The Arabidopsis phi class glutathione transferase AtGSTF2: binding and regulation by biologically active heterocyclic ligands

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Synopsis
The plant-specific phi class of glutathione transferases (GSTFs) are often highly stress-inducible and expressed in a tissue-specific manner, suggestive of important protective functions. To date, these functions remain unknown, although roles in the binding and transport of reactive metabolites have been proposed. Using a sensitive and selective binding screen, we have probed the Arabidopsis thaliana GSTFs for natural product ligands from bacteria and plants. Uniquely, when overexpressed in bacteria, GSTF2 and GSTF3 bound a series of heterocyclic compounds including lumichrome, harmane, norharmane and indole-3-aldehyde. When screened against total metabolite extracts from A. thaliana, GSTF2 was also found to selectively bind the indole-derived phytoalexin camalexin as well as the flavonol quercetin-3-O-rhamnoside. In each case, isothermal titration calorimetry revealed high affinity binding (typically $K_d < 1 \mu M$), which was enhanced in the presence of glutathione and by the other heterocyclic ligands. With GSTF2, these secondary ligand associations resulted in an allosteric enhancement in glutathione conjugating activity. Together with the known stress responsiveness of GSTF2 and its association with membrane vesicles, these results are suggestive of roles in regulating the binding and transport of defence-related compounds in planta.
Introduction

In plants, glutathione transferases (GSTs; EC 2.5.1.18) are a diverse group of proteins, with the respective super-families described in Arabidopsis thaliana [1], rice [2, 3], poplar [4], maize and soybean [5]. Plant GSTs exist in seven distinct subfamilies, termed the phi (F), tau (U), theta (T), zeta (Z), lambda (L), dehydroascorbate reductase (DHAR) and TCHQD classes [1, 6]. Whereas the DHARs and GSTZs have roles in ascorbic acid and tyrosine metabolism respectively [6], the endogenous functions of the other classes, notably the large and plant-specific groups of phi and tau GSTs, are poorly understood [7]. This is in contrast to the known roles of GSTFs and GSTUs in catalysing the conjugation of xenobiotics with the tripeptide glutathione (GSH), with the resulting detoxification determining the selectivity of several major herbicides [8].

Using Arabidopsis as a model system, the functional genomics of the GSTFs and GSTUs are currently the subject of considerable interest. Proteomic and transcriptomic studies in Arabidopsis plants and cultures have shown that these proteins accumulate in response to xenobiotics [9, 10], plant hormones [11], infection [12, 13], as well as illumination and environmental stress [14]. In the few instances where progress has been made in defining function, the Arabidopsis AtGSTs have been found to be involved in signalling and transport, rather than in conjugating natural products. For example, AtGSTF12 is involved in anthocyanin and proanthocyanidin accumulation [15], while AtGSTU20 modulates responses to light reception [16]. Based on these observations, the latest evidence suggests that plant GSTs have evolved regulatory non-catalytic functions, which are most likely an extension of their ability to selectively bind biologically active ligands. For example, tau class GSTs isolated from Arabidopsis and maize (Zea mays) have recently been shown to selectively bind fatty acid and porphyrin natural products respectively. When AtGSTUs were incubated with plant extracts, family members bound oxidized fatty acid derivatives in a protein-ligand specific manner [17]. Binding partners included unstable oxylipins with known roles as stress signalling agents [18, 19]. Similarly, the expression of maize ZmGSTUs in the chloroplasts of Arabidopsis plants resulted in the accumulation of porphyrins due to these proteins binding unstable porphyrinogen precursors [17, 20]. In both cases, similar types of metabolites were found to hyper-accumulate in bacteria when these same enzymes were over-expressed in Escherichia coli. Thus, when ZmGSTU enzymes were over-expressed in E. coli, the bacteria accumulated porphyrin intermediates in a de-regulated manner, due to their
binding of the heme precursor harderoporphyrinogen [20]. When the Arabidopsis tau AtGSTUs were over-expressed in E. coli, a range of unusual oxidised fatty acids were determined, which showed similarities in terms of chain length and oxygenation to the metabolites identified in binding studies with plant extracts [17]. These studies have demonstrated that a combination of determining metabolite perturbation following GST expression in bacteria and the characterisation of bound ligands on incubation with plant extracts are powerful and complementary approaches in identifying classes of chemicals which are selectively recognized by these proteins. Importantly, the identification of binding partners by ‘ligand fishing’ can determine new functions for GSTs. For example, using such approaches we have recently identified a sub-set of flavonols as specific binding partners of Arabidopsis and wheat lambda GSTs that are associated with a novel glutathionyl-flavonol reductase activity [21].

