Roles for Stress-inducible Lambda Glutathione Transferases in Flavonoid Metabolism in Plants as Identified by Ligand Fishing*

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The glutathione transferases (GSTs) of plants are a superfamily of abundant enzymes whose roles in endogenous metabolism are largely unknown. For example, the lambda class of GSTs (GSTLs) have members that are selectively induced by chemical stress treatments and based on their enzyme chemistry are predicted to have roles in redox homeostasis. However, using conventional approaches these functions have yet to be determined. To address this, recombinant GSTLs from wheat and Arabidopsis were tagged with a Strep tag and after affinity-immobilization, incubated with extracts from Arabidopsis, tobacco, and wheat. Bound ligands were then recovered by solvent extraction and identified by mass spectrometry (MS). With the wheat enzyme TaGSTL1, the ligand profiles obtained with in vitro extracts from tobacco closely matched those observed after the protein had been expressed in planta, demonstrating that these associations were physiologically representative. The stress-inducible TaGSTL1 was found to selectively recognize flavonols (e.g. taxifolin; $K_d = 25$ nM), with this binding being dependent upon $S$-glutathionylation of an active site cysteine. In the case of the wheat extracts, this selectivity in ligand recognitions lead to the detection of flavonols that had not been previously described in this cereal. Subsequent in vitro assays showed that the co-binding of flavonols, such as quercetin, to the thiolated TaGSTL1 represented an intermediate step in the reduction of the respective $S$-glutathionylated quinone derivatives to yield free flavonols. These results suggest a novel role for GSTLs in maintaining the flavonoid pool under stress conditions.

The glutathione transferase (GST) superfamily of proteins in plants can be divided into seven groupings based on sequence similarity, namely the phi, tau, zeta, theta, dehydroascorbate reductase (DHAR), lambda, and tetrachlorohydroquinone dehalogenase-like (TCHQD) classes (1). In the majority of cases, the endogenous roles of these proteins are unknown. Attempts to unravel the function of GSTs using reverse genetic approaches has only rarely proved successful (2), with even multiple knock-outs of related genes giving negligible perturbations in metabolic or physiological phenotype (3). The most plausible reason for this inability to disrupt phenotype is that at any given time, the expression of multiple GSTs with overlapping functions effectively masks the loss of function of individual members. Defining the roles for these abundant and stress responsive proteins using methods other than molecular genetics therefore represents a major challenge in functional genomics.

Whereas disrupting the expression of plant GSTs normally gives no measurable perturbation in metabolite contents in planta (3), these proteins do have the ability to bind natural products with high affinity. For example, we have determined that members of the tau (U) class of GSTs bind porphyrin intermediates and fatty acids derived from bacteria and plants both in vitro and in vivo (4–5). Whereas most of the ligands identified were substrate analogs rather than physiological binding partners, the studies did point to the potential of using advanced ligand-fishing approaches to identify physiological intermediates residing in the active sites of GSTs. Such bound entities could either represent substrates, reaction intermediates, or ligands being transported. The identification of these ligands could then provide a useful starting point for deducing the function of these proteins.

To test this ligand-fishing approach, we have looked for putative substrates of lambda (L) class GSTs. These GSTLs are one of the smaller groupings within the superfamily in plants and differ from the more numerous phi and tau GSTs involved in the classical glutathionylation of xenobiotics, in possessing a catalytic cysteine in place of the usual serine residue. The presence of this active site cysteine suggests that the GSTLs must use the co-substrate glutathione (GSH) to catalyze redox reactions, rather than acting to activate the thiol for nucleophilic substitution or addition reactions as is the case with the phi and tau enzymes. Consistent with roles in redox biochemistry, GSTLs catalyze GSH-dependent thiol exchange reactions with glutathionylated mixed disulfides in vitro (6). However, based on the activity of other well characterized GSH-dependent thiol transferases, this activity is unlikely to reflect their roles in vivo and an alternative stress-responsive function for these proteins is suggested, with cytosolic GSTLs strongly induced in Arabi-
dopsis, maize, and wheat in response to chemical treatments (6–8).

The primary GSTLs selected for ligand fishing screening in this study were the enzymes from wheat (*Triticum aestivum*). These *TaGSTLs* are of particular interest as some are strongly induced by safeners, which are a group of agrochemicals which enhance herbicide detoxification, and hence selectivity in this major crop (7–9). For example, the induction of the lambda GST originally termed cla30 and now renamed *TaGSTL1*, was among the strongest of all those differentially expressed genes determined in a subtractive library screen following treatment of wheat with the safener cloquintocet mexitil (7).

