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Functional Divergence in the Glutathione Transferase Superfamily in Plants

IDENTIFICATION OF TWO CLASSES WITH PUTATIVE FUNCTIONS IN REDOX HOMEOSTASIS IN *ARABIDOPSIS THALIANA**

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David P. Dixon, Benjamin G. Davis‡, and Robert Edwards§

From the School of Biological and Biomedical Sciences, University of Durham, Durham DH1 3LE and ‡Dyson Perrins Laboratory, Department of Chemistry, University of Oxford, South Parks Road, Oxford OX1 3QY, United Kingdom

Searches with the human Omega glutathione transferase (GST) identified two outlying groups of the GST superfamily in *Arabidopsis thaliana* which differed from all other plant GSTs by containing a cysteine in place of a serine at the active site. One group consisted of four genes, three of which encoded active glutathione-dependent dehydroascorbate reductases (DHARs). Two DHARs were predicted to be cytosolic, whereas the other contained a chloroplast targeting peptide. The DHARs were also active as thiol transferases but had no glutathione conjugating activity. Unlike most other GSTs, DHARs were monomeric. The other class of GST comprised two genes termed the Lambda GSTs (GSTLs). The recombinant GSTLs were also monomeric and had glutathione-dependent thiol transferase activity. One GSTL was cytosolic, whereas the other was chloroplast-targeted. When incubated with oxidized glutathione, the putative active site cysteine of the GSTLs and cytosolic DHARs formed mixed disulfides with glutathione, whereas the plastidic DHAR formed an intramolecular disulfide. DHAR *S*-glutathionylation was consistent with a proposed catalytic mechanism for dehydroascorbate reduction. Roles for the cytosolic DHARs and GSTLs as antioxidant enzymes were also inferred from the induction of the respective genes following exposure to chemicals and oxidative stress.

In eukaryotes, the cytosolic glutathione transferases (GSTs,[1] EC 2.5.1.18) are a diverse family of proteins that share a similar three-dimensional structure and possess a well defined glutathione-binding domain at their active sites (1). In plants, all the GSTs described to date are dimers composed of 25-kDa subunits, and on the basis of sequence similarity and gene organization, they appear to have evolved from a common ancestral GST into four distinct classes, namely the Phi, Tau, Zeta, and Theta GSTs (2). The two largest classes are the plant-specific Phi and Tau GSTs. Both classes have major roles in herbicide detoxification (3, 4). In addition, these GSTs have less well characterized roles in endogenous metabolism including functioning as glutathione peroxidases counteracting oxidative stress (5, 6) and also acting as flavonoid-binding proteins (7), stress signaling proteins (8), and regulators of apoptosis (9). In contrast, the smaller Zeta and Theta classes of GSTs are also found in animals and fungi, indicating conserved and essential functions for these enzymes in all eukaryotes. Thus, Zeta GSTs in *Arabidopsis*, animals, and fungi catalyze the glutathione-dependent isomerization of maleylacetoacetate to fumarylacetoacetate, an essential step in the catabolism of tyrosine (10), whereas Theta class GSTs act as potent glutathione peroxidases detoxifying organic hydroperoxides formed during oxidative stress (2).

Cumulatively, these studies point to a functional divergence in the GST superfamily in plants in which individual GSTs use GSH as either a co-substrate or co-enzyme in catalysis. Significantly, all four classes of plant GSTs identified to date contain a conserved serine residue within their active site which is central to stabilizing the charged thiolate form of GSH used to drive conjugation and peroxidase and isomerase reactions (11). In this respect, although the reactions driven by these enzymes are diverse, their mechanism of catalysis remains essentially conserved (12).

While searching the *Arabidopsis* data bases for other members of the extended GST family, we have identified genes encoding GST-like proteins, which contain the conserved GSH binding domain, but where the active site serine residue is replaced with cysteine. Because the cysteine residue cannot serve the same catalytic role as a serine residue at the active site, this suggests that these new members of the plant GST family may have evolved functions that employ an alternative reaction mechanism to that used by the four GST classes previously identified. To test this hypothesis we have cloned and expressed the members of these two additional types of the extended GST family in *Arabidopsis*, and we assayed the enzyme activity of the recombinant proteins. In the course of these studies we have determined that the active site cysteine of these novel GSTs forms transient mixed disulfides with GSH, and we propose that this reaction is involved in their catalytic function, which relates to counteracting oxidative stress. We also report on the regulation of the respective genes following exposure of *Arabidopsis* plants to conditions invoking chemical and redox stress.

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§ To whom correspondence should be addressed: School of Biological and Biomedical Sciences, University of Durham, Durham DH1 3LE, UK. Tel.: 44-191-374-2428; Fax: 44-191-374-2417; E-mail: Robert.Edwards@durham.ac.uk.

[1] The abbreviations used are: GST, glutathione transferase; AA, ascorbic acid; BHP, *t*-butyl hydroperoxide; BSO, L-buthionine-(*SR*)-sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EAA, L-erythroascorbic acid; ESI, electrospray ionization; GSH, glutathione; HED, 2-hydroxyethyl disulfide; MS, mass spectrometry; NAA, naphthalene acetic acid; SDHA, semi-dehydroascorbate; SET, single electron transfer; THK, thiohemiketal; TOF, time of flight; DTT, dithiothreitol.

TABLE I
Oligonucleotide primers used for cloning and semi-quantitative PCR
The primer combinations were used to amplify the products indicated (nomenclature based on Ref. 2).

Name	Sequence	Product
og2	gagagaggatcctcgac[t]$_{17}$	
og9	cgcactgagagaggatcctcgag	
T7	taatacgactcactataggg	
DHAR1a DHAR1b	cgcgcgccatggctctggaaatctgtgtgaaagtt cgcagatctcataacggtgcatagtttc	*At*DHAR1
DHAR4a DHAR4b	cgcgcgccatgggcatcgaagtctgcgtg cgcggatccaagacaataatgcatcacac	*At*DHAR4
DHAR2a DHAR2b	cgcgcgccatggctctagatatctgcgtg cgcagatctagagaagcatggatccac	*At*DHAR2
DHAR3a	cgcgcgccatggcgacggcggcgagtcctc	*At*DHAR3 (with og9)
DHAR1-his	gcggcggtcgacaggttaaccttgggagc	*At*DHAR1-his
DHAR2-his	gcggcgctcgagcgcattcaccttcgattc	*At*DHAR2-his
DHAR3-his	gcggcggtcgacacccataacctttggtctcc	*At*DHAR3-his
ERD11a ERD11b	cgcgccatggcaggaatcaaagttttc catcttctgatcgataaatagtttg	*At*GSTF3
GST8a GST8b	cgcgccatggcgaacgaggtgattcttc catcttaagtccgaaccatatgac	*At*GSTU2
GST5a GST5b	cgcgccatggctgagaaagaagaagtgaag ttcttaagaagatctcactgtctc	*At*GSTU1
ATZ1 ATZ3	ttgtttaccatggcgaattccggcgaag gcaacaggatcctcacagaatcagatggtggaag	*At*GSTZ1
ATT1 ATT2	cgcgccggcatatgatgaagctcaaagtgtatg gcgcggatccttagatcttggattgaagacc	*At*GSTT1
AtL1a AtL1b	caaatcctctccatggctctatc ggcgcggatccttcataagccatcatggcatcg	*At*GSTL1
AtL2b AtL2c	cgccgcccatggctgttgtagagtcaagtcg gcgcggatccggagaaccatctggttag	*At*GSTL2
GSTL1-his	gcggcgctcgagcatgaatctcttgaagtaattg	*At*GSTL1-his
GSTL2-his	gcggcgctcgagacgtgcttctgcttgg	*At*GSTL2-his
Actin1a Actin1b	gatcctaaccgagcgtggttac gacctgactcgtcatactctgc	Actin

EXPERIMENTAL PROCEDURES

Plant Treatments—For chemical induction studies, *Arabidopsis thaliana* (Columbia) seedlings were grown for 19 days as root cultures in 60 ml of sterile media (13). Chemical treatments were added to the media in 0.6 ml of sterile water containing 100 mM GSH (pH 7), 100 mM L-buthionine sulfoximine (BSO), 100 mM ascorbate, or 100 mM *t*-butyl hydroperoxide (BHP). Xenobiotic treatments were added in 0.6 ml of ethanol containing 40 mM 1-chloro-2,4-dinitrobenzene (CDNB), 20 mM fluorodifen, 10 mM dichlormid, 10 mM NAA, or 10 mM 2,4-dichlorophenoxyacetic acid. Control treatments consisted of 0.6 ml of solvent carrier only. After a 24-h treatment, the plants were frozen in liquid N_2 and stored at −80 °C pending analysis.