Using the combination of metabolic perturbation following bacterial expression and ligand fishing using plant extracts we now report on the identification of a new set of binding partners of the Arabidopsis phi class protein AtGSTF2. This GST has been identified in a range of studies as being regulated by pathogen attack, plant hormones, heavy metals and xenobiotics [10, 12, 22]. In contrast, to date its functional roles have remained unknown, despite its strong association with plant stress responses.

**Experimental**

*Materials*

Camalexin was prepared as described [23], with its properties agreeing with those previously determined [24]. 1-Acetyl-β-carboline was synthesised from tryptophan and methylglyoxal [25].

**GST cloning, expression, purification and ligand analysis**

Constructs for the bacterial expression of N-terminally Strep-tagged AtGSTs were synthesised and expressed in E. coli as described previously [1], with any disruption to metabolism in the cultures determined by HPLC-MS as described [17]. Site-directed mutagenesis was performed using overlap extension PCR and appropriate mutagenic oligonucleotide primers. Strep-tagged proteins were purified using standard procedures [1]. For expression of untagged GSTF2, the coding sequence was excised from pET-STRP3 with Ndel and SalI, and ligated into pET-24a (Novagen) Ndel and XhoI sites, generating pET24a-F2. Untagged GSTF2 was expressed as for tagged proteins and purified from soluble extracts using Orange A agarose (Millipore), with elution using 5 mM GSH in Hepes-buffered saline (HBS; 20 mM Hepes–NaOH, 150 mM NaCl, 1 mM EDTA, pH 7.5). To remove GSH and any bound ligands, purified AtGSTF2 was buffer exchanged into HBS acidified to pH 5.5 with acetic acid then repeatedly concentrated by ultrafiltration prior to dilution with acidified HBS. Finally, AtGSTF2 was desalted into the required buffer by gel filtration through a HiTrap desalting column (GE Healthcare). As an alternative method, AtGSTF2 was extensively dialysed against HBS prior to use.
Enzyme and ligand binding assays

GST activity was determined with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [26]. GST(CDNB) assays in the presence of ligands were performed using 1 mM GSH in Hepes-buffered saline (HBS; 20 mM Hepes, 150 mM NaCl, 1 mM EDTA pH 7.6) for consistency with the binding studies. Isothermal titration calorimetry (ITC) experiments were performed at 25 °C in HBS buffer and analysed using a VP-ITC instrument with Origin 7.0 software (GE Healthcare). In addition to titrating ligand into protein, control titrations of ligand into buffer and buffer into protein were performed. Ligand concentrations were determined from absorbance values using extinction coefficients which were empirically determined for the ligands in HBS as \( \varepsilon_{348}(\text{norharmane}) = 3.98 \text{ mM}^{-1} \), \( \varepsilon_{348}(\text{harmane}) = 4.44 \text{ mM}^{-1} \), \( \varepsilon_{352}(\text{lumichrome}) = 9.13 \text{ mM}^{-1} \) and \( \varepsilon_{317}(\text{camalexin}) = 1.23 \text{ mM}^{-1} \). For other ligands, literature extinction coefficients were used, while the concentration of GSH was determined gravimetrically. The content of Strep-AtGSTF2 protein was based on a calculated \( \varepsilon_{280} \) of 20.34 mM\(^{-1}\).

Gel filtration was performed using a Superdex 200 10/30 GL column (GE Healthcare), with HBS plus 2.5 mM DTT, and 1 mM GSH as running buffer at a flow rate of 0.5 ml/min. The effect of ligands on the elution of AtGSTF2 was tested by adding 10 µM lumichrome to the running buffer. Protein elution was monitored by determining the absorbance at 280 nm, after calibrating the column with aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen and ribonuclease A.

