sc gst          ---.-----.-E--E.IK-------TK-LAYTH                   --GAN-SF.T-V--
Nt parB         ---.-----.FE---.-K-------TQ-IAHVY                   --G-.-SF.T-V--
Hm gst          ---.-----.FE---.-K-------TQ-IAHTY                   --G-.-SF.T----
At gst2         ---.-----.FE---.-K-------TQ-IAHRY                   -----.ETF.T-T--
At ERD11        ---.-K---.FE---.FKI------TQ-IAHEF                   ---S.-HF.T-V--
At ERD13        Q--.-KI-V.-V---.YKI------MR-IAEKY                   ----.--V.S----
At PM239x14     H--.-VI-V.-E-E-GTKIY-----SR-LVAKY                   ----.NDF.T----

Dc gst1         N-L.-Y--V.-VH--.IVIAD-L--IM-LEE-F                   -AT-.-EV.G----

Nt C7           --IHKKI-V.-IHNG.KPIC--LI-VE-IDEVW                   -FG-.ERF.GFV-M
Nt 107          --IHKKI-V.-IHNG.KPIC--IIAVE-IEEVW                   -FG-.-SF.GFV-I
Eg par          --VHKKI-V.-IHNG.KPVC--HI-VQ-IDETW                   -FG-.ERF.GFL-V
Gm GxI          --IHKKI-V.-IHNG.KPIC--AI-VQ-IDEVW                   FYGD.-TF.GFV--
Np msr          --VHKKI-I.-IHNG.KPIC--LN-LE-IDEVW                   -FG-.-N-.GFV-V
Nt parA         --VHKKI-I.-IHNS.KAIC--LN-LE-IDEVW                   -FG-.-N-.GFV-V
Vr MII-4        --VHKKV-V.FVHGD.KLP.--LV-VE-IDETW                   FFG-.EEL.G-V-I
Gm hsp26A       --VHKKV-V.FVHNE.QPIA--LV-VE-IDETW                   FFV-.EEF.G-V-I
St gst1         --IHKKI-V.-IHNG.KCIC--MV-LE-IDEAF                   CFV-.-KF.GF--I
Nt 103          --VHKKV-V.-IHNG.KPIV--MV-LE-IDETF                   FFV-.-KF.GF--I
At 103-1a       --IHKKV-V.-VHNG.KTIL--HV-LE-IDETW                   -FV-.KTV.GFL-F
At 103-1b       S-IHKKI-V.-VHNG.KTII--HV-LE-IDETW                   -FG-.KTV.GFL-F

Zm Bz2          --VY-KI-VL-LP-G.RAIC--AV-VQ-IEDVA                   FFS-G-AAPG-L--

Os CIP          --VHKSV-V.-LHAG.RRERVAGHRAVHRRGLA                   V-Q-.EAV.LR-TA
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Fig. 2. Alignment of two conserved regions of plant GST isozymes. *A*, region from amino acid residue 50 to 79 (numbering according to the maize GST27 sequence). *B*, region from amino acid residue 155 to 166. * indicates amino acid residues that are absolutely or strongly conserved in all theta class isozymes. = indicates amino acid residues whose conservation is unique to either the theta or tau class plant GST isozymes. - indicates the presence of an identical amino acid residue. The absence of an amino acid residue is indicated by a *dot*. For references, see Table I. The sequences are grouped according to their phylogenetic class, the first 11 representing the theta class and the second 12 the tau class. The three other sequences have unique characteristics. For details, see text.

sures, for example, selection for herbicide resistance in cultivated maize.

Conserved Regions

It is clear from an alignment of all available full-length primary protein sequences that there are several conserved regions in all GST isozymes. One major region is located between amino acid residues 50 and 80 and a second region between amino acid residues 155 and 165 in the plant GST sequences (numbering of amino acid residues will be according to the maize GST27 sequence throughout the text, unless stated otherwise). Close inspection of these regions has indicated that they can be used to characterize the different classes of mammalian GSTs by the presence of specific unique amino acid residues. As noted above, the theta class is set apart by the presence of a conserved glutamic acid around amino acid residue 70, where the other four classes have a conserved glutamine, which is shown to interact directly with glutathione. The plant theta and tau classes both have the conserved glutamic acid at this position.

