Selective Binding of Glutathione Conjugates of Fatty Acid Derivatives by Plant Glutathione Transferases*§

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Proteomic studies with Arabidopsis thaliana have revealed that the plant-specific Tau (U) class glutathione transferases (GSTs) are selectively retained by S-hexylglutathione affinity supports. Overexpression of members of the Arabidopsis GST superfamily in Escherichia coli showed that 25 of the complement of 28 GSTUs caused the aberrant accumulation of acylated glutathione thioesters in vivo, a perturbation that was not observed with other GST classes. Each GSTU caused a specific group of fatty acyl derivatives to accumulate, which varied in chain length (C6 to C19a), additional oxygen content (0 or 1), and desaturation (0 or 1). Thioesters bound tightly to recombinant GSTs (Kd ~ 1 μM), explaining their accumulation. Transient expression of GSTUs in Nicotiana benthamiana followed by recovery by Strep-tag affinity chromatography allowed the respective plant ligands to be extracted and characterized. Again, each GST showed a distinct profile of recovered metabolites, notably glutathionylated oxophytodienoic acid and related oxygenated fatty acids. Similarly, the expression of the major Tau protein GSTU19 in the endogenous host Arabidopsis led to the selective binding of the glutathionylated oxophytodienoic acid-glutathione conjugate, with the enzyme able to catalyze the conjugation reaction. Additional ligands identified in planta included other fatty acid derivatives including divinyl ethers and glutathionylated chlorogenic acid. The strong and specific retention of various oxygenated fatty acids by each GSTU and the conservation in binding observed in the different hosts suggest that these proteins have selective roles in binding and conjugating these unstable metabolites in vivo.

In plants, the glutathione transferases (GSTs) are a superfamily of proteins with the dominant Phi (F) and Tau (U) classes having largely undefined functions in endogenous metabolism (1). This is in contrast to their relatively well studied role in catalyzing the glutathione conjugation of herbicides (2). Because of their importance in determining herbicide selectivity, these proteins have been purified from a range of major crops including maize (3), soybean (4), and wheat (5, 6). One approach which has been commonly applied to isolate these proteins is affinity chromatography using a range of glutathione (GSH) derivatives as ligands (7). These ligands often display a surprising degree of selectivity in capturing specific GSTs. Thus, in wheat extracts, whereas GSH-agarose effectively isolated GSTFs, S-hexylglutathione (GS-hexyl) proved to be a selective ligand for GSTUs (5). Similarly, glutathionylated dyes have proved to be highly selective for specific GSTs in maize (8).

In each application, the use of GSH derivatives as affinity ligands has been developed through serendipity, with limited attention directed to the potential significance of such binding selectivity in relating to the nature of in vivo substrates and reaction products. At a molecular level, crystallographic studies with both plant GSTFs (9) and GSTUs (10) have revealed a conserved active site architecture consisting of a GSH binding domain that orients the thiol toward an adjacent hydrophobic binding pocket containing the putative alkyl- or aryl-bearing acceptor. However, the nature of any such hydrophobic substrates in plants remains largely unknown, with little evidence that natural products accumulate as glutathione conjugates (11).

To address the significance of selective binding to glutathionylated derivatives, members of the GST superfamily from Arabidopsis thaliana have been screened for their ability to bind conjugated metabolites in vivo. Two methods have been used. In the first approach, the GSTs in Arabidopsis plants which bind GS-hexyl have been identified using affinity chromatography coupled with proteomics. In the second approach, the ligands which associate with these enzymes in vivo have been identified in a two-tiered screen. In the first instance, GSTs have been individually expressed in Escherichia coli, and the crude extracts from the recombinant bacteria were assessed for their aberrant accumulation of alkylated glutathione derivatives. Here, we reasoned that the lack of compartmentalization in bacteria would allow the GST to interact with any endogenous metabolic intermediate with disruptions in metabolism then directly monitored. A similar approach has recently demonstrated that specific maize GSTUs derail heme metabolism in recombinant E. coli by selectively binding porphyrinogen intermediates (12). In the secondary screening, we have then taken Strep-tagged GSTs with interesting profiles of ligand binding activity in E. coli and transgenically overexpressed them in Nicotiana benthamiana and/or Arabidopsis. The proteins were then recovered using the affinity tag, and bound ligands were identified by HPLC-MS.