Results

Ligand binding to AtGSTFs in E. coli

As an extension to our previously reported studies with the AtGSTUs [17], all the members of the AtGSTFs that expressed as soluble Strep-tagged recombinant proteins (AtGSTs F2-F10 and F14) were transformed into E. coli as described previously [1]. After growth under inducing conditions to stationary phase, the bacteria were harvested and solvent-extracted prior to analysis by HPLC, coupled with UV and MS detection. Unlike the GSTUs, the expression of GSTs F4, F5, F6, F7, F8, F9, F10 and F14 did not give any major perturbations in metabolic profiles as compared with the control bacteria. In contrast, examination of the metabolome profiles from E. coli expressing AtGSTF2 [AGI code: At4g02520] and AtGSTF3 [AGI code: At2g02930] revealed an identical group of five novel UV-absorbing peaks (1 to 5; Fig. 1A), which were not observed in the extracts from the control bacteria. In each case, these novel metabolites were tentatively identified by tandem and accurate mass electrospray MS as N-containing heterocyclic natural products (Fig. 2); namely norharmane (1), harmane (2), lumichrome (3), indole-3-aldehyde (4) and 1-acetyl-β-carboline (5). Identities were subsequently confirmed by comparing their spectroscopic properties with those of authentic standards. To distinguish between metabolite accumulation due to a selective binding to the GST and perturbations caused by a non-specific ectopic protein expression, Strep-tagged AtGSTF2 and AtGSTF3 were affinity-purified from E. coli and then extracted with methanol and analysed for co-purifying ligands. Compounds 1
to 4 were recovered bound to both AtGSTF2 and AtGSTF3 (Fig. 1B,C), showing that their accumulation was due to their selective associations with the respective proteins. In view of the identical ligand binding profiles obtained with the two Arabidopsis GSTFs, further detailed characterization was focused on AtGSTF2, which on the basis of its known regulation and stress inducibility was the best characterized of the two proteins [12].

Identification of Arabidopsis ligands of AtGSTF2
To complement and extend the bacterial screening, plants were screened for compounds that bound strongly to AtGSTF2. Two global metabolite profiling approaches were adopted, namely an in vivo method where the Strep-tagged enzymes were plant-expressed and bound ligands identified after affinity purification of the proteins and an in vitro method where the bacterially-expressed, purified enzymes were incubated with plant extracts [21]. In both cases, a Strep-tagged AtGSTU19 protein was used as a 'ligand-fishing' control. For in vivo ligand fishing, Strep-tagged AtGSTF2 was stably expressed in Arabidopsis, but poor expression levels hampered protein and ligand recovery. As a result, this method was not pursued further. Instead the in vitro method was employed, with extracts from Arabidopsis passed over affinity-immobilised AtGSTF2, which had been purified from bacteria grown in M9 medium (see below). As a further control, an additional phi-class enzyme (AtGSTF6) was included. This approach readily identified plant-derived ligands of AtGSTF2 that were not ligands of AtGSTU19 (Fig. 3). Surprisingly, AtGSTF6 pulled down a near-identical profile of ligands to AtGSTF2. In particular, the abundances of two compounds 6 and 7 (Fig. 2), were enhanced at least 100-fold on binding to AtGSTF2 and AtGSTF6 compared with their retention by the AtGSTU19 control. Compound 6 had a distinctive UV/Vis absorbance spectrum and was tentatively identified as the well known Arabidopsis flavonoid quercetin-3-O-rhamnoside (quercitrin). Compound 7 had a much weaker UV signature that nevertheless gave abundant ions by MS, and was tentatively identified as the indole-derived antifungal phytoalexin camalexin. The identities of both compounds were subsequently confirmed by comparison with authentic standards. AtGSTU19 previously bound metabolites from tobacco [17] and also specifically bound Arabidopsis metabolites that the AtGSTFs did not, though the characterisation of these is beyond the scope of this report.