To identify the functions of these stress-inducible wheat proteins, the strategy adopted in the current study has been to clone and express affinity-tagged *TaGSTLs*, then immobilize the proteins and look for retained ligands after incubation with plant (tobacco, wheat) extracts. For comparison we have also utilized *AtGSTLs*, the homologous proteins previously identified in *Arabidopsis thaliana* (6). In each case, the ligands retained by these GSTLs have been identified by high resolution mass spectrometry, with compounds whose binding is dependent on the presence of GSH, being of particular interest.

**EXPERIMENTAL PROCEDURES**

**Enzyme Cloning and Expression**—The cDNA sequence of *TaGSTL1* was available from previous studies (7). Full-length coding sequences for the other family members, *TaGSTL2* and *TaGSTL3*, were deduced from assembled ESTs. In each case, the GSTLs were PCR-amplified from a wheat (cv. Hunter) seedling cDNA library (9). The primer pairs used were *TaGSTL1_Nde* (gcg cgc cat atg gcc gct gca gc) and *TaGSTL1_Sal* (gcg cgc gct gac agg cag cca ccc tta tcc), *TaGSTL2_Pac* (gcg cgc tta att cac cat atg gcc gca cca gc) and *TaGSTL2_Sal* (gcg cgc gct gac gcc tca tga gtt tct tcc tc), *TaGSTL3_Nde* (gcg cgc cat atg gcc acc gcc gtt cca cc) and *TaGSTL3_Xho* (gcg cgc ccc gag cca ttc cct gcc atg c), and *TaGSTL4_Pac* (gcg cgc tta att cac cat atg gcc gcc gtc tgt cct g) and *TaGSTL4_Sal* (gcg cgc gct gcc gca cca tcc tta gcc atc cca ag). Products were cloned after restriction enzyme digestion into the bacterial expression vector pET-STRP3 (10). Cloned *Arabidopsis* *AtGSTLs* were available from previous studies (10). In each case, the recombinant enzymes were expressed in *Escherichia coli* strain Tuner(DE3) pRARE and purified as described (10). Site-directed mutagenesis of *TaGSTL1*, to replace each of its component three cysteines (Cys-38, Cys-41, and Cys-53) with serine residues, was performed using overlap extension PCR with KOD DNA polymerase (Merck Chemicals Ltd., Nottingham, UK) and suitable oligonucleotides. For plant expression, sequences were subcloned into the BIN-STRP3 vector (10).

**Enzyme Assays**—GST activity toward xenobiotics was determined with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reduction of *S*-glutathionyl-glutathione was determined in 0.1 M KPO₄ pH 6.5 containing 5 mM DTT, using 16 μM substrate (from stock solution in sodium citrate, pH 3.5). The reaction was monitored by following the increase in A₃₅₀ due to quercetin formation (using ε₃₅₀ = 17.4 mM⁻¹ cm⁻¹). Similar assays were used to test for modification of 2′-S-glutathionyl-rutin, except that reaction products were analyzed by LC-MS.

**Ligand Fishing**—Recombinant *Strep*-tagged GSTLs were first immobilized on 1 ml of *Strep*-tactin Sepharose columns (Stratech Scientific Ltd., Soham, UK). Plant tissue was homogenized in 5 (v/w) 60% methanol and extracted at 20 °C for 30 min prior to clarification by filtration and centrifugation (10,000 × g, 20 min, 4 °C). The extract was then dried under vacuum. The resulting powder was re-suspended in HBS (20 mM HEPES-NaOH, 150 mM NaCl, 1 mM EDTA (pH 7.5) to a final concentration of 1 g of original tissue ml⁻¹, with a small amount of avidin added to complex any biotin present. The clarified extract was then either used directly, or after the addition of either 5 mM DTT, or 1 mM oxidized glutathione (GSSG) and 5 ml loaded onto the GSTL-affinity column. After washing, the GSTLs and associated ligands were eluted as described (5). For the *in vivo* capture of ligands, the *Strep*-tagged enzymes were transiently expressed in unwounded *Nicotiana benthamiana* leaves prior to affinity recovery and analysis (5).