PCR Amplification, Cloning, and Expression of GST-like Sequences—The combined root and shoot tissue of 3-week-old *Arabidopsis* (Columbia) plants were used as the source RNA for cDNA synthesis (10). *At*GST sequences were amplified from cDNA by reverse transcriptase-PCR using *Taq* DNA polymerase and 30 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 90 s, with the combinations of specific primers detailed in Table I. For *At*DHAR4 and *At*GSTL2, the 5′ primers were designed to allow expression of the mature polypeptides without their putative transit peptides. The purified products were ligated into *Nco*I/*Bam*HI-digested pET-11d (Novagen) for expression in *Escherichia coli.* The coding sequences of the *At*DHARs were also subcloned into the expression vector pET24 by PCR using the respective DHARna and DHARn-his primers (Table I) to generate the C-His-tagged fusions (10). Site-directed mutagenesis of DHAR1-his was carried out by PCR using oligonucleotide primers containing the required mutations. To express the recombinant proteins, cultures of *E. coli* harboring the pET constructs were treated at mid-log phase with 1 mM isopropyl-1-thio-β-D-galactopyranoside and after 3 h harvested and lysed by ultrasonication. Lysates from the bacteria transformed with pET-11d were applied onto GSH affinity columns (14), whereas His-tagged proteins from pET24 transformed *E. coli* were recovered by nickel affinity chromatography (10). The recombinant GSTs were further purified by anion exchange chromatography using a 1 ml of UNO Q1 (Bio-Rad) column eluted with 20 mM Tris-Cl, pH 7.8, containing a linearly increasing concentration of NaCl (0–0.5 M; total volume 25 ml) at 1 ml/min. Purified proteins were analyzed by SDS-PAGE and gel permeation chromatography as described previously (10), as well as by mass spectrometry.

Semi-quantitative PCR—To determine the effect of various chemical treatments on GST mRNA levels, total RNA (300 ng RNA/μl) was used as a template for Moloney murine leukemia virus-reverse transcriptase (Promega). The resulting cDNA samples were then normalized to similar contents of actin1, as judged by the intensity of the respective PCR product obtained using actin1 primers (Table I). To quantify the abundance of each mRNA, each cDNA template (1 μl) was used in a 20-μl PCR using *Taq* DNA polymerase in the presence of 45 mM Tris-Cl, pH 8.8, 11 mM ammonium sulfate, 4.5 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 5 μM EDTA, 0.1 mg/ml bovine serum albumin, and 1 mM of each dNTP. Actin1 primers (each 0.2 μM) were used together with primers (each 0.5 μM) for the GST gene to be amplified (Table I). For each set of primers, a PCR master mix was used to minimize differences between reactions for different templates, with each reaction run in duplicate. Following amplification (28–30 cycles of 94 °C for 20 s, 58 °C for 30 s, and 72 °C for 60 s), products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified using a Bio-Rad Gel Doc 2000 system with supplied Quantity One analysis software. Actin1 primers amplified a product of about 530 nucleotides, whereas the GST primer sets detailed (Table I) amplified a product of about 650 nucleotides, with the similar size of the amplification products selected to minimize differences between primer sets. Control reactions using a range of template concentrations confirmed that for each set of primers, the ratio of PCR products derived from actin and GST was independent of template concentration and that quantification was reliable. The identity of each PCR product was then confirmed by sequencing.

Enzyme Assays—GST activity toward CDNB, glutathione peroxidase

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DHAR1                                                       MALEICVKAAVGAP
DHAR2                                                       MALDICVKVAVGAP
DHAR3   MISLRFQPSTTAGVLSASVSRAGFIKRCGSTKPGRVGRFVTMATAASPLEICVKASITTP
DHAR4                                                       MGIEVCVKAASGAP

             *
DHAR1   DHLGDCPFSQRALLTLEEKSLTYKIHLINLSDKPQWFLDISPQGKVPVLKIDD--KWVTD
DHAR2   DVLGDCPFSQRVLLTLEEKKLPYKTHLINVSDKPQWFLDISPEGKVPVVKLDG--KWVAD
DHAR3   NKLGDCPFCQKVLLTMEEKNVPYDMKMVDLSNKPEWFLKISPEGKVPVVKFDE--KWVPD
DHAR4   DVLGDCPFGQRILLTLEDKKLPYKTHLIDVSLKPDWFLAISPKGKLPLVKFDEDENWVAD

DHAR1   SDVIVGILEEKYPDPPLKT-PAEFASVGSNIFGTFGTFLKSKD-SNDGSEHALLVELEAL
DHAR2   SDVIVGLLEEKYPEPSLKT-PPEFASVGSKIFGAFVTFLKSKD-ANDGSEKALVDELEAL
DHAR3   SDVITQALEEKYPEPPLAT-PPEKASVGSKIFSTFVGFLKSKD-SGDGTEQVLLDELTTF
DHAR4   SDLIVGIIEEKYPEPSLVTFPPEFASVGSKIIGAFVMFLTSKDHANDGSDMALLDELEAL

DHAR1   ENHLKSHDGPFIAGERVSAVDLSLAPKLYHLQVALGHFKSWSVPESFPHVHNYMKTLFSL
DHAR2   ENHLKTHSGPFVAGEKITAVDLSLAPKLYHLEVALGHYKNWSVPESLTSVRNYAKALFSR
DHAR3   NDYIKDN-GPFINGEKISAADLSLAPKLYHMKIALGHYKNWSVPDSLPFVKSYMENVFSR
DHAR4   DHHLKTHVGPFVAGDKVTVVDLSLAPKLYHLETTLGHFMDWCVPESLTNVRDYMKVLFSL

DHAR1   DSFEKTKTEEKYVISGWAPKVNP
DHAR2   ESFENTKAKKEIVVAGWESKVNA
DHAR3   ESFTNTRAETEDVIAGWRPKVMG
DHAR4   ESFEKTKAAKEYLIASWAPKLDV
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FIG. 1. **Alignment of peptide sequences of *At*DHARs.** *Single underlined* residues indicate intron positions; the putative 42-residue transit peptide of *At*DHAR3 is also *underlined.* The conserved catalytically active cysteine residue is shown by an *asterisk.* Residues identical and conserved between sequences are marked with *black* and *gray bars*, respectively.

activity toward cumene hydroperoxide (15), and the GSH-dependent dechlorination of dichloroacetic acid (10) were determined as described previously. Thiol transferase activity toward 2-hydroxyethyl disulfide (HED) was measured in 0.1 M Tris-Cl, pH 7.8, containing 0.25 mM NADPH, 1 mM GSH, 0.6 units/ml glutathione reductase, and 2 mM EDTA and was performed at 30 °C. After a 3-min equilibration with 0.7 mM HED, enzyme was added, and the resulting decrease in absorbance at 340 nm due to NADPH oxidation ($\epsilon = 6.2\ mM^{-1}\cdot cm^{-1}$) was recorded. DHAR assays were performed over 60 s at 30 °C and contained 90 mM potassium phosphate buffer, pH 6.5, 5 mM GSH, and 0.5 mM DHA. Enzyme activity was determined by measuring the increase in absorbance at 265 nm due to the formation of ascorbate ($\epsilon = 14.0\ mM^{-1}\cdot cm^{-1}$), after correcting for spontaneous DHA reduction (16). In the case where ascorbate was added to the assays, DHAR activity was determined from the rate of GSH oxidation by adding 1.25 units/ml glutathione reductase and 0.2 mg/ml NADPH. The coupled oxidation of NADPH was then determined from the decrease in absorbance at 340 nm over 60 s. To determine the effect of *S*-glutathionylation and *S*-alkylation on enzyme activities, purified recombinant GSTs were incubated for 10 min on ice with or without 1 mM oxidized GSH (GSSG). The enzyme preparations were then treated with or without 10 mM iodoacetamide (10 min, 4 °C). Following desalting using a 5-ml HiTrap desalting column (Amersham Biosciences) to remove any GSSG and iodoacetamide, and protein preparations were assayed for enzyme activity.

Protein S-Glutathionylation—Samples (200 μl) of recombinant proteins (0.5 mg/ml) dissolved in 20 mM Tris-Cl, pH 7.5, were treated at 4 °C for 30 min with either 2 mM DTT or 2 mM GSSG. To ensure that adducts formed were due to mixed disulfide formation, an additional GSSG-treated sample was then treated with 20 mM DTT at 4 °C for 30 min to release GSH. Proteins were desalted into 0.4 ml of 2 mM Tris-Cl, pH 7.5, using a 5-ml HiTrap desalting column (Amersham Biosciences), and an equal volume of acetonitrile/formic acid (100:1, v/v) was added. The protein sample was then injected directly into a Micromass LCT time-of-flight (TOF) mass spectrometer, using electrospray ionization (ESI) at a flow rate of 0.1 ml/min. Operating in positive ion mode, mass spectrometry (MS) data were collected in the mass range 500–2000 Da and analyzed using the supplied MassLynx software, with multiply charged peaks deconvoluted using the MaxEnt1 plugin after calibration with horse heart myoglobin. Trypsin digests were performed in 1 mM Tris-Cl, pH 7.5, 50% v/v acetonitrile at 37 °C, using sequencing grade modified trypsin (Promega), and analyzed by MS as described for the parent polypeptides.