The two classes of plant GSTs can also be characterized by the presence of several uniquely conserved amino acid residues in the two conserved domains (see Fig. 2). In the first conserved domain there is a first triplet of amino acid residues absolutely conserved only in the class tau sequences in position 53–55, histidine-lysine-lysine (see Fig. 2*A*). A second triplet is in position 60–62, where the class theta sequences have a strongly conserved aspartic acid-glycine-aspartic acid triplet, whereas the class tau sequences have a strongly conserved histidine-asparagine-glycine triplet (see Fig. 2*A*). A third triplet, arginine-alanine-isoleucine at position 69–71, is absolutely conserved in the class theta sequences only. In the second conserved domain an absolutely conserved leucine, at position 156, in the theta class sequences is replaced by strongly conserved phenylalanine in the tau class (see Fig. 2*B*). An intriguing last amino acid residue clearly setting the plant class tau sequences apart form the class theta sequences is located 3 residues upstream from the aspartic acid residue, at position 165, which is completely conserved in all GST sequences known to date. In the class tau sequences it is a conserved glycine (G), whereas in the class theta sequences it is either a serine (S) or a threonine (T). Interestingly, in all but one of 150 sequences of GSTs this residue is either a G, an S, or a T, as if nature is telling us something here! Outside of the conserved domains there are several more uniquely conserved amino acid residues that confirm the existence of two separate classes of GSTs in plants. The class-specific conservation of specific amino acid residues indicates that there are different evolutionary constraints for the two classes,

suggesting that there are differences in either structure or activity of the two classes.

Although the phylogenesis indicates that the rice CIP is related to the *gst* sequences it does not show the strict conservation of essential amino acids observed for the other isozymes in the two conserved regions (see Fig. 2). It lacks several of the absolutely conserved amino acid residues observed in all GSTs, and it is doubtful whether it is active as a GST. It seems that it is diverged from a *gst* sequence but has evolved to serve a more specialized function in cold protection. The tau class maize *Bz2* seems to be somewhat unique as well. It has an extra amino acid residue in the first conserved domain and although it is clearly quite similar to the tau class sequences it lacks several of the conserved amino acid residues that characterize this class. It also has two additional amino acid residues in the second conserved domain, one of which it shares with the theta class *S. cucubalus gst.* Interestingly, the maize *Bz2* protein is the only tau class isozyme for which an in planta function has been described (Marrs et al. 1995), which will be discussed below.

Gene Structures

Genes encoding several of the plant GST isozymes identified have been isolated. An analysis of their primary structure shows that they can be divided into three types based on the number and the position of their introns. The first type contains two introns at conserved positions and includes the maize GSTI (Shah et al. 1986), the wheat *gstA1* and *gstA2* (Dudler et al. 1991), and the *S. cucubalus gst* (Prändl and Kutchan 1992). The second type includes only the two GSTs from carnation (Itzhaki and Woodson 1993) which each have nine introns. The third type includes the soybean *Gmhsp26A* gene (Czarnecka et al. 1988), the maize *Bz2* gene (Nash et al. 1990), the potato *gst1* gene (Taylor et al. 1990), as well as the tobacco *Nt103* (van der Zaal et al. 1991), *Nt107* (Droog 1995), and *Nt114* genes (Droog 1995), and the *Arabidopsis At103* genes (van der Kop 1996). All of these genes have one intron at a conserved position. This division coincides perfectly with the two major classes identified by comparison of the primary protein sequences, class theta genes having two introns and class tau genes one. It also is in line with the unique position in the phylogenesis of the carnation *gst* genes. It also supports the notion that at least two genes, leading to class tau and class theta, existed before the separation of plants or early in the development of the different plant species.