Extracts containing recovered protein and bound ligands were concentrated to 50 μl using a Vivaspin 2 centrifugal concentrator (Sartorius Stedim, Epsom, UK). Protein was precipitated by centrifugation after the addition of 2 vol of methanol, with the supernatant analyzed by HPLC-MS, and the pellet dissolved in 50% acetonitrile, 0.5% formic acid prior to MS (5). In brief, ligand mixtures were resolved by reversed phase HPLC, with the eluate analyzed by photodiode array detection and electrospay mass spectrometry in series. For ions of interest, accurate mass and relative isotope abundance data were used to generate candidate molecular formulae. Subsequent MS/MS analyses and comparison with previously identified plant products enabled tentative identifications to be made that, where possible, were confirmed using authentic standards.

**Ligand Binding Analysis**—The binding affinity of *TaGSTL1* for chosen ligands was measured by isothermal titration calorimetry (ITC) in HBS buffer using a Microcal VP-ITC instrument (GE Healthcare UK Ltd., Amersham Biosciences, UK). In each case, 100 μM ligand was titrated into 10 μM *TaGSTL1* at 25 °C. Ligand concentrations were determined gravimetrically, while *TaGSTL1* concentration was calculated from its absorbance at 280 nm. Where additives (GSH, DTT, GSSG) were used, these were added to both protein and ligand. Thermal data for each injection peak were integrated and fitted to a one binding site model using the supplied Origin software.

**Synthesis of S-Glutathionylated Flavonoids**—Quercetin (in ethanol) and aqueous GSH were slowly mixed to final concentrations of 500 μM and 625 μM, respectively, in 20 mM Tris-Cl, pH 7.5, containing 625 μM H₂O₂, and 1 μM horse radish peroxidase (HRP). The reaction was allowed to proceed for 5–10 min until no further loss of yellow coloration was observed and the mixture applied to a Strata-X reversed phase SPE cartridge (Phenomenex, Macclesfield, UK). After washing with water, the conjugate was eluted with 25% methanol, concentrated by lyophilization and purified by semi-preparative reversed-phase C₁₈ chromatography. Rutin and taxifolin conjugates were pre-
Flavonoid Substrates of Lambda Glutathione Transferases

| TABLE 1 | Enzyme activities of TaGSTLs as thiol transferases towards HED, as glutathione transferases towards CDNB and as DHAR |
|----------------|-----------------------------------|
| Enzyme         | Activity towards substrate         |
|                | nkat/mg                           |
| TaGSTL1        | HED                               |
|                | 337 ± 9                           |
| TaGSTL2        | CDNB                              |
|                | 28 ± 1                            |
| TaGSTL3        | DHAR                              |
|                | 106 ± 2                           |
| TaGSTL1 C38S   |                                    |
|                | 449 ± 8                           |
| TaGSTL1 C41S   |                                    |
|                | 5 ± 0.5                           |
| TaGSTL1 C53S   |                                    |
|                | 322 ± 9                           |

pared essentially similarly, with the SPE purification step omitted. Purified compounds were analyzed by 1H NMR immediately after dissolution in D2O buffered either with 20 mM KPO4 pD 7.0 or sodium citrate pD 3.5 on a Varian VNMRS-700, with resonances reported relative to HDO at 4.75 ppm.