Sequence Analysis—Phylogenetic analysis was performed on polypeptide sequences aligned with ClustalW (17) using PHYLIP (Phylogeny inference package, J. Felsenstein, Department of Genetics, University of Washington, Seattle). PROTDIST was used to calculate evolutionary distance between sequences and NEIGHBOR (using the UPGMA (unweighted pair group method with arithmetic mean) method of clustering) to calculate the tree.

RESULTS

Identification and Classification of Two New Groups of GSTs in A. thaliana—The strategy adopted in the current study was to use proteins that have been ascribed recently to be outlying members of the GST superfamily in vertebrates and to look for sequences showing similarity in the *Arabidopsis* genome. In the first screen, the sequence of the recently reported human Omega class GST (*h*GSTO1) was used (18). In a BLAST search, the *h*GSTO1 polypeptide sequence identified a sequence (GenBank™ accession number AB037970) in rice (*Oryza sativa*) encoding a protein with dehydroascorbate reductase (DHAR) activity (19). Subsequent searches of the *Arabidopsis* genome data base with the rice DHAR identified four related sequences, which on alignment showed between 60 and 70% identity with the rice gene. The *Arabidopsis* genes were named *dhar1*, *dhar2*, *dhar3*, and *dhar4*, encoding the putative proteins *At*DHAR1, *At*DHAR2, *At*DHAR3, and *At*DHAR4, respectively (Fig. 1). The *dhar1* and *dhar2* sequences were well represented in the *Arabidopsis* EST data bases. In contrast, no *dhar4* ESTs were identified, and only one *dhar3* EST was found. Significantly, although the *dhar1*, *-2*, and *-3* genes possessed two introns in conserved positions, the *dhar4* gene contained no predicted introns, suggesting it was a pseudogene. The sequences of *At*DHAR1, *At*DHAR2, and *At*DHAR4 encoded polypeptides of 213, 213, and 217 amino acid residues, respectively, with respective predicted molecular masses of 23.6, 23.4, and 23.9 kDa. Unlike the other *At*DHARs, the deduced polypeptide sequence for *At*DHAR3 contained an N-terminal polypeptide extension (Fig. 1). Analysis of this extension using TargetP (20, 21) suggested that it contained a 42-residue transit peptide, which would target *At*DHAR3 to the chloroplast or possibly to the mitochondrion.

The BLAST search with *h*GSTO1 also identified the proteins In2-1 in maize (22) and Cla30 in wheat (23), which resemble

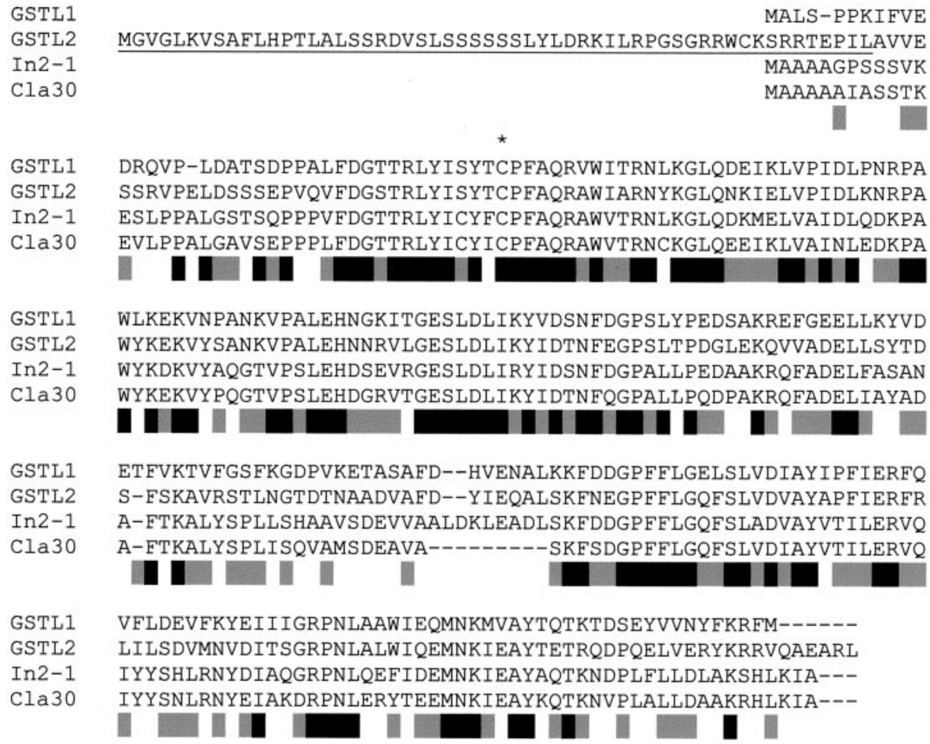

FIG. 2. **Alignment of the peptide sequences of the *At*GSTLs (GSTL1, GSTL2) with Lambda GSTs from maize (In2-1) and wheat (Cla30).** The putative polypeptide transit polypeptide of *At*GSTL2 is *underlined* and the position of the putative active site cysteinyl residue is indicated by an *asterisk*. Residues that are identical and conserved between sequences are marked with *black* and *gray bars*, respectively.

GSTs but lack GSH conjugating activity toward xenobiotics (24). BLAST searches with *In2-1* and *Cla30* sequences as queries showed that they were more similar to mammalian Omega GSTs than to the other classes of plant GSTs. However, although *In2-1* and *Cla30* shared a similar active site motif (CPFA) with the Omega GSTs (18), their overall sequences were sufficiently divergent to put these genes into a separate class that we have termed the Lambda (L) GSTs. Searching the *Arabidopsis* data base with the *In2-1* sequence identified two closely related Lambda GST genes, *gstl1* and *gstl2*. The proteins corresponding to these predicted coding sequences were termed *At*GSTL1 and *At*GSTL2, respectively (Fig. 2). The *gstl1* gene was present on BAC F9G14 (GenBank™ accession number AL162973) and was also represented by an EST (GenBank™ accession number AI995850). However, the annotated genomic clone was missing one intron when compared with the EST sequence. The corrected gene sequence contained 8 introns and a single open reading frame encoding a 27.2-kDa polypeptide composed of 237 amino acid residues (Fig. 2). The other gene, *gstl2*, was present on BAC T15C9 (GenBank™ accession number AL132970). The gene had 8 putative introns that gave an mRNA with a single open reading frame encoding a polypeptide of 292 residues (Fig. 2). Comparison of *At*GSTL1 with *At*GSTL2 showed the two polypeptide sequences to be very similar, but the *At*GSTL2 contained a long N-terminal extension. Analysis (20, 21) showed that this extension was most likely a transit peptide, probably targeting *At*GSTL2 to the chloroplast. Following processing, the resulting mature *At*GSTL2 polypeptide was composed of 237 amino acid residues and had a predicted molecular mass of 27.0 kDa.

Cloning and Heterologous Expression of Arabidopsis DHAR Genes—The coding sequences of *dhar1*, *-2*, *-3*, and *-4* were amplified by PCR using combinations of specific primers. All four genes were successfully amplified from cDNA prepared from total RNA isolated from whole *Arabidopsis* plants, although *dhar4* gave a much less abundant product than that obtained with the other primer combinations, and may have been amplified from contaminating genomic DNA. The products were initially cloned into pET-11d to give the respective pET-DHAR*n* constructs and then were sequenced. pET-DHAR1 and pET-DHAR3 had deduced polypeptide sequences identical to those predicted from the genomic sequences of *dhar1* and *dhar3*, respectively. The deduced sequence of pET-DHAR2 contained 1 amino acid substitution compared with that anticipated from published genomic and EST sequences such as GenBank™ accession number NM_106182. The pET-DHAR4 sequence contained four mis-sense substitutions compared with GenBank™ accession number NM_123018 (A to G at base 175, T to C at base 280, G to A at base 349, and A to G at base 560). The *At*DHAR2 substitution was later shown to be a PCR-induced error. However, the four substitutions in *At*DHAR4 most likely arose from minor differences in the gene sequences of different *Arabidopsis* ecotypes. The *At*DHAR1 nucleotide sequence was identical to the coding sequence of GenBank™ accession number AY039590, and the *At*DHAR3 sequence was identical (except for a silent substitution of T for A at base 159) to bases 106–759 of GenBank™ accession number AF301597.