AtGSTF2-ligand binding studies
ITC was used to examine the affinity and stoichiometry of binding of the ligands identified in E. coli and Arabidopsis to AtGSTF2. In preparing the proteins for ITC studies, it became apparent that when the Strep-tagged AtGSTF2 was purified from E. coli under standard conditions, the protein could not be used in titration assays, due to the high levels of pre-bound ligands. To minimize the likelihood of such contamination, AtGSTF2 was expressed in bacteria grown in M9 minimal broth instead of the normal ‘rich’ medium used, to reduce the availability of preformed indole compounds. In an alternative approach, when AtGSTF2 was purified from bacteria grown in rich medium, the protein was either extensively dialysed prior to use, or acidified to pH 5.5 with acetic acid to precipitate the protein and release associated ligands, prior to rapid desalting.
and renaturation. Binding studies with lumichrome, norharmane and harmane showed that all three ligands bound with high affinity to AtGSTF2, with the association being strongly promoted in the presence of 1 mM GSH (Table 1). However, increasing the concentration of GSH to 5 mM did not further enhance ligand binding. In contrast, indole-3-aldehyde bound with more moderate affinity, while other N-containing heterocyclic molecules including riboflavin, kinetin, trans-zeatin, indole-3-acetic acid, harmine and tetrahydroharmane carboxylic acid showed no obvious binding by ITC. To rule out any effect of the Strep-tag on ligand association, untagged native AtGSTF2 was purified from recombinant bacteria using Orange A affinity chromatography [27]. When assayed by ITC with lumichrome, binding to the native protein was essentially identical to that observed with the Strep-tagged form, confirming the affinity tag did not affect the results obtained. It was then of interest to determine the stoichiometry of ligand binding to AtGSTF2, which normally associates to form homodimers [28]. In the case of norharmane, harmane and indole-3-aldehyde each ligand bound in an approximately 1:1 ratio with the AtGSTF2 polypeptides, consistent with one molecule of ligand binding per GST monomer. In contrast, lumichrome bound to AtGSTF2 with a lower stoichiometry (0.36:1). To examine the possibility that this binding-ratio arose from AtGSTF2 forming trimeric or other unexpected oligomers, the protein was analysed by gel filtration chromatography in the presence and absence of lumichrome. In both cases, AtGSTF2 eluted as a single peak immediately following an ovalbumin standard (43 kDa), consistent with the protein remaining as a dimer on ligand binding. It was concluded that a single lumichrome molecule was binding per protein dimer, although the determined stoichiometry was sufficiently low that inaccuracies in determining active protein and ligand concentrations could not satisfactorily account for the deviation from the expected 0.5:1 ratio for associations with a dimer.

Further binding studies with AtGSTF2 focused on the optimal ligands harmane and lumichrome, and the effect of their binding on GST activity towards the model substrate CDNB. In both cases stoichiometric binding by each ligand enhanced the rate of CDNB conjugating activity by 60%. Kinetic analysis showed the addition of harmane (52.9 µM) did not cause a major decrease in substrate binding affinity, with the apparent Kₘ toward GSH being unchanged (15.5 µM), while the apparent Kₘ toward CDNB increased slightly from 1.6 mM to 2.4 mM. Instead, the observed activation was due to an increase in turnover (k₉) in each case. There was no obvious explanation for this non-competitive activation, but possibilities include enhanced catalysis, a reduction in the binding affinity for the dinitrobenzylglutathione product of CDNB conjugation, or displacement of an unobserved non-competitive inhibitor from the enzyme active site. The observed increase in apparent Kₘ(CDNB) pointed to decreased product binding being the most likely explanation. To confirm that the association of these ligands with the protein did not involve covalent modification, electrospray mass spectrometry of intact, ligand-treated AtGSTF2 was performed. These experiments demonstrated that the protein had not undergone any irreversible modifications.

The binding of other ligands to AtGSTF2 was then examined. Firstly, the effect of lumichrome and GSH on the binding of harmane to AtGSTF2 was
investigated using ITC (Table 1). The presence of lumichrome and GSH both increased harmane binding affinity, but by different mechanisms. Lumichrome reduced the stoichiometry and associated heat release of harmane binding to \textit{AtGSTF2} by one third, while GSH showed no such effect. Complementary binding studies also demonstrated that both lumichrome and harmane increased binding affinity for GSH from $K_d = 22 \, \mu M$ to $K_d = \sim 5 \, \mu M$ (Fig. 4, Table 1). From these studies it was clear that the binding of ligands and GSH to \textit{AtGSTF2} was cooperative. The binding of the phytoalexin camalexin to \textit{AtGSTF2} showed a different response, with an apparent unusual stoichiometry of 1.45:1. Intriguingly, camalexin prevented any thermally visible binding of harmane to \textit{AtGSTF2}. Similarly the presence of harmane prevented any ITC-detectable association with camalexin, confirming that the two ligands shared a (presumably single) binding site. Quercitrin showed stoichiometric binding to \textit{AtGSTF2}, albeit with a somewhat weaker affinity ($K_d = 6 \, \mu M$) than determined with the heterocyclic ligands. Binding by quercitrin was abrogated by pre-binding of harmane, again suggesting that both ligands were binding at the same site.