RESULTS

Identification, Cloning, and Expression of GSTLs—Using the sequence of TaGSTL1 = Cla30 (7) for homology searching, over 100 related sequences were identified in the full-length and EST datasets derived from Triticum species. The sequences clustered into 5 distinct clades, based on phylogenetic analysis of the DNA sequences. For each clade, ESTs were assembled to form the full-length contigs TaGSTL1 (clade 1, EBI accession Y17386), TaGSTL2 (clade 2, ESTs CJ684065, and CJ583943), TaGSTL3 (clade 3, CJ808737, CJ60389, CJ657451, CJ808410, and CJ552837) and TaGSTL4 (clade 4, CA502651 and CA486301). Clade 5 sequences were also identified, as exemplified by EST CA485755, but lacked sufficient sequence conservation for further cloning. The full-length contigs were PCR-amplified from wheat seedling cDNA. Products were obtained for TaGSTs L1, L2 and L3, but not for clade 4. In the case of TaGSTL2, the cDNA was recovered without the putative N-terminal chloroplast targeting sequence identified in the respective ESTs. TaGSTs L1, L2, and L3 were then cloned into the bacterial expression vector PET-STRP3 to generate N-terminal Strep II tag fusions (10). On sequencing (supplemental information), TaGSTL1 was found to be identical to Cla30 (7), while TaGSTL2 was essentially identical to EBI accession CJ718665, albeit missing five N-terminal alanine residues due to mispriming with an oligonucleotide designed to amplify the subset of ligands also found in the full-length sequence of TaGSTL1. TaGSTL3 was identical to EST CA486301. Clade 5 sequences were also identified, as exemplified by EST CA485755, but lacked sufficient sequence conservation for further cloning. The full-length contigs were PCR-amplified from wheat seedling cDNA. Products were obtained for TaGSTs L1, L2 and L3, but not for clade 4. In the case of TaGSTL2, the cDNA was recovered without the putative N-terminal chloroplast targeting sequence identified in the respective ESTs. TaGSTs L1, L2, and L3 were then cloned into the bacterial expression vector PET-STRP3 to generate N-terminal Strep II tag fusions (10). On sequencing (supplemental information), TaGSTL1 was found to be identical to Cla30 (7), while TaGSTL2 was essentially identical to EBI accession CJ718665, albeit missing five N-terminal alanine residues due to mispriming with an oligonucleotide designed to amplify a closely related sequence. TaGSTL3 was identical to EST CA486301 (C terminus from CJ552837). Each GSTL was expressed in E. coli as described (5) and purified.

Characterization of GSTL Activities—In the absence of known in vivo activities for GSTLs, potential side activities were assayed to help characterize the enzymes. In particular, GSTLs from Arabidopsis have previously been shown to possess thiol transferase activity (6). In addition, the dehydroascorbate reductase (DHAR) class of GSTs also contains an active site cysteinyl residue (6). It was therefore of interest to determine if the GSTLs also showed DHAR activity. Enzyme assays (Table 1) showed that all three native wheat enzymes were active as thiol transferases, confirming that these GSTLs can catalyze redox reactions. In contrast, only TaGSTL1 showed appreciable activity as a DHAR, suggesting that this was not the in vivo activity for GSTLs. Studies with the Arabidopsis GSTLs had identified the importance of an active site cysteine in the thioltransferase activity (6). Similarly, TaGSTL1 activities both as a thioltransferase and DHAR were abolished on the mutagenesis of the presumed active site cysteinyl residue to generate the C41S substitution (Table 1). Mutation of either of the two other cysteine residues present in the TaGSTL1 sequence did not abolish these activities. Uniquely, the C41S mutant possessed low but measurable glutathione transferase activity toward CDNB, an activity associated with phi and tau GSTs containing a serine active site residue, confirming the critical importance of this residue in determining enzyme function.

In each case, the MS-measured masses of purified TaGSTLs were as predicted from their coding sequences, except that each showed a varying degree of +305 Da modification. This addition was symptomatic of mixed disulfide formation with glutathione and termed thiolation (6). Whereas the majority of TaGSTL1 ionized as a mono-glutathionylated species, TaGSTs L2 and L3 showed near quantitative thiolation. This modification was reversed following treatment with 10 mM DTT in all cases. The availability of the Cys-Ser mutants of TaGSTL1 allowed for the dissection of the site of disulfide formation. Whereas both the C38S and C53S mutants showed a reduction in glutathionylation, mixed disulfide formation was only abolished in the case of the C41S substitution. The Cys-41 residue of TaGSTL1 was therefore the primary site of glutathionylation and the reactive catalytic residue in TaGSTL1.

Identification of TaGSTL Ligands—In previous studies (5), Strep-tagged GSTs were transiently expressed in N. benthamiana leaves and bound ligands then identified by HPLC-MS after extracting the affinity-purified fusion proteins. This method (in vivo ligand fishing) worked well but was relatively laborious and was limited to species such as N. benthamiana that allowed straightforward transient expression of recombinant proteins. To study the interaction between wheat GSTLs and their endogenous ligands a second, in vitro, method was therefore developed. Bacterially expressed GSTLs were immobilized on a Strep-tactin column and subsequently exposed to metabolite extracts from the plant species of interest. After allowing any ligands present to bind to the immobilized protein, the affinity-eluted protein-ligand samples were then analyzed as detailed for the in vivo method.