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2 The abbreviations used are: GST, glutathione S-transferase; GSTU, Tau-class GST; GSTF, Phi-class GST; ESI, electrospray ionization; GS-hexyl, S-hexylglutathione; GSH, glutathione; HPLC, high performance liquid chromatography; MS, mass spectrometry; OPDA, 12-oxo-10,15(9)-phytodieneoic acid; TOF, time of flight.
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EXPERIMENTAL PROCEDURES

Chemicals—12-Oxo-10,15(Z)-phytodienoic acid (OPDA) was obtained from Larodan Fine Chemicals AB, Malmö, Sweden. S-Myristoyl glutathione was synthesized from myristoyl chloride and GSH as described for the palmitoyl derivative (13) and used without further purification ($\varepsilon_{251 \text{ nm}} = 4.6 \text{ mM}^{-1}\text{cm}^{-1}$). The glutathione conjugate of chlorogenic acid (2-S-glutathionyl-chlorogenic acid) was synthesized using horseradish peroxidase and $\text{H}_2\text{O}_2$ (14).

Bacterial Metabolite Profiling—Arabidopsis GSTs were cloned and expressed in E. coli as N-terminal Strep-tagged polypeptides (15). Stationary phase bacterial cultures (10 ml) were centrifuged and washed in water (1 ml), and the pellets were extracted in 150 $\mu$l of methanol (22 °C, 10 min). Samples (10 $\mu$l) were injected onto a C$_{18}$ column (Waters Acquity UPLC BEH, 2.1 mm $\times$ 100 mm) and eluted with a gradient of 5–100% acetonitrile in 0.1% formic acid at 0.2 ml min$^{-1}$ over 9 min. The eluate was subsequently analyzed with an Acquity diode array detector and a Waters Q-TOF Premier mass spectrometer operating in positive electrospray ionization (ESI) mode (cone voltage 25 V). A collision energy ramp (25–40 eV) was used to fragment ions for further analysis. The TOF detector was calibrated with sodium formate using leucine encephalin as lock-spray standard for dynamic calibration correction.

Plant Metabolite Profiling—Strep-tagged GSTs were transiently expressed in N. benthamiana using agroinfiltration (12). Before harvest, leaves were wounded by pinching with forceps and proteins extracted 1 h later. For Arabidopsis studies, plants were stably transformed using floral dipping (16). In both cases the Strep-tagged GSTs were purified by affinity chromatography (12), with bound ligands recovered from the concentrated protein in 2 volumes of methanol. Extracts were then analyzed by liquid chromatography-MS as described for bacterial metabolite profiling but using a 1 $\times$ 100-mm column running at 0.1 ml min$^{-1}$ to enhance sensitivity. Additional analyses using negative mode ESI were also performed. To confirm their integrity, precipitated proteins were redissolved in 50% CH$_3$CN, 0.5% formic acid, and their intact masses were determined by ESI-TOF MS (15). Protein purity was determined by SDS-PAGE, with gels stained with colloidal Coomassie Brilliant Blue G250.

Assays and Binding Studies—GST activity toward 1-chloro-2,4-dinitrobenzene was determined as described (17) but using 1 mM GSH and HBS (20 mM HEPES-NaOH, 150 mM NaCl, 1 mM EDTA, pH 7.6) as buffer. GST activity toward OPDA was assayed at 30 °C in a total volume of 20 $\mu$l composed of 100 mM Tris-Cl, pH 6.5, 17 $\mu$m OPDA, 1 mM GSH, and 4.5 $\mu$m enzyme. Reactions were quenched with 10 $\mu$l of 10 mM N-ethylmaleimide, and the contents were then stabilized and solubilized by the addition of 10 $\mu$l of 1% formic acid and 40 $\mu$l of CH$_3$CN. Products were analyzed by MS using the metabolite profiling methodology, and the appearance of glutathionylated conjugates and the loss of parent OPDA were quantified by comparison with standards of known concentration. Binding studies were performed using a Microcal VP-ITC isothermal titration calorimeter. For thioester binding, S-myristoyl-GSH (100 mM in DMSO) was diluted 500-fold into HBS containing 1 mM GSH and injected in 5-$\mu$l pulses at 25 °C into a cell containing 20 mM recombinant GSTU7 in HBS, 1 mM GSH, and 0.2% DMSO. On each injection the released heat was measured, with a similar experiment performed without GSTU7 to measure the signal because of ligand dilution. For OPDA binding/conjugation, 137 $\mu$m OPDA in HBS was injected into HBS containing 11 $\mu$m GSTU19 and 1 mM GSH. In both experiments a one-binding site model was fitted to the background-subtracted data to calculate binding parameters.