\textit{Mutagenesis}

To better understand the results obtained from binding studies, the active sites of \textit{AtGSTF2} and \textit{AtGSTF3} were examined, making use of the available crystal structure for \textit{AtGSTF2} (PDB accession 1BX9). Of particular interest were residues around the active site that were conserved in these two GSTs but altered in related enzymes that did not show the selective binding to harmane or lumichrome and could therefore be involved in the ligand binding observed. Phe-123 was a promising candidate since this residue is substituted with an isoleucine in \textit{AtGSTF4} and \textit{AtGSTF5}, being enzymes that do not bind these heterocycles. On generating the mutant \textit{AtGSTF2-123I}, the purified enzyme showed considerably higher CDNB-conjugating activity than the parent \textit{AtGSTF2}. In addition, unlike \textit{AtGSTF2}, the conjugating activity of the mutant was inhibited by high levels of harmane (Fig. 5). As compared to \textit{AtGSTF2}, \textit{AtGSTF2-123I} bound harmane and lumichrome with only slightly reduced affinity and with similar heat release (Table 1), showing that this mutation had not disrupted ligand binding. However, overall a lower binding stoichiometry was observed, suggesting that only about half the protein was active, which was indicative of the inefficient conformational folding of the mutated protein.

\textit{Discussion}

The functional characterisation of GST superfamily members in Arabidopsis and other plants remains challenging, at least in part due to the potential functional redundancy resulting from the expansion of GST family gene numbers. Genetic approaches have had limited success at deducing GST function. For example, \textit{AtGSTF12} disruption caused an obvious pigment phenotype in Arabidopsis seeds that could be linked to transport of anthocyanins and proanthocyanidins [15], although even here the precise function of the GST remains unknown. Altering the expression of other GSTs to perturb function has generally been unsuccessful. For example, the RNAi
knockdown of multiple GSTFs followed by detailed analysis failed to detect any major disruption in metabolism or physiology [29]. Instead, information on plant GST function has come through unexpected associations with other proteins of interest, such as the discovery of AtGSTU20 interacting with phytochrome signalling components [16]. In an attempt to apply an alternative systematic strategy to define GST function we have developed a ligand fishing approach that uses global metabolite profiling of extracts purified by virtue of their affinity to a protein of interest, to identify tightly-binding ligands. We have already shown that this approach works to identify not only ligands of lambda-class GSTs, but also to subsequently identify a natural function for these enzymes [21]. Here, we have applied this approach to Arabidopsis phi-class GSTs, in particular AtGSTF2, raising some interesting possibilities concerning the function of this protein in planta.

An initial metabolite profiling screen of bacterially expressed AtGSTFs identified AtGSTF2 (and its close homologue AtGSTF3) as causing an unusual accumulation of heterocyclic compounds in the host bacteria, due to their tight binding as ligands to the ectopically expressed proteins. None of the identified ligands were bacterial in origin and instead originated from the rich culture medium and showed similarities to plant natural products. The four indole derivatives identified were probably formed through tryptophan reacting with other medium components during the autoclaving of the medium [25, 30]. Lumichrome was most likely present as a photodegradation product of the riboflavin present in yeast extract [31]. In each case, harmene, norharmane and lumichrome all bound to AtGSTF2 far more tightly than had been described for previously identified interactions of this protein with auxins and flavonoids [32]. Although lumichrome was not identified as an AtGSTF2 ligand in the Arabidopsis extracts, it is a well known plant metabolite with an established activity as a rhizobial signalling agent [33]. It is therefore conceivable that AtGSTF2 could regulate the biological activity and associated photoactivation of lumichrome in planta by being involved in its transport and storage. Intriguingly, AtGSTF2 is known to be up-regulated in response to pathogens, with riboflavin implicated in the induction of systemic disease resistance in plants [34], most likely following its transformation to lumichrome. The identified indole derivatives harmene, norharmane and 1-acetyl-β-carboline are unusual natural products that are not known to occur in Arabidopsis. However, Arabidopsis does contain a variety of indole-derived defence-related metabolites that these ligands could be mimicking, and the availability of pure standards made these useful for in vitro binding studies.