To look for GSTL ligands, both in vivo and in vitro ligand fishing methods were used, and their results subsequently compared. TaGSTL1 and its point mutants C38S and C41S were transiently expressed in N. benthamiana and affinity purified. Additionally, E. coli-expressed TaGSTL1 and TaGSTL1-C41S were immobilized on the Strep-tactin matrix and challenged with a metabolite extract from N. benthamiana to allow direct comparison with the in vivo experiment. When the bound ligands from the in vivo expression experiments were analyzed by HPLC-MS, the control extraction gave a very clean profile (Fig. 1A). TaGSTL1 extracts contained 5 UV absorbing peaks (1, 2, 3, 6, and 8) not observed in the controls, with the same subset of ligands also found in the TaGSTL-C38S mutant. In contrast, the TaGSTL-C41S mutant bound a simpler set of compounds with peak 7 being the only major ligand retained. This peak was subsequently identified as an artifact corresponding to the reagent HABA (4-hydroxyazobenzene-2’-car-
boxylic acid), which was used in the regeneration of the affinity columns. In all cases the yields of GSTL proteins were similar (Fig. 1, inset). Similar ligand profiles were obtained when the same proteins were exposed to the plant extracts in vitro (Fig. 1B), with some additional peaks observed (4 and 5) because of a higher overall sensitivity. Again, TaGSTL1 gave a much more complex profile than TaGSTL1-C41S.

The ligands present in the samples derived from both methods were then further analyzed by tandem MS which, coupled with UV spectral and accurate mass information allowed the compounds of interest to be tentatively identified (Table 2 and Fig. 2, supplemental Fig. S1). The compounds which were selectively retained by TaGSTL1, but not by TaGSTL1-C41S, notably peaks 2, 3, 4, 6, and 8, were of particular interest. These were all identified as flavonoids derived from kaempferol or quercetin. In the case of rutin (1), kaempferol-3-O-rutinoside (2), and taxifolin (3) the identities of these metabolites were confirmed by comparison with authentic standards.

TaGSTL1 Flavonoid Binding Is Sensitive to Enzyme Glutathionylation and Extends to Wheat-derived Ligands—The results obtained with the wild type and mutant enzymes demonstrated that Cys-41 of TaGSTL1 was critical for ligand binding. Because this residue was also the site of S-glutathionylation, the relationship between mixed disulfide formation and flavonoid binding was investigated. Binding studies with plant extracts were performed with TaGSTL1 that had been pretreated with either DTT (Cys-41 unmodified), or GSSG (Cys-41 glutathionylated). In each case, MS was used to confirm that the GST was modified as expected. The immobilized GSTLs were exposed to the plant extracts and bound ligands were identified by HPLC-MS. These plant extracts were obtained from plants treated with chemicals known to induce the expression of GSTLs to maximize the likelihood of identifying physiologically relevant ligands, based on the known strong induction of GSTLs under these conditions. Thus, extracts from safener-treated wheat shoots (7) and Arabidopsis plants sprayed with AgNO₃ (public microarray data) were tested. These studies revealed that extracts from different plant species gave very different results, but in all cases glutathionylated TaGSTL1 bound more chromophores than the fully reduced enzyme (Fig. 3). This result was clearly seen with N. benthamiana extracts where TaGSTL1, which had been pretreated with DTT bound hardly any metabolites compared with the high yields of ligands recovered with the GSSG-treated protein. With the wheat extracts, which are most likely to contain the physiological substrates/ligands for TaGSTL1, both DTT- and GSSG-treated enzymes bound a series of metabolites that were tentatively identified as tricin-derived flanonolignans (11). However, these compounds were not considered to be specific GSTL ligands as similar binding was observed with the unrelated GSTs AtGSTF2, AtGSTF6, and AtGSTU19 (data not shown). In contrast, GSSG-treated TaGSTL1, but not the DTT-treated protein, selectively retained a more polar group of

![FIGURE 1. Reversed phase HPLC analysis of solvent extracts from Strep-tagged TaGSTL1 and point mutants. A, GSTLs purified from transiently transformed N. benthamiana; B, GSTLs purified from E. coli and challenged with plant extract. Inset: SDS-PAGE analysis of purified proteins with M, molecular mass markers (sizes in kDa indicated), 1, TaGSTL1; 2, TaGSTL1-C38S; 3, TaGSTL1-C41S; 4, p19 control. Each experimental lane corresponds to 0.6 g of infiltrated tissue.](image-url)
Flavonoid Substrates of Lambda Glutathione Transferases