Proteomics—For the Arabidopsis studies, 12-day-old root cultures were used as a protein source (18). For each purification, 4 g of wet weight tissue was extracted by grinding in 20 ml of 20 mM Tris-Cl, pH 7.5 (buffer A), with proteins precipitated at 4 °C by the addition of ammonium sulfate to 80% saturation. The protein pellets were redissolved in 10 ml of buffer A and applied to 1-ml affinity columns containing either GSH (GE Healthcare) or GS-hexyl (Sigma) as ligands. After removing unbound protein with buffer A, GSTs were eluted with buffer A containing either 5 mM GSH (GSH affinity) or 5 mM GS-hexyl (GS-hexyl affinity). Each purified sample was concentrated to ~100 $\mu$l using a 10-kDa-cutoff Vivaspin 2 device (Sartorius Stedim, Epsom, UK), and polypeptides were recovered by precipitation with 4 volumes of acetone. Each entire sample was analyzed by two-dimensional-PAGE on a 12% acrylamide gel (18), and polypeptides of interest were excised and identified by peptide mass fingerprinting (19).

RESULTS

Proteomic Analysis—To identify GST family members that preferentially bind S-alkyl glutathione derivatives, crude protein samples from Arabidopsis root cultures were subjected to affinity chromatography using GS-hexyl as the ligand. In parallel, the extracts were also applied to GSH-agarose, an affinity purification method typically used in Arabidopsis proteomic studies (20–22). GST and GS-hexyl chromatography recovered very different profiles of GSTs (Fig. 1). Although the use of GSH as ligand purified GSTFs and also two GSTUs, GS-hexyl affinity chromatography purified Tau class enzymes in greater numbers and with much increased yield along with GSTF2, which was only poorly recovered using the GSH columns. Unlike the GSH affinity columns, the GS-hexyl ligand also purified a number of truncated GST polypeptides generated by proteolysis of the parent proteins. This simple screen demonstrated that Arabidopsis GSTUs were selectively retained by an S-alkyl-glutathione ligand. Attention was, therefore, focused on the GSTUs for their ability to bind conjugates of hydrophobic compounds in vivo.

Metabolic Screening in E. coli—The Arabidopsis Strep-tagged GSTU expression library (15) was used to test all family members for their ability to bind endogenous bacterial ligands and, hence, perturb metabolite accumulation in the recombinant bacteria. Metabolites from bacteria expressing GSTUs were solvent-extracted, and for each extract time-resolved mass chromatograms were analyzed using pairwise comparisons to look for substantially altered ion intensities. These perturbations were then confirmed in independent experiments. Metabolites of interest were analyzed by tandem MS, identifying a group of related metabolites that accumulated as major products in 17 of the 28 GSTUs screened. Lesser amounts of
similar compounds were also identified in extracts from most of the remaining bacteria expressing other GSTUs. These compounds were not detected in E. coli expressing any other class of GSTs, confirming that these were Tau-specific ligands (data not shown). Common features of the GSTU-associated metabolites included the presence of a glutathione moiety as shown by characteristic fragmentation by tandem MS, and the presence of an acyl chain as judged by a high positive mass defect (Fig. 2). By analogy, this inferred that the related compound (Fig. 2). The highly abundant \( m/z \) 518.29 (MH\(^+\)) ion from GSTU7-overexpressing cultures was chosen for further characterization. Fragmentation of this ion gave neutral losses of 75, 129, 147, 204, and 232 Da, with fragment ions at \( m/z \) 76 and 179, which are all characteristic of glutathione conjugates (Fig. 2). In contrast, no fragmentation of the putative acyl moiety was observed. High resolution MS and isotope modeling of this ion identified the most likely molecular formula as \( C_{24}H_{44}N_2O_S \), corresponding to \( \text{GSH} + C_4H_9O + H^+ \). The acyl oxygen was not apparently because of a keto group, as judged by the negative reaction of the compound with 2,4-dinitrophenylhydrazine. However, the metabolite was quantitatively removed after treating with hydroxylamine, suggesting the presence of a thiourea group. The compound was, therefore, tentatively identified as S-myristoylglutathione (Fig. 3). This assignment was then confirmed by synthesizing an authentic S-myristoylglutathione standard whose properties matched the isolated compound (Fig. 2). By analogy, this inferred that the related metabolites observed in the other GSTU-expressing bacteria were also S-acylated glutathione derivatives, albeit differing in chain length, the degree of desaturation, and presence of hydroxyl groups in the acyl moiety.