Protein Structures

Crystal structures of several class alpha, mu, pi, and theta GST enzymes are known, and even though the primary sequence homology is limited the overall peptide folds were found to be very similar (Wilce and Parker 1994 and references therein). However, each class was found to exhibit unique features as well, particularly around the active site and at the COOH terminus. Recently the three-dimensional structure of the first plant GST was resolved, that of the *Arabidopsis* class theta *gst2* encoded protein (Reinemer et al. 1996). Despite the very limited overall sequence identity between plant GSTs and mammalian isozymes (Droog et al. 1995a) they appear to be topologically similar. The *Arabidopsis* GST subunit has the characteristic modular structure with two distinct domains, connected by a linker segment. The enzyme forms a globular dimer of two identical subunits with a GST-typical large cleft formed in the center, which is open to the active sites and the bulk solvent. Each subunit binds two molecules of the competitive inhibitor S-hexylglutathione. The glutathione peptide of one inhibitor occupies the G-site, or GSH binding site, with multiple interactions, similar to those observed for other GSTs, and this is termed the productively binding inhibitor. The glutathione of the second inhibitor exhibits only weak interactions and is termed the unproductively binding inhibitor. The hexyl moieties of both inhibitors are oriented parallel and fill the H-site, or hydrophobic substrate site, of the enzyme's active site.

Translating the structural data obtained for the *Arabidopsis* GST to the other class theta plant GSTs predicts that they are all structurally very closely related, especially in domain I (Reinemer et al. 1996). All seven residues involved in binding glutathione are conserved or conservatively replaced. The variation in domain II is much larger, and residues lining the H-site are not strictly conserved, although most are replaced conservatively.

Considering the conserved structures of all GSTs for which the three-dimensional structure has now been determined, it seems likely that other plant GSTs will also have a similar structure. Several amino acid replacements however do suggest that the class tau plant GSTs might have a slightly different domain I structure than the class theta enzymes. For example, the 3_{10} element might be shorter, and the His-40 and Lys-41 residues binding glutathione in this element in the *Arabidopsis* class theta GST are not present at a similar position in class tau GSTs. Interestingly, there is a strictly conserved histidine-lysine pair positioned in the element connecting the 3_{10} element with strand β_3 in class tau GSTs. These residues would be in a similar position to the Glu-53 and Val-54 of the *Arabidopsis* class theta GST structure, which are also involved in glutathione binding. These differences indicate that class tau GSTs have some unique features around the active site, substantiating that they can be separated into a new class distinct from class theta. It should be emphasized here again that the class theta encompasses a large group of GSTs with limited homologies, and the three-dimensional structures of class theta members might be more variable than observed and

expected for the any of the other classes. It is very likely that as more sequences become available more subclasses of theta will be recognized.

Comparison of the two exons of the class tau GSTs separately shows that the homology in the first exon is much higher than in the second exon, ranging from a 7 to a 45% difference. This probably reflects more stringent evolutionary constraints on the first domain of the GST subunit, as the first exon encodes domain I and the element linking domain I and II. More strict conservation of the NH_2-terminal region or domain I is a general feature of all GST isozymes. Remarkably, the linker element seems to be strongly conserved as well in the class tau GSTs, contrary to the class theta GSTs. This might be related to the different gene structure of the class theta GSTs, which have two introns and three exons that do not coincide with the domains. The exons in class theta plant GSTs do seem to coincide with structural elements, as exon I ends in a region and exon III starts in a region that shows low structural similarity when plant and nonplant crystal structures are compared (Reinemer et al. 1996).

Biological Activities

Attempts have been made to relate the results from the phylogenesis to differences in function of the different types of GST subunits. There is however insufficient information to allow any significant conclusions to be drawn. For example, in both class tau and class theta both (auxin-) inducible and constitutive subunits occur. Herbicide detoxification activity has been shown for most, but not all, class theta subunits. Activities toward the common substrate CDNB have been found to be both low and high for members of each of the two classes. Unfortunately, information on endogenous substrates and on the functional significance of inducibilities is still lacking. The situation in plants appears to be largely similar to that in the much more studied mammals. There seem to be preferential substrate(s) for most classes, but activities are overlapping, and not all isozymes of the same class necessarily behave identically.

A first step toward identifying endogenous substrates might be the characterization of a maize GST that can conjugate cinnamic acid (CA) and other phenylpropanoids (Dean et al. 1995). This enzyme is reported to have some unique features; it is monomeric rather than dimeric and not only accepts GSH but also cysteine as a sulfhydryl source. Whether this enzyme will share any homology to the class tau or theta plant sequences remains to be investigated. In French bean and several other legumes a CA-conjugating GST activity has also been characterized (Diesperger and Sandermann 1979, Edwards and Dixon 1991). The activity in French bean showed cross-reactivity to antibodies raised against maize class theta GST subunits. The only plant GST for which additional activity as a GSH peroxidase, observed for several mammalian GST isozymes, has been shown is the *Arabidopsis* PM239x14 (Bartling et al. 1993). This does suggest that the function of some plant GSTs might be the reduction of toxic fatty acid hydroperoxides formed during normal cellular metabolism or as a result of oxidative stress.