The sequence substitutions were not corrected in the initial attempts to express the pET constructs in *E. coli,* and following induction with isopropyl-1-thio-β-D-galactopyranoside, the crude bacterial lysates were analyzed for polypeptide composition by SDS-PAGE and for enzyme activity. As compared with extracts from *E. coli* harboring the empty pET construct, bacteria expressing pET-*At*DHAR1 and pET-*At*DHAR3 accumulated appreciable amounts of recombinant polypeptides of 23 and 27 kDa, respectively. Expression of pET-*At*DHAR2 and pET-*At*DHAR4 resulted in the accumulation of large quantities of insoluble polypeptide, with a mass of around 24 kDa in each case. After correcting for the low levels of endogenous enzyme activity due to the GSTs in *E. coli*, no conjugating activity toward CDNB or glutathione peroxidase activity toward cumene hydroperoxide could be determined in any of the soluble fractions from the recombinant *E. coli*. Crude cell-free extracts from the *At*DHAR1 and *At*DHAR3 preparations showed considerable GSH-dependent DHAR activity (between 2 and 4 microkatals·mg^{-1} protein), whereas in extracts from bacteria harboring the pET vector alone, DHAR activity could not be

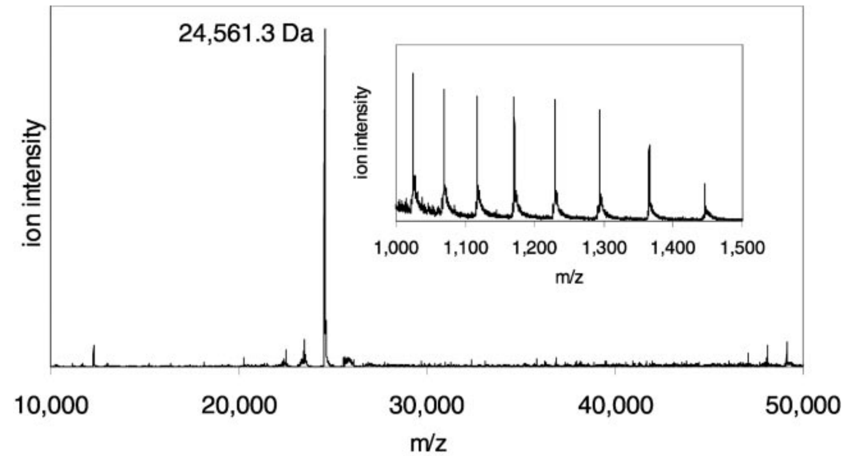

FIG. 3. **Example of mass spectrum obtained by ESI-MS demonstrating the purity of *At*DHAR1-his.** Main figure shows spectrum after deconvolution, whereas the *inset* shows data before deconvolution.

determined above the chemical rate. In the bacteria expressing *At*DHAR2, DHAR enzyme activity could just be determined in the soluble fraction (10 nanokatals·mg^{-1} of protein) demonstrating the presence of minor amounts of soluble active protein. However, no DHAR activity could be determined in the soluble fraction from the bacteria expressing *At*DHAR4. In view of the difficulty in obtaining *At*DHAR4 in soluble form and the uncertain status of *dhar4* as an expressed gene, further characterization of this recombinant protein was not undertaken.

Attempts to purify the recombinant DHAR polypeptides from crude bacterial lysates by GSH affinity chromatography proved unsuccessful, so the *At*DHAR1, *At*DHAR2, and *At*DHAR3 sequences were sub-cloned into the C-terminal His tag expression vector pET-24 to give pET24-DHAR1, pET24-DHAR2, and pET24-DHAR3, respectively. For pET24-DHAR2, the *At*DHAR2 sequence was re-isolated by PCR, and its fidelity with the coding sequence of GenBank™ accession number NM_106182 confirmed. Expression of pET24-DHAR1, pET24-DHAR2, and pET24-DHAR3 resulted in the production of the soluble polypeptides *At*DHAR1-his, *At*DHAR2-his, and *At*DHAR3-his, respectively, all of which migrated as 26-kDa polypeptides when analyzed by SDS-PAGE. The His-tagged proteins were individually purified by nickel affinity chromatography followed by anion exchange chromatography, and their purity was confirmed by SDS-PAGE and ESI-TOF MS (Fig. 3).

Characterization and Catalytic Mechanism of AtDHARs—The purified His-tagged recombinant proteins had high DHAR activities (Table II). None of the purified enzymes showed detectable GSH conjugating activity toward standard xenobiotic GST substrates such as CDNB, 4-nitrobenzyl chloride, and benzyl isothiocyanate (14). The DHARs also showed no activity as dichloroacetic acid dehalogenases or glutathione peroxidases. However, when assayed with the model substrate HED, the *At*DHARs all possessed thiol transferase activity (Table II). This activity was greatest after pre-incubating the HED with GSH for 3 min, suggesting the enzyme used the spontaneously formed 2-mercaptoethanol-glutathione disulfide as substrate, rather than HED itself. When assayed as either a DHAR or thiol transferase, *At*DHAR2-his was considerably less stable than the other enzymes, rapidly losing activity in solution at 4 °C or following precipitation with ammonium sulfate. As a result this enzyme was not characterized further with respect to physical properties and enzyme activities. To determine whether the recombinant DHARs were monomers or multimeric proteins, the purified proteins were analyzed by gel filtration chromatography. With both *At*DHAR1-his and *At*DHAR3-his, protein and DHAR activity eluted as a single peak with molecular masses of 29 and 32 kDa determined, respectively (data not shown). It was concluded that the *At*DHARs were monomeric proteins, and under the conditions used for chromatography, we were unable to obtain any evidence that these polypeptides associated together to form multimers. The purified recombinant *At*DHAR-his fusions were then subjected to kinetic analysis. *At*DHAR1-his had an apparent K_M(DHA) of 0.26 mM, whereas *At*DHAR3-his had a K_M(DHA) of 0.50 mM. Both enzymes had a low affinity for GSH when assayed with 0.5 mM DHA, with an apparent K_M(GSH) of about 10 mM in both cases. The kinetics of *At*DHAR1-his were then examined in more detail. The enzyme appeared to conform to Michaelis-Menten kinetics with respect to each substrate, suggesting that the rate-limiting step(s) required 1 molecule of DHA and 1 molecule of GSH. Tests for product inhibition showed that ascorbic acid was not inhibitory at concentrations of up to 5 mM, even when low concentrations of DHA or GSH were used. In contrast, the co-reaction product GSSG gave uncompetitive inhibition with respect to DHA, with 2.5 mM GSSG reducing DHAR activity by 73% when assayed in the presence of 1.25 mM GSH. GSSG showed more complex inhibition with respect to GSH, giving rise to strong positive co-operativity with this substrate when added at 2.5 mM.

TABLE II
Enzyme activities associated with purified recombinant His-tagged AtDHARs and AtGSTLs

Enzyme	Thiol transferase activity[a]	DHAR activity
	nanokatals · mg^{-1} protein	
*At*DHAR1-his	116	15,600
*At*DHAR1-his-C6S	21	8,300
*At*DHAR1-his-C20S	0	0
*At*DHAR2-his[b]	15	2,000
*At*DHAR3-his	131	4,400
*At*GSTL1-his	41	0
*At*GSTL2-his	69	0

[a] Thiol transferase activity determined with HED as substrate.
[b] Activities for *At*DHAR2-his were determined using freshly prepared recombinant protein, but due to the instability of this enzyme the true DHAR and thiol transferase activities may be underestimated.

Because all the *At*DHARs contained a cysteinyl residue in place of a serine residue as an active site residue (Fig. 1), samples of purified *At*DHAR1-his and *At*DHAR3-his with the activities detailed in Table II were incubated with 10 mM iodoacetamide, and the effect on activity was determined. In both cases, the resulting *S*-alkylation abolished all DHAR activity. To determine whether such a catalytic cysteinyl group could undergo *S*-glutathionylation, recombinant *At*DHAR1-his and *At*DHAR3-his were pre-incubated with GSSG to promote protein-glutathione mixed disulfides prior to *S*-alkylation. After desalting, the preparations were then directly assayed for DHAR activity. As compared with the untreated enzymes, the

TABLE III
Mass ions of recombinant polypeptides following treatment with GSSG to promote S-glutathionylation followed by reduction with DTT
Assuming parent and *S*-glutathionylated polypeptides ionize with similar efficiencies, their relative abundances are given in % in parentheses.

Polypeptide	Mass of parent ion (% abundance)		
	Untreated	+ GSSG	+ GSSG + DTT
*At*DHAR1-his	24,561.3	24,867.3	24,561.9
*At*DHAR1-his-C6S	24,545.6	24,851.4	24,545.8
*At*DHAR1-his-C20S	24,546.2	24,546.1	24,545.9
*At*DHAR2-his	24,341.7	24,341.1 (14%) 24,952.5 (86%)	24,341.9 (75%) 24,647.3 (25%)
*At*DHAR3-his	25,124.5	25,122.8 (90%) 25,427.9 (10%)	25,124.6
*At*GSTL1-his	28,101.9	28,407.6	28,102.1
*At*GSTL2-his	27,822.8	28,128.5	27,823.1

GSSG pre-treated *At*DHAR1-his retained 77% of its activity, whereas *At*DHAR3-his maintained 95% of its activity.