To confirm that the accumulation of these conjugates was because of binding to GSTU, thus preventing further metabolism, rather than accumulation in solution due to an alternative metabolic perturbation, S-myristoylglutathione and GSTU7 were subjected to isothermal titration calorimetry. Tight, stoichiometric binding was demonstrated with \( K_d \) = 900 nM. Similarly, GSTU7 activity toward 1-chloro-2,4-dinitrobenzene was near quantitatively inhibited in the presence of stoichiometric (or higher) concentrations of S-myristoylglutathione.

The apparent high specificity shown by each GST toward the various acyl moieties was of particular interest. Clear preferences were observed between GSTs for the chain length and presence of hydroxyl groups, with each protein showing a distinct and reproducible binding fingerprint of different conjugated acyl moieties (Fig. 4 and supplemental table). The liquid chromatography-MS analysis also showed that each GST varied markedly in the total amounts of thioesters accumulated (Fig. 4). For example, GSTU9 and GSTU10 accumulated large amounts of long chain (C\(_{16}\), C\(_{18}\)) acyl derivatives, whereas GSTU13 accumulated similar compounds but at much lower levels. In contrast, GSTU6 and GSTU16 showed preference for shorter chain length acyl substituents (mainly C\(_6\) to C\(_{14}\)). Expression of GSTU26 and GSTU28 caused accumulation of non-hydroxylated acylated conjugates, whereas GSTU24 and GSTU25 overexpressors exclusively accumulated hydroxy-acylated glutathione conjugates.

**Identification of GST Ligands in Plants**—Having identified a range of fatty acid ligands of GSTUs in bacteria, it was then of interest to determine whether similar ligands could be observed associated with these proteins in plants. Based on the bacterial screens, the GSTs chosen for study were 1) GSTU9 and GSTU10 because of their high level accumulation of long chain acyl conjugates, 2) GSTU25 and GSTU28 because of their differential selectivity toward hydroxy-containing conjugates, and 3) GSTU19 because of its broad ligand selectivity in E. coli and its natural abundance in Arabidopsis (Fig. 1). GSTUs were transiently overexpressed in N. benthamiana as Strep-tagged fusion proteins (15). Because many GSTs are stress-inducible (11), it was decided to enhance the abundance of defense-related metabolites by pinching transfected leaves with forceps 1 h before extraction to simulate wounding. Processing of 40 g of leaf tissue generated ~1 mg of tagged GST (28–30 kDa), recovered at high purity (supplemental Fig. S1). Minor high molecular weight bands observed on SDS-PAGE were identified by peptide mass fingerprinting as Strep-GST polypeptide oligomers. GSTU9 and GSTU10 preparations also contained a number of low molecular weight bands corresponding to GST degradation products. Intriguingly, GSTU28 preparations contained a minor 28-kDa band that was identified as the tobacco Tau class GST termed C-7 (23). This suggested that heterodimerization occurs between the host GSTU subunits and Arabidopsis GSTU28. When the recovered Strep-GST fusion proteins were analyzed by ESI-MS under denaturing conditions (after solvent extraction; see below), in every case the mass of the intact polypeptide was consistent with cleavage of the N-terminal methionine and quantitative N-acetylation of the revealed alanine amino group of the Strep tag. Similar processing of the Strep-tagged GSTs has also been identified when these proteins were expressed in E. coli (15).
However, there was no other evidence of further post-translational modification.