GS-X Pump and Detoxification

Although plant GSTs were originally discovered as being involved in detoxification of herbicides in maize, it seems very likely that they all have evolved from a common ancestor that had an endogenous substrate. For most plant GST isozymes analyzed, no endogenous substrates are known. Recent results obtained with the maize *Bronze-2* gene might point to a new direction to look for possible substrates and functions of plant GST isozymes. The maize *Bz2* gene was found to code for a GST isozyme that conjugates glutathione with cyanidin-3-glucoside, the last genetically defined step in the synthesis of anthocyanins (Marrs et al. 1995). This glutathione conjugation marks it for transport to the vacuole and leads to the appearance of red and purple pigments. Absence of this activity leads to accumulation of cyanidin-3-glucoside in the cytoplasm and a bronze color of tissues. In mammalian cells transport of glutathione conjugates is mediated by a so-called GS-X pump located in the cell membrane (Ishikawa 1992). Plant cells also possess such a GS-X pump, just as yeast cells (Li et al. 1995, Martinoia et al. 1993) but it is located in the vacuolar membrane. Contrary to mammalian cells that excrete the glutathione conjugates, plant and yeast cells eliminate unwanted glutathione conjugates by transporting them into the vacuole, a process referred to as storage excretion. So through the concerted action of GSTs and the GS-X pump, plant cells can confer a common structural determinant on hydrophobic compounds, rendering them more water soluble, and thereby eliminating them from the cytosol. These results clearly open new ways to look at the function of other plant GSTs and to study the possible role of auxins. Interestingly, the last steps in anthocyanin biosynthesis involve cytochrome P-450 monooxygenases, glucosyltransferases, and glutathione *S*-transferases, the same enzymes that are involved in detoxification of xenobiotics.

As discussed above, a comparison of the primary sequences of the *Bz2* protein with those of the other plant GSTs suggests that it may belong to the tau class, although it is clearly a divergent member. The *Arabidopsis* EST6 seems to represent the ortholog in *Arabidopsis* of the maize *Bz2*, indicating that this isozyme is not specific for maize. The class tau GSTs, or a subset thereof, might thus represent isozymes that operate in sequestering

structurally similar but functionally diverse molecules into the vacuole.

Direct Interaction of Auxins with GSTs

Two of the plant GSTs identified to date were originally isolated as auxin-binding proteins, the Hmgst1 (MacDonald et al. 1991) and the *Arabidopsis gst2* (Zettl et al. 1994), indicating the possibility of a direct physical interaction between auxins and GSTs. Several studies have been performed to test the effect of auxins on in vitro GST activities using CDNB as a substrate and GSH as the cofactor. For Hmgst1 and several other GSTs the effects of auxins on the in vitro GST activity have been studied. IAA, NAA, 2,4-D, 2,3-D, and 2,4,5-T were all found to inhibit the activity of Hmgst1 against CDNB. The inhibition by 2,4-D was found to be competitive toward CDNB, whereas IAA acted noncompetitively (Bilang and Sturm 1995). The potato *gst1* was found to be inhibited by IAA, 1-NAA, and 2-NAA, and found to bind the photoaffinity-labeled 5-azido-[7-^{3}H]indole-3-acetic acid (Hahn and Strittmatter 1994). Inhibition by IAA was competitive toward CDNB. The activity of the *Arabiopsis gst5* was reported to be inhibited by IAA, 2,4-D, 1-NAA, and 2-NAA (Watahiki et al. 1995). However, in contrast to the other studies, here a competitive inhibition of all four auxins toward the cofactor glutathione was reported. The tobacco NT103 and NT107 (GST1-1 and GST2-1) proteins were not only inhibited by 2,4-D but also by other non-auxin-chlorinated compounds (Droog et al. 1995a). Here, IAA only showed a minimal inhibition and could not be tested at higher concentrations because of its absorption at the same wavelength as the product of the reaction with CDNB. Inhibition by 2,4-D was shown to be competitive toward CDNB. Interestingly, in these last studies the phenylacetic acid derivative ethacrynic acid, a known substrate and inhibitor of mammalian GSTs, was also tested and found to be inhibitory in a competitive manner toward CDNB at a nearly 1,000-fold lower concentration than that reported for 2,4-D.