To determine the stoichiometry and site of *S*-glutathionylation of the *At*DHARs, the two cysteinyl residues at positions 6 and 20 (or their equivalent), which are conserved in all DHAR family members (Fig. 1), were targeted for site-directed mutagenesis to the respective serine residues. The two cysteines of *At*DHAR1-his, at residues 6 and 20, were independently mutated to serines to give the C6S and C20S His-tagged mutant proteins, respectively, which were purified and assayed for DHAR activity. Whereas C6S retained around 50% of the activity of the parent *At*DHAR1-his, C20S possessed no detectable activity (Table II). Similarly, C6S retained thiol transferase activity whereas C20S did not (Table II). Parent *At*DHAR1-his and the C6S and C20S proteins were then incubated on ice for 30 min with either 2 mM DTT, to give fully reduced protein to serve as a control, 2 mM GSSG to promote mixed disulfide formation, or 2 mM GSSG followed by a further incubation with 20 mM DTT to re-reduce any glutathionylated residues. Following desalting, the proteins were analyzed by ESI-TOF MS (Table III). The DTT-reduced proteins gave molecular masses within 1 Da of those predicted from the respective sequence after taking into account cleavage of the N-terminal methionine. Treatment of *At*DHAR1-his with GSSG increased the mass of all the polypeptide present by 306 Da, consistent with the formation of a single mixed disulfide with GSH. This was confirmed by demonstrating the displacement of the GSH following treatment with DTT. Similarly, the C6S mutant underwent reversible *S*-glutathionylation. In contrast, treatment with GSSG had no effect on the mass of the C20S mutant.

By having established the site of *S*-glutathionylation in *At*DHAR1-his at Cys-20, it was then of interest to define the stoichiometry of *S*-glutathionylation in the other cytosolic enzyme *At*DHAR2 and the putative plastidic form *At*DHAR3. When *At*DHAR2-his was *S*-glutathionylated as detailed for *At*DHAR1-his, the dominant peak showed an increased mass of 610.7 Da, as compared with the parent polypeptide (Table III). This shift in mass corresponded to the *S*-derivatization of the parent polypeptide with two molecules of GSH, and because *At*DHAR2-his has only two cysteine residues, both must have been modified. This double modification was rapid and complete, even at pH 5, suggesting that both cysteines were relatively reactive. Following reduction with 10 mM DTT, the majority of the *S*-glutathionylated *At*DHAR2-his was converted to the parent form. However, a proportion was converted to an intermediate form of molecular mass 305 Da larger than the parent, corresponding to a *At*DHAR2-his derivatized with a single GSH molecule. Treatment with lower concentrations of DTT (1 mM), followed by 10 mM iodoacetamide treatment, gave a polypeptide that was quantitatively singly *S*-glutathionylated and singly alkylated (as determined by ESI-TOF MS). Trypsin digestion fragmented this polypeptide between the 2 cysteine residues, and subsequent MS analysis showed that the fragment containing Cys-6 was *S*-glutathionylated, whereas the fragment containing Cys-20 was alkylated.

The *S*-glutathionylation studies with *At*DHAR3-his gave a rather different result. Rather than forming a GSH adduct, treatment with GSSG caused a shift in molecular mass of −2 Da (Table III). Subsequent treatment of the GSSG-treated protein with DTT restored the polypeptide to its original mass. This result was consistent with GSSG treatment promoting the formation of an intramolecular disulfide bond in *At*DHAR3-his. *At*DHAR3-his contains an additional cysteine (Cys-28) in close proximity to the active site Cys-25 (equivalent to Cys-20 in *At*DHAR1-his) which is not observed in the other *At*DHARs and which could account such disulfide formation (Fig. 1). *At*DHAR3-his was treated with iodoacetamide with and without a prior treatment with GSSG. Following desalting, the resulting protein was then analyzed by MS and also assayed for DHAR activity. Iodoacetamide treatment after incubation for between 15 s and 20 min with GSSG did not significantly reduce DHAR activity, and MS analysis showed that the major species present was *At*DHAR3-his with an intramolecular disulfide, some of which was also *S*-glutathionylated at the third cysteine residue. No evidence for iodoacetamide modification was found, showing that the intramolecular disulfide formed very rapidly on GSSG treatment. In contrast, iodoacetamide treatment of freshly DTT-reduced *At*DHAR3-his completely abolished DHAR activity and produced a polypeptide modified by 2 or 3 iodoacetamide alkylations as determined by MS (data not shown). Trypsin digestion of untreated *At*DHAR3-his and iodoacetamide-treated *At*DHAR3-his, which had been alkylated both with and without a pre-treatment with GSSG, allowed the sites of modification to be determined. In particular two tryptic fragments were of interest, an N-terminal fragment 1 containing Cys-11 and the adjoining fragment 2 containing an internal lysine residue resistant to cleavage, and Cys-25 and Cys-28. For fragment 1, masses 56 and 305 Da higher than the parent mass could be determined following treatment with iodoacetamide and GSSG/iodoacetamide, respectively. For fragment 2, iodoacetamide treatment gave a fragment 112 Da larger than predicted, indicative of double alkylation, but iodoacetamide treatment following GSSG treatment gave a fragment 2 Da smaller than predicted, indicative of an intramolecular disulfide bond. It was therefore concluded that GSSG treatment of *At*DHAR3-his caused partial *S*-glutathionylation of Cys-11 and disulfide bond formation between Cys-25 and Cys-28.

Cloning and Heterologous Expression of Arabidopsis Lambda Class GSTs—By using cDNA prepared from total