The affinity-purified GSTs were solvent-extracted, and the bound ligands were analyzed by HPLC-MS. Each GST retained a distinct range of metabolites, as illustrated by the differences observed in total UV absorbance and mass spectral profiles (Fig. 5, supplemental Fig. S2). As found in the bacterial ligand screens, many of these differential ions were glutathione conjugates, as judged by the presence of the GSH m/z 308.09 ion on collision-induced dissociation (supplemental Fig. S2). The profiles of recovered metabolites were analyzed to determine components that were significantly altered in intensity between samples. As well as directed analysis of mass ions associated with novel UV-absorbing compounds, a further undirected screen to highlight mass ions unique or highly enriched in GST versus control samples was used. The likely identities of compounds of interest were then investigated by a combination of tandem and high resolution MS with isotopic distribution modeling to deduce likely molecular formulae (Table 1). Major ions associated with the GSTUs were m/z 600.295, which corresponded to C_{28}H_{46}N_{3}O_{9}S (or GSH/H\textsubscript{11001} C_{18}H_{30}O_{3}/H\textsubscript{11001} H), and m/z 602.307 (GSH + C_{18}H_{30}O_{3} + H\textsuperscript{+}). These metabolites were particularly abundant in the GSTU25 and GSTU28 preparations. For the m/z 600 ion, two time-resolved ion peaks were present, with the first matching the glutathione conjugate of OPDA (Fig. 3, supplemental Fig. S3). The second m/z 600 peak was unidentified but was likely to be the glutathione conjugate of an isomer of the closely related keto fatty acid oxo-octadecatrienoic acid (KOTrE) (Fig. 3).

The m/z 602 ions were also resolved into a series of peaks, which was consistent with them corresponding to a series of isomers. Based on their mass and potential for multiple m/z 602 peaks, these derivatives were most likely the glutathione conjugates of oxo-octadecadienoic acid (KODE; Fig. 3).

In addition to these “common” ligands, each GST showed a characteristic metabolite binding profile, with some binding partners reliably determined within groupings of the GSTUs. For example, GSTU9 and GSTU10 and to a lesser extent GSTU28 bound a pair of hydrophobic UV-absorbing metabolites (absorbance maxima ~250 nm) that gave ions at m/z 291.20 (M-H\textsuperscript{+}) and m/z 293.21 (M-H\textsuperscript{+}), corresponding to C_{18}H_{29}O_{3} and C_{18}H_{30}O_{3}, respectively. These characteristics matched the divinyl ether fatty acids colnelenic acid and colneleic acid (Ref. 24 and Fig. 3). This identification was supported by tandem MS,
with ion fragmentation observed at either side of the ether group (supplemental Fig. S4). GSTU9 and GSTU10 also bound a range of other metabolites, including a strong preference for non GSH-conjugated oxylipin-like compounds (Table 1).

As demonstrated in the E. coli expression studies, GSTU19 interacted with a wide range of ligands. These ligands included GSH-conjugated oxylipins and uniquely large amounts of a phenolic metabolite tentatively characterized as 2-S-glutathionylchlorogenic acid. This identity was subsequently confirmed by comparison with a synthesized standard (supplemental Fig. S5). A second related compound, probably representing a structural isomer of this conjugate, was also retained by GSTU19. This related conjugate, but not 2-S-glutathionylchlorogenic acid, was also bound by GSTU25, demonstrating the high degree of selectivity shown by the GSTUs.

As compared with the other enzymes, GSTU25 and GSTU28 accumulated shorter chain length conjugates. In the case of GSTU25, one of these was identified as S-hexanol-glutathione. The specificity for shorter chain alkyl moieties matched that observed in E. coli, where GSTU25 showed a strong preference for shorter chain length hydroxy-fatty acids (Fig. 4).

Fatty Acid Ligands of Glutathione Transferases

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Ligands of GSTU19 in Arabidopsis and Their Enzymic Formation—Based on the results obtained in the binding screens in E. coli and N. benthamiana, it was then of interest to determine the nature of the endogenous ligands for these proteins in their originating host Arabidopsis. For this investigation, GSTU19, which is one of the best described and more abundant GSTs in Arabidopsis (15, 18, 20), was selected for further study. Arabidopsis plants were transformed with Strep-tagged GSTU19, and lines expressing high levels of the transgene were selected and propagated. For controls, untransformed Arabidopsis and plants overexpressing the Phi-class Strep-GSTF2 were used. MS analysis of the ligands bound to GSTU19 expressed in the foliage identified similar m/z 600.3 and m/z 602.3 oxylipin-GSH conjugates to those recovered.
Fatty Acid Ligands of Glutathione Transferases