The data obtained for the inhibition of the in vitro activity of several plant GSTs by auxins relate well to results obtained for the effect of 2,4-D on mammalian isozymes. In both rat (Vessey and Boyer 1984) and human (Singh and Awasthi 1985) 2,4-D and 2,4,5-T were found to inhibit several isozymes in different ways. Both competitive and noncompetitive inhibition toward both CDNB and GSH was observed. In the absence of knowledge on endogenous substrates for most plant GSTs it is worth mentioning that plant polyphenols are also recognized as inhibitors of rat liver GST isozymes (Zhang and Das 1994).

What is clear from the observations discussed above is that there seems to be a direct physical interaction between at least some auxins and some GSTs. The data indicate that not all auxins will bind all GSTs and point to the possibility of auxins binding to different sites of different GSTs. Mammalian GSTs are well known for their broad substrate specificities and the large variety of molecules that they can bind at different positions. The situation in plants seems to be not very different. These data do not necessarily mean that auxins function as substrates for GSTs. It is well known that in addition to their catalytic activity some animal GSTs can also function as ligand-binding proteins and thereby facilitate the intracellular storage and transport of a variety of hydrophobic nonsubstrate compounds, including hormones, metabolites, and drugs. This suggests the possibility that plant GSTs might be involved in the transport or storage of auxin.

Regulation of GST Gene Expression

In general, plant *gst* genes have been found to be induced by a wide variety of stimuli, pointing to the need for the activity of GSTs under various conditions. Although the induction of individual genes in some cases seems to be restricted to just one stimulus or tissue, i.e. the ethylene-responsive flower petal senescence-related carnation *gst1* gene (Meyer et al. 1991b), it is frequently observed that multiple stimuli can induce the same *gst* gene. In those cases where regulation of *gst* gene expression has been studied by detailed promoter analysis it has been observed that seemingly unrelated stimuli can lead to activation of the same gene. The soybean *Gmhsp26-A* gene is induced by a wide variety of agents, among which are auxins, nonauxin analogs, other plant hormones, heavy metals, hydrogen peroxide, DTT, GSH, salicylic acid (SA), and jasmonic acid (Ulmasov et al. 1995). At least some of these inducers work through a single element in the promoter, termed the *ocs* element, and it has been suggested that this element might function similarly to AP-1 sites in some animal *gst* genes (Ulmasov et al. 1994). The tobacco *Nt103, Nt107,* and *Nt114* genes were also found to be induced, to varying degrees, by a wide variety of agents, including auxins, auxin transport inhibitors, cytokinins, ABA, heavy metals, SA, H_2O_2, GSH, ethanol, ethacrynic acid, and pathogen infection (Boot et al. 1993, Droog 1995). Flooding of seedlings also had an effect and in general increased the response to various agents, possibly because of anaerobiosis (Ushimaru et al. 1992). Similar results, showing an induction by auxins, SA, wounding, and copper, as well as bacterial, fungal, and viral infection, have been described independently for the tobacco gene *str246c* (Gough et al. 1995), which is identical to *Nt114-4.* The *Nt103, Nt107,* and *Nt114* genes all contain an *as* element in their promoter, which is related to the *ocs* element in the *Gmhsp26-A* promoter, and it has been shown that the

response to most inducers is conferred by this element (Droog 1995, Droog et al. 1995b, van der Zaal et al. 1996). The potato *gst1* gene is specifically induced in a localized manner after infection of host plants with various pathogenic or symbiotic organisms, whereas expression in uninfected plants is confined to root apices and senescing leaves (Strittmatter et al. 1996). The results discussed above certainly are not meant to imply that all of the GST genes will have just one common element regulating their expression. It is much more likely that multiple regulatory elements are present in the promoters of most GST genes, some of which will react to specific signals and some of which to more general stress-related signals. This makes the promoters of these genes very interesting to study signal transduction pathways and the way in which they are specific or overlapping for different signals.