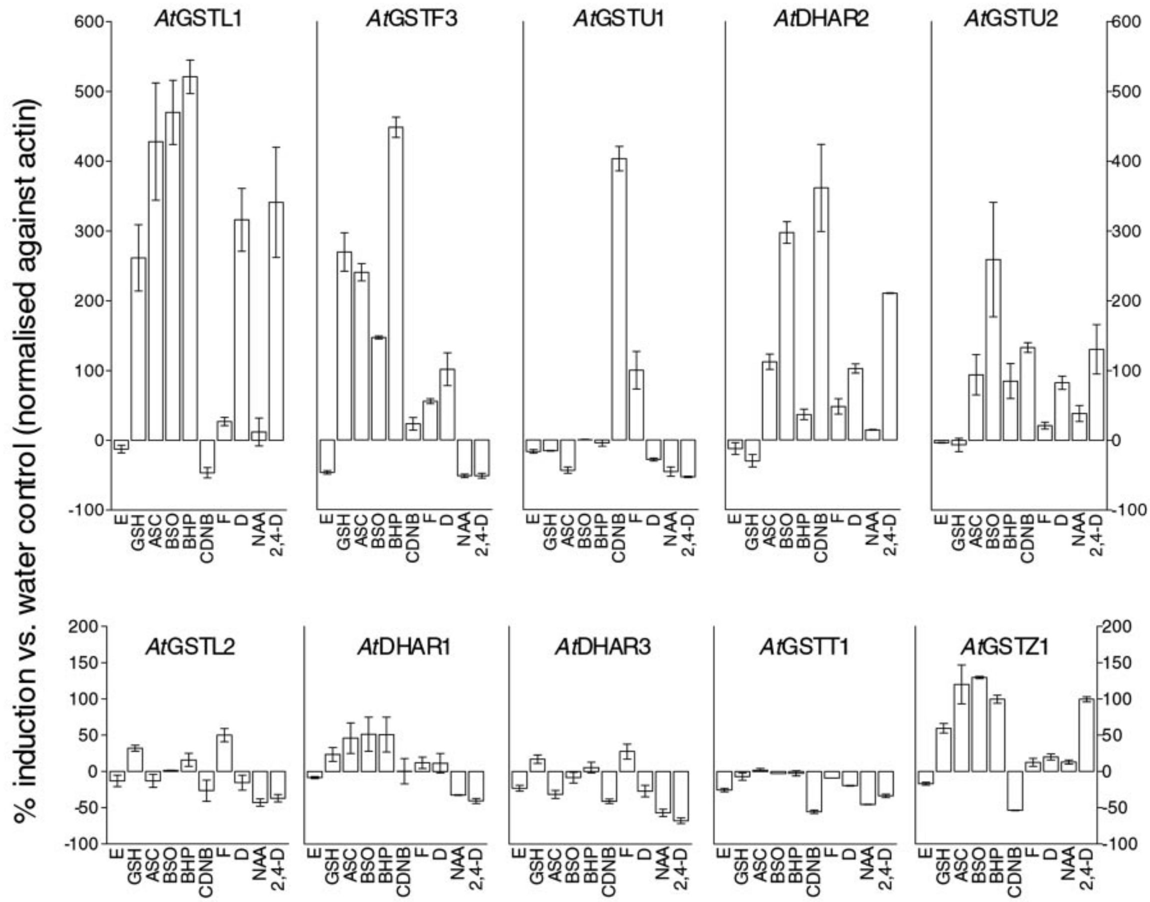

FIG. 4. **Induction of *At*DHARs, *At*GSTLs, and representatives of the Phi (GSTF), Tau (GSTU), Zeta (GSTZ), and Theta (GSTT) classes of GSTs in *Arabidopsis* root cultures treated for 24 h with compounds that perturb the redox environment or xenobiotics known to induce GSTs in cereals.** Chemical treatments were 1% v/v ethanol (*E*), 1 mM glutathione (*GSH*), 1 mM ascorbic acid (*ASC*), 1 mM BSO, 1 mM *tert*-butyl hydroperoxide (*BHP*), 0.4 mM CDNB, 0.2 mM fluorodifen (*F*), 0.1 mM dichlormid (*D*), 0.1 mM NAA, and 0.1 mM 2,4-dichlorophenoxyacetic acid (*2,4-D*). For each gene the % expression is represented relative to expression in cultures exposed to a control treatment with sterile water. Values are means of duplicated PCRs with the *error bars* showing the variation in the replicates.

RNA from whole *Arabidopsis* plants as a template, reverse transcriptase-PCR was used to amplify the respective coding sequences of *gstl1* and *gstl2* (Table I). The amplification products were then cloned into the pET-11d expression vector to give constructs pET-GSTL1 and pET-GSTL2. The *At*GSTL1 nucleotide sequence was identical to GenBank™ accession number NM_120356, whereas the *At*GSTL2 sequence was identical to bases 166–879 of GenBank™ accession number NM_115362, except for the introduced N-terminal methionine codon. Expression of both constructs in *E. coli* resulted in recombinant polypeptides of 28 kDa accumulating in the soluble fraction. In common with the recombinant DHARs, neither GSTL could be purified from the lysates using GSH affinity chromatography, so the *gstl* sequences were sub-cloned into pET-24d to express the respective *At*GSTL1-his and *At*GSTL2-his fusion proteins. Following nickel-affinity purification, gel filtration chromatography demonstrated that both proteins eluted as monomers under the conditions used, with relative molecular masses of 31 kDa for *At*GSTL1-his and 34 kDa for *At*GSTL2-his being determined. The purified preparations had no glutathione peroxidase or GSH conjugating activity toward the substrates tested and no DHAR or dichloroacetic acid dehalogenase activity. However, both *At*GSTLs were active as thiol transferases with HED as substrate (Table II).

Both *At*GSTLs contained a single cysteinyl residue, present at the putative active site (Fig. 2). When the GSTLs were incubated with iodoacetamide all thiol transferase activity was abolished. When the *At*GSTLs were incubated with GSSG and then analyzed by ESI-TOF MS, both polypeptides were found to undergo reversible mixed disulfide formation with a single molecule of GSH (Table III).

Expression of GSTs in Arabidopsis Root Cultures Exposed to Chemical Treatments—Sterile *Arabidopsis* root cultures were exposed to a range of chemical treatments, and semi-quantitative PCR was used to monitor the relative abundance of transcripts encoding the members of the *At*DHAR and *At*GSTL families as compared with that of representatives of the Phi (*At*GSTF3), Tau (*At*GSTU1, *At*GSTU2), Theta (*At*GSTT1), and Zeta (*At*GSTZ1) classes of *Arabidopsis* GSTs. Three groupings of chemical treatments were used. First, agents that directly perturb the redox potential in the cell, namely the oxidant *t*-butyl hydroperoxide (BHP), the reductants ascorbic acid (AA) and GSH, and the GSH synthesis inhibitor BSO. Second, cultures were treated with xenobiotics known to induce the expression of GSTs in cereals (14), namely the GST substrate 1-chloro-2,4-dinitrobenzene, the diphenylether herbicide fluorodifen, and the herbicide safener dichlormid. Finally, in view of the known inducibility of many plant GSTs to auxin treatment (3), the cultures were also exposed to 2,4-dichlorophenoxyacetic acid and NAA. To ensure comparability in the results, the intensity of the reverse transcriptase-PCR-amplified products was normalized against an internal standard, namely the PCR product derived from the *actin1* gene whose mRNA is reportedly constitutively expressed in *Arabidopsis* (25). In each case, significant induction was assumed when PCR indicated at least a doubling in *At*GST transcript abundance (Fig. 4).

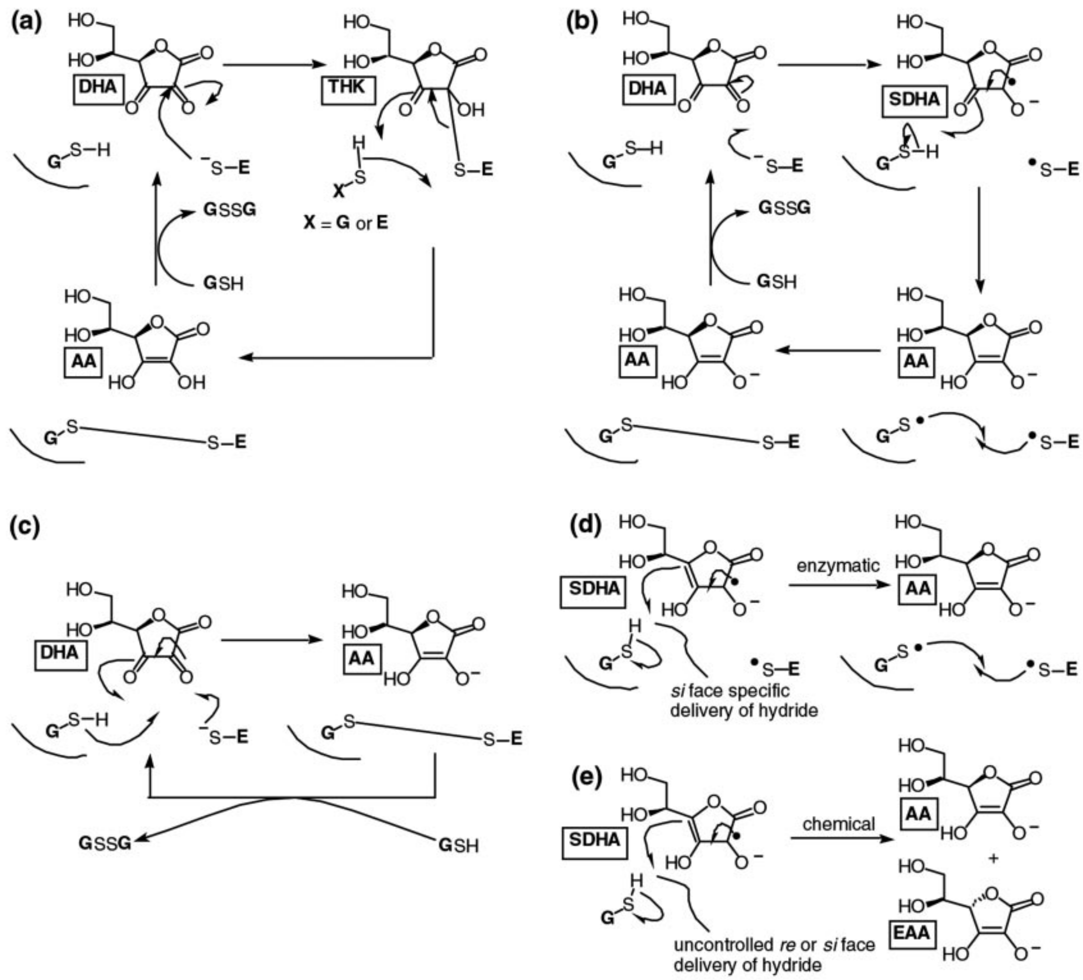

FIG. 5. **Possible catalytic mechanisms for DHAR.** *a*, the previously proposed DHAR mechanism of Wells and co-workers (33, 34) invoking the intermediacy of a thiohemiketal THK. *b*, proposed stepwise DHAR mechanism involving SET to give stabilized radical SDHA followed by hydride abstraction to give AA and mixed glutathione-enzyme disulfide. The enzyme is subsequently returned to its reduced active state by external GSH. *c*, a concerted alternative electron transfer mechanism. *d*, *si* face-specific hydride delivery to C-4 of the enolic tautomeric form of SDHA is another alternative 2nd step in the SET mechanism. *e*, the previously observed chemical reduction of SDHA to both AA and EAA by GSH (40) is consistent with nonspecific hydride delivery to both *re* and *si* faces of C-4 of the enolic tautomer of SDHA.