TABLE 2
Identification of metabolites isolated from TaGSTL1 binding experiments

| Compound | m/z (MH⁺) | Identification                                      |
|----------|-----------|-----------------------------------------------------|
| 1        | 611.159   | Rutin (= quercetin 3-O-rutinoside)                   |
| 2        | 595.161   | Kaempferol 3-O-rutinoside                            |
| 3        | 305.063   | Taxifolin (= dihydroquercetin)                        |
| 4        | 289.077   | Dihydrokaempferol                                    |
| 5        | 584.275   | Tricoumaroylspermidine?                              |
| 6        | 301.083   | Isokaempferide                                        |
| 7        | 243.080   | HABA (column regenerant)                              |
| 8        | 315.089   | Kaempferol dimethylether                              |
| 9        | 625.176   | Quercetin methylether-3-O-rutinoside                  |
| 10       | 565.117   | Unknown                                              |
| 11       | 639.204   | Unknown                                              |
| 12       | 569.201   | Unknown                                              |
| 13       | 616.291   | GSH + C₁₈H₂₈O₄ (oxylipin conjugate)                   |
| 14       | 618.307   | GSH + C₁₈H₃₀O₄ (oxylipin conjugate)                   |
| 15       | 600.290   | GSH + C₁₈H₂₈O₄ (oxylipin conjugate)                   |
| 16       | 355.101   | Chlorogenic acid                                      |
| 17       | 463.123   | Kaempferol methylether-O-hexoside                     |

Flavonoid Substrates of Lambda Glutathione Transferases—Having determined GSH-dependent binding of flavonoids with TaGSTL1, it was then of interest to determine if other GSTLs were similarly selective. The other wheat and Arabidopsis GSTLs were incubated with extracts from N. benthamiana and the presence of ligands determined under conditions where the proteins were either reduced or glutathionylated (supplemental Figs. S2 and S3). Only AtGSTL1 showed substantial flavonoid binding that was specific to glutathionylated enzyme, with an
**Abundant peak (17) consistent with being an O-methylether of kaempferol conjugated with a hexose sugar being selectively retained. Glutathionylated GSTL3 pulled down low yields of the hydrophobic flavonoid 8. In most cases, the only ions dependent on the enzyme glutathionylation state observed were the oxylipin-GSH conjugates similar to those found for TaGSTL1, which were only observed in the DTT-treated extracts. Other ligands identified included chlorogenic acid 16, which bound to TaGSTL3 independent of glutathionylation state and to AtGSTL2, where binding was enhanced with the glutathionylated form of the enzyme.

Flavonoid Binding by TaGSTL1 Requires Glutathionylation and Disrupts Enzyme Activity—Isothermal titration calorimetry (ITC) and enzyme assays were used to characterize the observed glutathionylation-sensitive binding of flavonoids to TaGSTL1, using the commercially available ligands taxifolin, kaempferol-3-O-rutinoside and rutin (supplemental Fig. S4, Table 3). For taxifolin, tight binding was observed in the presence of GSSG, with only weak interactions determined with the DTT-treated protein. Unexpectedly, GSH treatment of TaGSTL1 also promoted taxifolin binding. However, MS studies showed that TaGSTL1 was efficiently glutathionylated in the presence of GSH, presumably due to its partial conversion to GSSG through chemical oxidation. These results confirmed that the tight binding of flavonoids required glutathionylation, with a taxifolin:enzyme binding stoichiometry of 1:1 determined once the degree of TaGSTL1 glutathionylation was taken into account.

The effect of flavonoids on the enzyme (DHAR) activity of TaGSTL1 was then examined. At stoichiometric amounts (1 μM each) of TaGSTL1 and flavonoid (taxifolin, quercetin, luteolin or apigenin), an 80% inhibition of DHAR activity was observed. With rutin and the isoflavone formononetin, inhibition was limited to 50%. At higher concentrations of flavonoids (10 μM), inhibition was near-quantitative in all cases. The high ligand binding affinity coupled with the requirement for high concentrations of protein to measure activity meant that meaningful \( K_r \) values could not be calculated, although the observed inhibition was consistent with the binding observed by ITC for the glutathionylated enzyme.