All of the above discussed genes belong to the class tau, and similar data on the theta class members seem to be lacking. Several theta class members are known to increase in mRNA levels upon herbicide and safener treatments, but usually these were the only treatments tested (Edwards and Owen 1988, Fuerst et al. 1993, Irzyk and Fuerst 1993, Mozer et al. 1983, Wiegand et al. 1986). The theta class wheat *gstA1*-encoded protein GST29 was found to be induced specifically by pathogen infection and not by herbicides (Mauch and Dudler 1993). The only other inducer for GST29 was glutathione. The same study indicated the presence of two other GSTs in wheat, GST25 and GST26, which cross-reacted with antibodies raised against maize class theta GSTs. These two subunits did respond to herbicides and xenobiotics but not to pathogen infection or glutathione. In maize, the GST29 subunit was found to be constitutively expressed and slightly induced by herbicide safener (Jepson et al. 1994). The GST27 in maize was found to be strongly induced by safener and only marginally by high doses of auxins (Jepson et al. 1994). Treatments leading to phytotoxic effects were observed to lead to significant induction of the GST27 subunit as well. The theta class auxin-binding *H. muticus gst* was found to be induced by 2,4-D but not by IAA or herbicides (Bilang and Sturm 1995).

The combined results on the analysis of *gst* promoters suggest that there might be a common factor in the signaling transduction pathway from the initial recognition of the stimulus to the activation of gene expression for many of the plant *gst* genes. They also suggest that the activity of the encoded proteins is needed under many different conditions. A proposed and indeed very likely model would be that gene expression of at least a subset of *gst* genes is activated by the occurrence of oxidative stress, and the activity of the encoded proteins is needed to protect cells against oxidative damage (Droog 1995, Droog et al. 1995a, Ulmasov et al. 1995). An involvement of stress is not only indicated by the wide variety of substances and treatments leading to activation of the *gst* genes but also by the usually high levels of inducers needed and the linearity in the dose-response curves. It is now well established that one of the first responses to pathogen infection is the occurrence of an oxidative burst (Levine et al. 1994). A more specific role of particular individual *gst* genes and gene products can clearly not be excluded and indeed seems likely. The potato *gst1* gene is reportedly only induced by pathogen infections, whereas the tobacco *Nt114-4* gene is induced by both pathogen infections and abiotic treatments. The existence of sizable gene families in different plant species also argues in favor of some form of specialization. Elucidation of endogenous substrates of specific GSTs will be needed before their role can be ultimately determined. It should again be stressed here that promoters and encoded proteins could have evolved separately to accommodate individual demands.

GSTs and Oxidative Stress

Oxygen is essential for aerobic life, yet reactive oxygen intermediates can be highly toxic to cells. Because of their highly reactive nature activated oxygen species (AOS) can lead to DNA, protein, and membrane damage (Halliwell and Gutteridge 1984). To protect themselves against AOS plants have developed a variety of enzymatic and nonenzymatic antioxidant mechanisms. During oxidative stress, the balance between the scavenging capacity of the antioxidant systems and the production of AOS is lost (Sies 1985). The potential for the production of AOS is greatly enhanced by a wide variety of environmental stresses, and it is thought that the ensuing damage results from the accumulation of these AOS to levels exceeding the antioxidant capacity of the cell.

Glutathione is an important antioxidant and plays a crucial role in the defense against AOS (Alscher 1989). GSH is the most abundant nonprotein thiol in plant cells and is present at millimolar concentrations (Foyer and Halliwell 1976). GSH functions both as a scavenger of free radicals and as a component of the GSH/ascorbate cycle. It also is a cofactor in the GST-mediated detoxification of electrophilic compounds. The ability of cells to withstand an oxidative stimulus is dependent at least in part upon the capacity for de novo GSH synthesis (May and Leaver 1993), and the frequently observed increase in the levels of GSH in response to an oxidative stimulus can play a crucial role in cellular protection. Extracellular GSH has been shown to act as an activator of the transcription of genes (Wingate et al. 1988). Hence, the observed increase of GSH during oxidative stress can serve two functions. GSH can act directly as an antioxidant and simultaneously activate a panoply of stress genes, including GSTs.