DISCUSSION

In addition to the existing Phi, Tau, Zeta, and Theta classes of GSTs, *Arabidopsis* also contains outlying members of the superfamily that have adopted new catalytic functions though the substitution of an active site serine to a cysteine (Cys-20 or equivalent). These GST-like proteins fall into two distinct groups based on sequence similarity. One group, the *At*DHARs, is indistinguishable from the dehydroascorbate reductases recently identified in rice and share the same functional activity (19). The other group classified as the new Lambda class of GSTs is similar to the *In2-1* gene (22) and its wheat homologue *Cla30* (23) previously identified as genes encoding proteins of unknown function that are induced by treatments with herbicide safeners (24). Although the two *Arabidopsis* Lambda GSTs did not show any DHAR or other activity normally associated with GSTs, these enzymes did have GSH-dependent thiol transferase activity, as did the *At*DHARs. Thiol transferase activity was also associated with the Omega class *h*GSTO1 protein which also contains a cysteine at its active site, though like the DHARs and GSTLs, the activity was modest as compared with that demonstrated with glutaredoxins (18). One potential function for a GSH-dependent thiol transferase would be to dethiolate-specific *S*-glutathionylated proteins that accumulate during oxidative stress (26). Such substrate-specific dethiolation would complement similar activities more normally associated with glutaredoxins, thioredoxins, and protein disulfide isomerases (27).

Although their activities as thiol transferases remain ambiguous, the *At*DHARs have important functions in ascorbic acid metabolism. DHAR (glutathione dehydrogenase (ascorbate), EC 1.8.5.1) catalyzes the GSH-dependent reduction of dehydroascorbate (DHA) to ascorbate (28), a reaction implicated in plant redox homeostasis for some time (29). However, such a DHAR has only recently been purified and cloned from rice (19). The distantly related *h*GSTO1 also possesses limited DHAR activity, although the significance of this in the metabolism of ascorbic acid in animal cells remains uncertain (18). In plants, DHA arises from the dismutation of semi-dehydroascorbate (SDHA), the major oxidized form of ascorbate with SDHA normally recycled back to ascorbate by an NAD(P)H-dependent SDHA reductase (29). However, if SDHA is allowed to accumulate then DHA accumulates, although the importance of this reaction *in planta* is the subject of some debate (30, 31). The isolation of DHARs predicted to be targeted to either the cytosol and chloroplast clarifies the compartmentalization of these enzymes in *Arabidopsis*. The DHAR cloned from rice was predicted to be a cytosolic protein (19), whereas a DHAR isolated and cloned from spinach was clearly plastidic (32), and

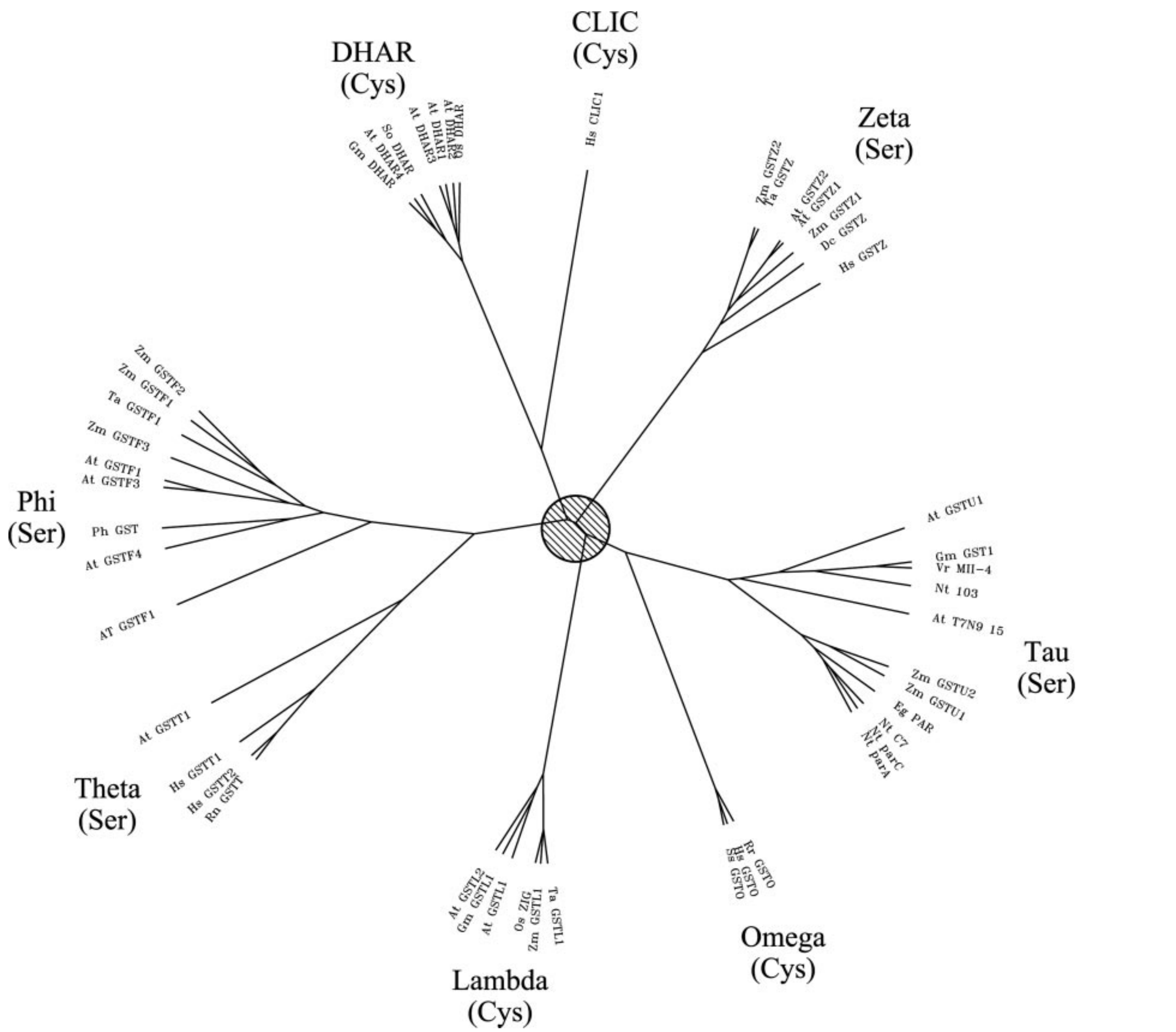

FIG. 6. **Dendrogram illustrating inferred phylogenetic relationship between plant GST classes and mammalian Theta class, Omega class, Zeta class, and chloride channel sequences.** Branches within the *central hatched circle* are not well supported. Residues in *parentheses* indicate the amino acid residue known or assumed to interact with the SH group of bound GSH for that class. GenBank™ data base accession numbers (or other source) for the sequences are as follows: Gm DHAR, AW509423; At DHAR1, AC024609; At DHAR2, AB026661; At DHAR3, AC025814; At DHAR4, AL391147; So DHAR, AF195783; Os DHAR, AB037970; Hs CLIC1, NM_001288; Zm GSTZ1 (U. S. patent US5962229); Zm GSTZ2 (U. S. patent US5962229); Ta GSTZ, AF002211; At GSTZ1, AC005312; At GSTZ2, AC005312; Dc GSTZ, M64268; Hs GSTZ, U86529; At GSTU1, D44465; Gm GST1, M20363; Vr MII-4, U20809; Nt 103, X56263; At T7N9 15, AC000348; Zm GSTU2, AJ010439; Zm GSTU1, Y12862; Eg PAR, U80615; Nt C7, X64399; Nt parC, X64398; Nt parA, D90215; Rr GSTO, AB008807; Hs GSTO, AF212303; Ss GSTO, AF188838; Ta GSTL1, Y17386; Zm GSTL1, X58573; Os ZIG, AF237487; At GSTL1, AL162973; At GSTL2, AL132970; Gm GSTL1 (U. S. patent US06063570); Rn GSTT, D38556; Hs GSTT2, Z84718; Hs GSTT1, Z84718; At GSTT1, AJ131580; At GSTF1, X68304; At GSTF4, D17673; Ph GST, Y07721; At GSTF3, D17672; At GSTF2, X75303; Zm GSTF1, X06754; Zm GSTF2, X79515; Zm GSTF3, AJ010295; Ta GSTF1, X56012.

two DHAR ESTs from *Medicago trunculata* (GenBank™ accession number AW694131) and soybean (GenBank™ accession number AW509423) also possess putative transit peptides. Biochemical evidence suggests that a significant proportion of DHAR activity resides in the plastid, acting to reduce the large amounts of ascorbate oxidized during hydrogen peroxide scavenging by ascorbate peroxidase (28).