GSTLs Reduce Glutathionylated Flavonoids—Because TaGSTL1 only tightly bound flavonoids if its active site Cys-41 residue was glutathionylated, this suggested that this ternary complex could be a trapped intermediate in catalysis. This together with the known GSH-dependent reductase activity of the enzyme suggested a potential redox role for the enzyme directed toward flavonoid derivatives. Potential substrates were the known glutathione conjugates of flavonoids, which are formed from the respective quinones reacting with GSH. To evaluate such compounds as substrates, quercetin was incubated with GSH at neutral pH in the presence of horseradish peroxidase (HRP). Based on the available literature, we expected to obtain quercetin quinone glutathionylated in its A-ring (12). However, analysis of the 2 reaction products by a combination of \(^1\)H NMR at pH 3.5 (supplemental Fig. S5), MS, and UV spectroscopy was instead consistent with the formation of diastereomeric C-ring conjugates (12). NMR of the same compounds at neutral pH gave spectra closely matching those of the described A-ring conjugates (13) except for the presence of an additional A-ring proton (supplemental Fig. S5).

The two synthesized C-ring glutathione conjugates of quercetin (Q-SG1 and Q-SG2) were tested as GSTL substrates. HPLC and spectrophotometric assays showed that both AtGSTL1 and TaGSTL1 reduced both Q-SG1 and Q-SG2 to quercetin using DTT as reductant, but showed a marked preference for Q-SG1 (Table 4). The C38S and C53S point mutants of TaGSTL1 also possessed this activity, while the C41S mutant was inactive. The remaining GSTLs also showed good activity, but did not show a significant preference between the two substrates. Whereas TaGSTL1 efficiently acted on the C-ring conjugates, the enzyme was inactive toward the similarly synthesized B-ring mono-glutathionylated derivative of taxifolin. Similarly, oxidation of rutin in the presence of GSH gave a compound identified as the B-ring conjugate 2’-S-glutathionyl-rutin that was not modified by any of the wheat GSTLs.
DISCUSSION

Whereas GSTLs have been identified as chemically inducible proteins in cereals and Arabidopsis, to date their functions have been unknown (1, 7–8). However, it has been suggested that they could perform redox roles due to the presence of a catalytic cysteinyl residue (1). In this study we have used Strep affinity-tagging of the inducible TaGSTL1 to identify natural ligands present in crude plant preparations and identified a group of flavonoids whose binding is dependent upon the glutathionylation of an active site cysteine residue. Intriguingly, the sensitivity of this ligand fishing approach has revealed the presence of a group of flavonoids in wheat which had not previously been described in this species. The specific requirement for the glutathionylation of the catalytic cysteine to promote this binding, compounded by the observation that incubation with the flavonoids resulted in the loss of the DHAR activity of the GSTLs made it reasonable to assume that these interactions were occurring at the active site of the enzyme. Flavonoids are well known to bind to a wide range of proteins and in many cases this can lead to an inhibition in their enzyme activity (14). However, while it is unlikely that all the flavonoids we have identified binding to wheat TaGSTL1 are true in vivo ligands, the identification of flavonols, which can undergo S-glutathionylation in the course of their metabolism does point to a potential functional role for these enzymes in reducing such adducts to release the free flavonol. In this respect, we hypothesize that the observed binding of a flavonoid within the active site in immediate proximity to the catalytic S-glutathionylated cysteine residue effectively represents an intermediate enzyme substrate complex. By way of confirmation, we could demonstrate that TaGSTL1 catalyzed the GSH-dependent reduction of an S-glutathionylated quinone derivative of quercetin, resulting in the release of the free flavonol in a scheme shown in Fig. 4. Under oxidizing conditions, flavonols such as quercetin are readily converted to reactive quinone intermediates which spontaneously react with glutathione to form adducts (15). Though flavonols are most commonly observed in plants as their respective glycosides, the inherent reactivity of the non-conjugated intermediates such as quercetin and the relative abundance of GSH in the cell does make the formation of the respective quinone conjugates very likely under oxidizing conditions. In animal cells, the formation of such quinones and the subsequent conjugation reactions have attracted attention from toxicologists due to the ability of the intermediates to form covalent adducts with cellular components (12). In the case of glutathionylation, such conjugation cannot be considered to be an effective route of detoxification, as for example quercetin adducts can spontaneously break down to re-release a reactive benzofuranone derivative (12). Instead, we speculate that by catalyzing the GSH-dependent reduction of the quinones, GSTLs effectively accelerate the removal of potentially toxic intermediates and help restore the antioxidant flavonol pool. Whereas a role for TaGSTL1 in detoxifying oxidatively damaged flavonoids, or chemically equivalent natural products, has yet to be functionally demonstrated in planta, it is consistent with the enzyme chemistry of the protein and its regulation. Thus, TaGSTL1, which showed such flavonol binding and reductive activity, is strongly up-regulated in wheat plants following treatment with safeners which both mimic oxidative stress and cause major perturbations in the flavonoid pool (16). Similarly, AtGSTL1, which also shared this binding and activity, was also strongly induced by chemical treatments in Arabidopsis (6, 17).