The binding studies with lumichrome and AtGSTF2 were mechanistically revealing, as they demonstrated that the association with one ligand caused the protein to both increase its enzymic conjugating activity and enhance its binding to secondary ligands, notably harmene. Both harmene and lumichrome enhanced AtGSTF2 enzyme activity, without affecting the affinity of the enzyme for its substrates. Further studies using the mutant AtGSTF2-123I showed that for both wild-type and mutant enzyme, low concentrations (< 5 µM) of harmene enhanced enzyme activity while higher levels further increased activity for the wild-type enzyme but slowly reduced activity for the mutant (Fig. 5). Based on the kinetic data, we postulate that binding of harmene/lumichrome to AtGSTF2
promotes the release of the DNB-GSH reaction product, thereby enhancing enzyme turnover. It is unclear whether the non-substrate ligands bind adjacent to the active site and directly influence catalysis, or bind further away and act as allosteric effectors. The competitive nature of ligand binding suggests these compounds associate at the same location. In addition, ligand and GSH binding are cooperative, suggesting interaction, and with the association enhancing catalysis without obviously altering substrate affinity. The two-phase effects seen on catalysis with increasing harmane concentration suggest the presence of a high-affinity harmane binding site that activates the enzyme and a much lower affinity binding site (or sites) that activates the wild-type enzyme but inhibits the 123I mutant. The nature of these binding sites awaits further investigation.

The association of AtGSTF2 with multiple ligands had previously been observed in structural biology studies, which showed each monomer to bind two molecules of the inhibitor S-hexylglutathione [28]. Biochemical results consistent with AtGSTF2 having separate binding sites for the ligands indole-3-acetic acid (IAA) and N-1-naphthylphthalamic acid (NPA) have also been reported [32]. The current study provides direct evidence of how ligand-binding interactions with AtGSTF2 could modulate signalling events, with one group of compounds (eg: lumichrome) influencing the binding to other biologically active natural products (eg: indole derivatives). Alternatively, such binding could directly modulate the conjugating activity of AtGSTF2 toward molecules involved in signalling, thereby altering their activity. Consistent with AtGSTF2 having a signalling role, previous studies have shown the respective gene to be responsive to diverse stimuli, including plant hormones (auxins, ethylene) and pathogen attack [12, 32]. A regulatory role for AtGSTF2 in development and stress tolerance has also been suggested by the observation that Arabidopsis plants expressing sense and antisense constructs of the orthologous gene from *Brassica juncea* showed subtle changes in flowering time, along with differences in shoot regeneration and stress resistance [35].

The binding of AtGSTF2 to indoles, such as camalexin in Arabidopsis extracts, and the β-carboline alkaloids harmane, norharmane and 1-acetyl-β-carboline, and indole-3-aldehyde in *E. coli* cultures was particularly interesting. AtGSTF2 was originally identified as a membrane-associated protein which could be labelled with azido-activated IAA in photoaffinity experiments [36]. Our own ITC studies showed that neither IAA, kinetin, nor trans-zeatin were tightly bound by AtGSTF2, supporting earlier suggestions that this protein is unlikely to have a physiological role in binding these plant hormones [32]. However, AtGSTF2 could bind other plant indole-derived metabolites sufficiently well to exert a physiological effect. For example the AtGSTF2 ligand indole-3-aldehyde is a known plant metabolite [37], with its application causing an inhibition of lateral bud growth [38]. More strikingly, tight (K_d = 1.2 μM) binding of AtGSTF2 to camalexin is very suggestive of a physiological role, perhaps in the intracellular transport of this reactive and defence-inducible secondary metabolite. A strong link between camalexin and glutathione already exists, with good evidence that the sulfur in camalexin is introduced through glutathionylation of a cytochrome P_{450}-activated precursor [39, 40]. This conjugation may occur spontaneously, but it is likely that the reaction is
more carefully regulated using a GST and as such, AtGSTF6 has been implicated in this role [39]. There is a strong link between AtGSTF2 and AtGSTF6 (see below) and it is possible that AtGSTF2 also catalyses glutathione conjugation of the camalexin precursor. A similar conjugation occurs during (indole) glucosinolate synthesis [41], providing another potential role for AtGSTF2. GSTs may therefore be involved both in the synthesis of these indole-derived defence compounds, and in the subsequent intracellular transport of these compounds, as illustrated using camalexin as an example in Fig. 6.