Our studies with the recombinant *At*DHARs clearly show that these enzymes require a reduced thiol group for enzyme activity, a characteristic also observed with the DHAR purified from rice (32). The identification of the conserved Cys-20 as a catalytically essential residue in the *At*DHARs explains their sensitivity to thiol-derivatizing chemicals. This cysteine forms mixed disulfides with GSSG, which protect the enzyme from being inactivated by iodoacetamide. Such *S*-glutathionylation of enzymes protects essential cysteinyl residues from irreversible oxidation to the sulfinic acid and sulfonic acid derivatives during redox stress. However, in the case of *At*DHAR1 we propose that the mixed disulfide is also a key intermediate in the catalytic mechanism (Fig. 5). Based on the known chemistry of DHA, AA, and thiolates, we propose the mechanism shown in Fig. 5*b*, which is in contrast to previous proposals (33, 34), which invoked the intermediacy of an enzyme-DHA thiohemiketal (THK) intermediate (Fig. 5*a*). A parallel mechanism (not shown) involving a GSH-DHA-THK intermediate has also been proposed (35). Whereas thiols and thiolates are sufficiently nucleophilic to create such THKs, the subsequent attack of another thiol (*2nd step*, Fig. 5*a*) required by these mechanisms is without chemical precedent. Similarly, we were unable to demonstrate by ESI-TOF MS the presence of a THK enzyme-bound intermediate following incubation of the *At*DHARs with DHA (data not shown), and no evidence for such an intermediate has been reported in other studies. Indeed, studies with dione systems analogous to DHA (36, 37) suggest that the formation of a THK or its GSH-counterpart would instead proceed to the formation of a dead-end thioketal-enzyme complex.

As an alternative mechanism we propose a single electron transfer (SET) from the key active site Cys-20 (Fig. 5*b*) (38, 39). Such a SET process would create SDHA, a known intermediate which as a stabilized radical gives rise to the powerful antioxidant properties of AA. Subsequent hydride abstraction by SDHA from the thiol side chain of GSH is consistent with the well known ability of thiols to act as hydride donors in radical reactions and would result in the regeneration of AA. The resulting thiyl radicals GS˙ and ES˙ would then rapidly terminate to form the mixed disulfide ESSG, determined by ESI-TOF MS. The enzyme would then be returned to its reduced active state by reaction with additional external GSH. We have also considered a related concerted model as being an alternative pathway of catalysis (Fig. 5*c*). Support for the intermediacy of SDHA, or its enolic tautomer, may be found in the reduction of DHA by GSH in the absence of DHAR. This results in the formation of both AA and its stereoisomer, the C-4 epimer EAA (40). This observation is consistent with the SET chemical mechanism shown in Fig. 5*e* involving nonspecific delivery of hydride to C-4 at either face of the enolic tautomer of SDHA. Recently, Cu(I) and Fe(II), known SET reductants, have also been shown to reduce DHA to AA and EAA (41). Together these observations suggest a potential variation to the 2nd step of our proposed mechanism (Fig. 5*b*), where stereoselective delivery of hydride to the *si* face of the enolic tautomer of SDHA results in the formation of AA (Fig. 5*d*). This would suggest a potential additional evolutionary benefit of DHAR-mediated DHA reduction, control of the stereochemistry of reduction as well as enhanced rate of reduction. Similar reaction mechanisms to that proposed for the *At*DHARs may also drive catalysis in other GST-like proteins containing cysteinyl residues in their active sites. Although a catalytic mechanism was not proposed, the Omega class GST *h*GSTO1 was found to contain a disulfide-bound GSH molecule on the active site cysteinyl residue of the crystallized protein (18). Similarly, disulfide exchange reactions are used in the catalytic mechanism of tetrachlorohydroquinone dehalogenase, a bacterial GST-related enzyme (42).

Our studies also demonstrated that the *At*DHARs could undergo *S*-glutathionylation at non-active site cysteines. For example, *At*DHAR2-his underwent a second *S*-glutathionylation at Cys-6. The Cys-6-SG disulfide was more recalcitrant to reduction than the active site disulfide, consistent with the lower reactivity of this residue. However, the corresponding Cys-6 residue in *At*DHAR1-his did not undergo *S*-glutathionylation, suggesting that this residue is either more reactive or more accessible in *At*DHAR2-his than in *At*DHAR1-his. In the case of the plastidic isoenzyme, *At*DHAR3-his underwent rather different modifications when treated with GSSG, with an intramolecular disulfide bond formed between Cys-25 and Cys-28 and the N-terminal Cys-11 residue undergoing partial *S*-glutathionylation. Similar arrangements of active site cysteinyl residues are also seen in other plastidic DHARs (GenBank™ accession number EST AW509423 from soybean and EST AF195783 from spinach) but not in other isoenzymes. The functional significance of intramolecular disulfide formation in DHAR catalysis in the chloroplast is unknown but may relate to the redox conditions in the compartment.

Dendrograms based on sequence analyses show the evolutionary relatedness of plant DHARs and GSTLs to other GSTs (Fig. 6). Intriguingly, data base searches showed that the four *At*DHAR sequences from *Arabidopsis* were also significantly similar to the mammalian intracellular chloride channels, including nuclear chloride channel-27 from humans (43) and p64H1 from rats (44). Sequence alignments showed that whereas the chloride channels possessed insertions totaling 22 residues relative to *At*DHAR1, the GSH binding region was well conserved between the two predicted proteins (data not shown).

Although identified from BLAST searches as resembling the Omega GSTs, it was clear from their sequence divergence that the *Arabidopsis* Lambda GSTs belonged to a separate class (Fig. 6). Although not closely related in terms of sequence similarity to DHARs, the GSTLs shared a common thiol transferase activity as well as similar physical characteristics. Both classes of protein contained a cysteinyl residue at the active site which underwent *S*-glutathionylation and are expressed as monomers, rather than as dimers as is typically the case with GSTs. The *At*GSTLs also shared some structural similarities to the GSTOs (19), both proteins containing N-terminal extensions of unknown function. In terms of their expression, DHARs and GSTLs also had the common feature of being composed of isoenzymic forms which were directed to both the chloroplast and the cytosol, with one cytosolic form of each, *At*DHAR1 and *At*GSTL1, being markedly induced in response to conditions likely to invoke oxidative stress. In contrast transcripts encoding the chloroplastic forms of *At*DHARs and *At*GSTLs were constitutively expressed and unaffected by stress. Collectively, these observations point to *At*GSTLs and *At*DHARs having complementary functions, probably in counteracting oxidative stress in both the cytosol and chloroplast.

The differential regulation of the transcripts encoding the different classes of GSTs was also suggestive of the different functions of the members of the *At*GST superfamily. In maize both Phi and Tau class GSTs are well known to be responsive to treatments with compounds that serve as GST substrates or herbicide safeners, which are compounds that enhance herbicide-detoxifying enzymes in cereals (45). In *Arabidopsis*, the safener dichlormid was only effective in inducing *gstl1*, suggesting that this safener, which is used to increase tolerance to chloroacetanilide herbicides in maize by enhancing the expression of multiple Phi and Tau GSTs (45), was far less active as a safener in *Arabidopsis*. The induction of *gstl1* was not seen with the GST substrates CDNB and fluorodifen; instead these compounds were most effective in inducing the Tau GST *gstu1*, a gene shown previously (46) to be regulated by auxin. This result suggested that GST induction by safeners and xenobiotic substrates of GSTs must proceed by distinct recognition/signaling pathways. These xenobiotic/safener-responsive signaling pathways are in turn subtly different from the regulatory system that responds to feeding with GSH, AA, and BSO, which are all likely to perturb the redox potential of the cell. For example, *gstl1* was induced by the safener and by the "redox" treatments but not by the GST substrates; *dhar2* was selectively induced by the redox treatments and by CDNB but not by dichlormid; and *gstf3* was responsive only to the redox treatments. This differential enhancement of different GST classes gives further insight into the relationship between responses to oxidative and xenobiotic stress in plants. Previous studies have concentrated on the induction of single *Arabidopsis* GST genes, notably the Phi GST *gstf6*, which was found to be regulated by multiple stress treatments such as pathogen attack (47), dehydration (48), and a variety of environmental stresses and wounding (49). Analysis of the promoter of the *gstf8* gene has demonstrated the presence of multiple *ocs* enhancer elements sites that could help account for the differential of plant GSTs by multiple stresses (50).

Our studies further illustrate the extraordinary functional diversity of the GST family of proteins that is evident in both plants and mammals. The mechanisms of functional evolution have been proposed as arising from domain swapping and mutagenesis based around an ancestral structural fold responsible for binding GSH (51). In the case of the DHARs and GSTLs in plants, it is interesting that such diversification has occurred independently of the evolution of the Omega GSTs in mammals (18). It will now be of interest to determine which plant GSTs have independently evolved to fulfil roles that have counterparts in mammals and that carry out plant-specific functions.

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