The observed flavonoid deglutathionylation reaction, although unusual, is not without precedent. For example LigG, a GST from the lignin-degrading bacterium Sphingomonas paucimobilis, was shown to possess “glutathione lyase” activity, removing GSH from α-glutathionyl-β-hydroxypropiovanillone (18). Similar activities have been demonstrated for aromatic conjugates, for example a purified enzyme from the fungus Phanerochaete chrysosporium with GST-like characteristics catalyzed “glutathione conjugate reductase” activity on S-glutathionyltrichloro-1,4-hydroquinone (19), and a similar “glutathionyl-hydroquinone lyase” activity is catalyzed by the GST-like enzyme PcpF from Sphingobium chlorophenolicum (20). Finally, the human omega GSTO1 can catalyze the conversion of S-(4-nitrophenacyl)glutathione to 4-nitroacetophenone.
(21). Where known, all these GST-like proteins have a reactive cysteine residue, a feature shared by GSTLs.

These studies showing GSTLs acting on glutathionylated flavonoid adducts shed new light on the nature of these reaction products and their selective use by TaGSTL1. Glutathione conjugates were formed with the flavonols quercetin and taxifolin, giving C-ring and B-ring adducts respectively (12). Whereas TaGSTL1 was able to reduce the quercetin conjugates, this was not the case with the taxifolin derivative, suggesting that the enzyme was specific for C-ring adducts. In the case of quercetin, two diastereomer conjugates were determined and termed Q-SG1 and Q-SG2. Unlike other family members, TaGSTL1 (and AtGSTL1) preferentially reduced Q-SG2 over Q-SG1, demonstrating high selectivity of the enzyme for its flavonol substrate. With respect to the Q-SGs, earlier literature described the conjugates of quercetin and related flavonoids as being A-ring adducts (13). However, our current results suggest this original assignment is not well supported, with the observed addition of water and large hypsochromic shift in absorption spectrum on conjugation being inconsistent with A-ring modification. Instead, we propose the spectral data are consistent with C-ring rearrangement and hydration followed by GSH addition. Such an oxidative rearrangement has been well characterized in the absence of GSH (22). Similarly, 1H NMR data for the Q-SG1 and Q-SG2 conjugates (13) is not inconsistent with re-assignment as the two diastereomic C-ring conjugates previously characterized as forming under acidic conditions (12). In particular the presented NMR spectra in the original report were truncated, such that the expected second H6/H8 proton would be missed. A signal from this proton may also have been at least partially quenched due to acid-promoted H/D exchange at H8 (23); indeed a reduction in signal of around 20% was observed for this proton in our hands and it is feasible that differences in preparation could increase this exchange. In other respects these spectra closely match those for Q-SG1 and Q-SG2 at neutral pD presented here, while the Q-SG spectra at pD 3.5 match those for the C-ring conjugates at pH 3.5 (12). Many of the published assignments of flavonoid-GSH conjugates as A-ring conjugates have been based on the precedent discussed (13) and should now be re-evaluated.

Whereas these studies pointed to potential functions for the stress-inducible TaGSTL1 in wheat and the homologous AtGSTL1 in Arabidopsis, the functions of other family members were less clear-cut. Interestingly, although TaGSTL2 and TaGSTL3 did not bind flavonoids in the ligand fishing experiments, both proteins were able to efficiently reduce the quercetin conjugates, showing no preference for one diastereomer over the other. Based on this lack of specificity and low binding affinity for flavonols, we speculate that while these other GSTs may have comparable deglutathionylating activities to TaGSTL1, they are more likely to act on non-flavonoid natural product substrates. The glutathionylation-specific binding of chlorogenic acid to AtGSTL2 suggests that other catechol-containing metabolites may become GSTL substrates on oxidation. The identification of such inherently unstable metabolites in planta is a major challenge. We are therefore concentrating current studies on the effect of disrupting GSTL expression in wheat on the turnover of redox-sensitive natural product pools under stress conditions.

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