AtGSTF2 was also shown, along with AtGSTF6, to selectively bind the flavonol quercitrin in Arabidopsis extracts, even though it was present in very low concentrations. AtGSTF2 has also been shown to interact with flavonoids in earlier studies [32], with the binding of kaempferol inhibiting associations with NPA. This binding was shown to be competitive, with 100 µM kaempferol inhibiting binding to either NPA, or IAA by two thirds [32]. In the current studies, the binding of AtGSTF2 to quercitrin was weaker than that observed with the N-containing ligands, suggesting that binding to camalexin and related molecules would be more likely than associations with flavonols. However, previous studies in Arabidopsis have shown that AtGSTF2 had an altered subcellular localisation in flavonoid-deficient mutants as compared with wild type plants, suggesting flavonol-binding was physiologically relevant [32]. It is feasible that AtGSTF2 functions to transport flavonoids under normal conditions, but in response to pathogen attack also transports the now abundant indole-derived phytoalexins, with its expression level increased to cope with the increased demand. The turnover of molecules for an intracellular transport process will be orders of magnitude slower than a typical enzyme catalytic turnover. Therefore intracellular transport proteins would be expected to be present at much higher abundance than enzymes in the same pathway. When coupled with the high levels of synthesis of camalexin during a defence response, very high amounts of transporter would be needed, and this is indeed observed for AtGSTF2 and also for other GSTs, which are easily affinity-purified from relatively small amounts of Arabidopsis tissue [17].

The ligand binding results for AtGSTF6 are intriguing. AtGSTF2 and AtGSTF6 are closely linked and their functions may well overlap. The two genes are closely related [1], and have closely matching expression patterns [40]. It is clear from the Arabidopsis ligand fishing results that the two enzymes bind similarly to indole and flavonol metabolites. It is perhaps surprising then that AtGSTF6 did not bind similar ligands to AtGSTF2 when expressed in E. coli, but it is reasonable to assume that differences between the proteins altered binding to non-target bacterial ligands without substantially altering binding to physiological ligands. It is therefore likely that the bacterial screens have missed other ligand-binding GSTs, and that these will only be detected using plant-based ligand fishing approaches.

GSTs are typically thought of as detoxifying conjugating enzymes. However, neither AtGSTF2 nor AtGSTF6 has been shown to form conjugates in planta, with the recombinant enzymes showing little activity towards model substrates such as CDNB [1]. None of the identified ligands appeared sufficiently electrophilic to allow GSH conjugation and while AtGSTF2 did not
catalyse the conjugation of any of the ligands identified, their binding to the
protein was shown to be promoted in the presence of GSH. This provides
further evidence that such GSTs retain a functional dependence on GSH even
when performing non-catalytic binding functions. While the studies described
here cannot unequivocally identify a function for AtGSTF2 and other GSTFs in
planta, they do give a mechanistic context for how this protein could perform a
regulatory role in responding to different classes of ligands through alterations
in structure and function. When considered along with the strong and rapid
induction of AtGSTF2 in response to pathogens [12] and its association with
plasma membrane vesicles [36] this does suggest that this protein performs a
regulatory transport function involving the export of small bioactive natural
products during plant stress.

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References

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Table 1. ITC-derived thermodynamic parameters for titration of the compounds listed into $\text{AtGSTF2}$ and its point mutant $\text{AtGSTF2-123I}$.

In each case, parameters were obtained from fits to a one set of sites binding model, with errors representing standard error in the fitted model. $N =$ ligand:GST binding stoichiometry. Certain experiments contained GSH (1 mM) and/or lumichrome (23.5 µM). $^\dagger$ Stoichiometry unreliably determined at low affinity, so fixed at 1:1.

<table>
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<th>Interaction (+ cofactors)</th>
<th>$N$</th>
<th>$K_s$ (µΜ⁻¹)</th>
<th>$\Delta H$ (kcal/mol)</th>
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<tbody>
<tr>
<td>GSTF2 + harmane</td>
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<td>0.86 ± 0.06</td>
<td>0.16 ± 0.02</td>
<td>-21.1 ± 2.1</td>
</tr>
<tr>
<td>GSTF2 + camalexin (+ GSH)</td>
<td>1.45 ± 0.02</td>
<td>0.84 ± 0.08</td>
<td>-9.3 ± 0.2</td>
</tr>
<tr>
<td>GSTF2 + GSH</td>
<td>$^\dagger$ 0.044 ± 0.001</td>
<td>-5.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>GSTF2 + GSH (+53 µM harmane)</td>
<td>$^\dagger$ 0.22 ± 0.03</td>
<td>-5.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>GSTF2 + GSH (+64 µM lumichrome)</td>
<td>$^\dagger$ 0.17± 0.02</td>
<td>-5.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>GSTF2-123I + harmane (+ GSH)</td>
<td>0.48 ± 0.06</td>
<td>2.84 ± 0.30</td>
<td>-15.2 ± 0.2</td>
</tr>
<tr>
<td>GSTF2-123I + lumichrome (+ GSH)</td>
<td>0.14 ± 0.01</td>
<td>3.57 ± 0.26</td>
<td>-35.9 ± 0.8</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Reversed-phase HPLC analysis of AtGST ligands from *E. coli*. A) Extracts from bacteria expressing AtGSTF12 (control; grey lower line) or AtGSTF2 (black upper line), showing accumulation of metabolites 1 to 5. B) Ligands from purified AtGSTF2, and C) AtGSTF3 were similarly analysed. Altered retention times between panel A and panels B and C were due to changing from a 2.1 x 50 mm column to a 2.1 x 100 mm column.