The induction of many GST genes by a large variety

of seemingly unrelated compounds and treatments can be most easily explained by a common step in the activated signal transduction pathways. A possible candidate for such a step might be a sufficiently large change in the oxidation state of the cell. Stress treatments can be envisioned to induce an oxidative burst like the one studied mostly in pathogen infections (Apostel et al. 1989, Doke and Ohashi 1988, Legendre et al. 1993, Mehdy 1994). During pathogen infections a biphasic oxidative burst can be observed, generated by activation of an NADPH oxidase, not unlike the situation observed in human neutrophils (Dwyer et al. 1996). The AOS that are generated can either directly or indirectly lead to the activation of defense genes, including GSTs, needed to protect the cells against oxidative damage. It is shown that AOS play an important role in the induction of SAR, often observed after pathogen infections (Chen et al. 1993), and the induction of many GSTs by pathogens is well documented. The rat GST Ya promoter contains an ARE element that mediates transcriptional activation by H_2O_2 and also by redox-labile electrophilic compounds, implicating it as a part of the signal transduction pathway that is activated upon the occurence of oxidative stress (Friling et al. 1992, Rushmore and Pickett 1990, Rushmore et al. 1991). Xenobiotics can induce the rat GST Ya gene either directly or after the activity of cytochrome P-450 enzymes have rendered them redox labile. This suggests that similar mechanisms might function in plant GST genes that are involved in detoxification of xenobiotics, such as herbicides, and which are induced by herbicide safeners.

Induction of defense genes, such as GSTs, by thiols, such as GSH, or oxidative stress can be the effect of a direct or indirect activation of a transcription factor. In animal cells, an unusual posttranslational modification involving oxidation-reduction regulates the DNA binding activity of the transcription factors AP-1 and NF-κB (Devary et al. 1991, Schreck et al. 1991). Interestingly, AP-1 binding is activated and NF-κB binding is inhibited by reducing agents, indicating that changes in redox state can both activate and inactivate gene expression. A factor regulating the redox state of AP-1, Ref1, itself subject to redox regulation, has also been isolated (Xanthoudakis and Curran 1992, Xanthoudakis et al. 1992), and AP-1 has been suggested to act as a secondary antioxidant-responsive factor (Meyer et al. 1993). The conservation of such a pathway controlling the oxidative stress response between plants and animals is suggested by the characterization of an *Arabidopsis* protein analogous to Ref1, Arp1, which was found to be able to activate AP-1 in vitro (Babiychuk et al. 1994).

The suggestion that changes in the oxidation state of the cell might lead to activation of GST genes raises the possibility that auxins also change the oxidatition state of the cell, thereby inducing the expression of specific GST genes. Analogous to the situation observed during pathogen infections, one of the effects of auxin might be a stimulation of an NADPH oxidase and the production of AOS. Alternatively, auxin might change the oxidation state in a different manner. It has been reported that auxins do not effect the NADPH oxidase (Morré and Brightman 1991) but do stimulate a plasma membrane-localized NADH oxidase in a way that can be inhibited by GSH (Brightman et al. 1988, Morré et al. 1995). The effect of auxins might also be at a different stage. AOS lead to membrane damage and the generation of hydroperoxides, and these might be the actual inducers of GST genes. Similarly, auxins might lead to the formation of hydroperoxides as a result of their effect on cell division and elongation. Alternatively, the effect of auxin might be more subtle, by acting on specific proteins in the oxidation state transduction pathway and thus regulating transcription.

Final Remarks

It is clear that the classification proposed here is based upon a still limited number of sequences and limited knowledge on the functional roles of plant GST isozymes. However, the existence of at least two major classes of GSTs in plants, theta and tau, is evident. The reasons for the observed induction by such a wide variety of stimuli for some genes and the seemingly specific induction of others and the way in which this is connected to their activities remain to be investigated. Hopefully this review will prove to be a positive contribution to both the study of auxin signaling transduction pathways and the role of GSTs in plants.

Acknowledgments. I thank all those colleagues who have contributed to this work by publishing their data and their ideas and apologize to those whose work has not been mentioned here because of space limitations. I also give special thanks to B. J. Taller for creating the opportunity to write this paper and to MRTC for its running support. This work was in part made possible by support from the Dr. W. Harry Feinstone postdoctoral fellowship and the Feinstone Microbiology research fund at the University of Memphis.

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