Figure 2. Structures of the identified AtGSTF2 ligands. Ions in bold are the parent mass ions (MH⁺), with associated collision-induced dissociation fragments.

Figure 3. Reversed-phase HPLC analysis of AtGSTF2 and AtGSTF6 ligands isolated from Arabidopsis extracts. Bacterially-expressed Strep-tagged enzymes were affinity immobilised and incubated with metabolite extracts from Arabidopsis. Ligands were recovered from affinity-eluted AtGSTF2 and AtGSTF6 (black lines) and compared with the compounds isolated from the control protein AtGSTU19 (grey line). Identified Arabidopsis-derived ligands are numbered (see Figure 2). Ion counts for the m/z 201 ion corresponding to camalexin (peak 7) are shown inset.

Figure 4. The heat released on titrating GSH into a solution of 13.41 µM AtGSTF2 in the presence of no additive (♦), 52.8 µM harmane (▲) or 64.3 µM lumichrome (■) as determined by ITC. The associated lines indicate best fit curves for a one set of sites binding model.

Figure 5. Effect of harmane on CDNB-conjugating activity of AtGSTF2 and its point mutant AtGSTF2-123I.

Figure 6. Potential roles for GSTs in the synthesis and delivery of camalexin in Arabidopsis. As well as a putative catalytic role introducing sulfur into the molecule, GSTFs may also be involved in transporting both the glutathione conjugate intermediate and the end product to their required locations.
Figure 1.

A 300 nm

B 300 nm

C 300 nm
Figure 2.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>UV spectrum</th>
<th>Ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Norharmane</td>
<td><img src="image" alt="Norharmane" /></td>
<td>169.078, 142.069, 115.057</td>
</tr>
<tr>
<td>2</td>
<td>Harmane</td>
<td><img src="image" alt="Harmane" /></td>
<td>183.094, 168.072, 142.068, 115.057</td>
</tr>
<tr>
<td>3</td>
<td>Lumichrome</td>
<td><img src="image" alt="Lumichrome" /></td>
<td>243.090, 216.080, 198.070, 172.091, 145.079, 130.068</td>
</tr>
<tr>
<td>4</td>
<td>Indole-3-aldehyde</td>
<td><img src="image" alt="Indole-3-aldehyde" /></td>
<td>146.063, 118.068, 91.057</td>
</tr>
<tr>
<td>5</td>
<td>1-Acetyl-β-carboline</td>
<td><img src="image" alt="1-Acetyl-β-carboline" /></td>
<td>211.090, 193.080, 169.080, 142.068, 140.053, 115.058</td>
</tr>
<tr>
<td>6</td>
<td>Quercetin-3-O-rhamnoside</td>
<td><img src="image" alt="Quercetin-3-O-rhamnoside" /></td>
<td>449.108, 303.053, 229.055, 129.052, 85.023, 71.044</td>
</tr>
<tr>
<td>7</td>
<td>Camalexin</td>
<td><img src="image" alt="Camalexin" /></td>
<td>[Not resolved], 201.053</td>
</tr>
</tbody>
</table>

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Figure 3.
Figure 4.
Figure 5.

![Graph](image)

**GSTF2**

**GSTF2-123I**

CDNB-conjugating activity (nkat/mg)

[harmane] (μM)
Figure 6.

Tryptophan

↓

(activated) Indole-3-acetaldoxime

↓

Glutathione-indole-3-acetonitrile

↓

GSTM-mediated transport?

Cysteine-indole-3-acetonitrile

↓

Camalexin

↓

GSTF-mediated transport?

Camalexin

(at site of action)