TABLE 1
Relative abundances of ligand mass ions for GSTs expressed in N. benthamiana

For each ion, the HPLC retention time (RT) is shown along with its m/z value (+ = MH⁺; − = M−H⁻), likely identity and ion abundance in the GST preparations tested. Abundances are not comparative between ions and are not strictly quantitative between GST preparations. Preparations containing relatively high levels of a particular ion are highlighted in bold. Con, control; -SG, S-glutathionyl derivative.

| m/z     | RT* | Identity                  | Peak area in each GST preparation |
|---------|-----|---------------------------|-----------------------------------|
| +408.18 | 3.2 | Hexanol-SG                | Con 1.8 U9 1.3 U10 3.9 U19 509 24.6 |
| +442.24 | 5.4 | ?                         | Con 44.7 U9 24.9 U10 3.1 U19 92.7 |
| +504.20 | 3.8 | C₁₆H₃₂O₇ + GSH            | Con 4.3 U9 1.6 U10 5.3 U19 66.8 |
| +513.21 | 4.0 | C₁₆H₃₂O₇ + GSH            | Con 5.3 U9 1.8 U10 3.7 U19 70.5 |
| +526.26 | 5.1 | C₁₆H₃₂O₇ + GSH            | Con 7.0 U9 1.5 U10 6.3 U19 13.0 |
| +536.17 | 4.0 | C₁₆H₃₂O₇ + GSH            | Con 10.5 U9 2.5 U10 3.7 U19 18.3 |
| +556.23 | 3.6 | C₁₆H₃₂O₇ + GSH            | Con 16.7 U9 4.0 U10 8.1 U19 13.0 |
| +572.26 | 4.5 | C₁₆H₃₂O₇ + GSH            | Con 669 U9 20.1 U10 3.7 U19 18.3 |
| +600.30 | 5.0 | OPDA + GSH                | Con 12.9 U9 27.7 U10 7.0 U19 20.1 |
| +606.30 | 5.25| C₁₆H₃₂O₇ + GSH            | Con 121 U9 35.9 U10 39.0 U19 70.1 |
| +602.31 | 4.85| Chlorogenic acid-SG        | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +602.31 | 5.1 | Chlorogenic acid-SG        | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +602.31 | 5.1 | Chlorogenic acid-SG        | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +616.29 | 4.8 | C₁₆H₃₂O₇ + GSH            | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +618.30 | 4.3 | C₁₆H₃₂O₇ + GSH            | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +626.21 | 2.9 | ? + GSH                   | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +627.23 | 2.5 | ?                         | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +640.25 | 5.1 | C₁₆H₃₂O₇ + GSH?            | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +648.22 | 3.5 | ? + GSH                   | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +794.26 | 5.5 | ? + GSH                   | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +815.28 | 4.0 | C₁₆H₃₂O₇ + GSH?            | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +836.38 | 4.6 | ?                         | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| +932.24 | 4.2 | ? + GSH                   | Con 12.7 U9 27.7 U10 34.6 U19 27.6 |
| -291.20 | 7.5 | HPOT (H₂O)                | Con 16.5 U9 22.4 U10 11.8 U19 176 |
| -293.21 | 8.9 | Colnelenic acid            | Con 21.6 U9 669 U10 892 U19 13.5 |
| -293.21 | 7.3 | Colnelenic acid            | Con 21.6 U9 669 U10 892 U19 13.5 |
| -307.19 | 7.95| C₁₆H₃₄O₄                 | Con 19.5 U9 500 U10 5.5 U19 23.8 |
| -327.22 | 4.9 | C₁₆H₃₂O₇                 | Con 14.3 U9 1.3 U10 30.9 U19 16.0 |

* d = doublet of peaks, t = triplet of peaks, m = multiple peaks.

DISCUSSION

Our results suggest that the binding of plant GSTUs to the S-alkylated glutathione derivatives utilized in affinity chromatography relates to their interactions with analogous conjugated products in vivo. This activity was widely exhibited in the Arabidopsis GSTUs, with 25 of the 28 class members leading to the aberrant accumulation of S-acylated glutathione derivatives of fatty acids when expressed in E. coli. Short-chain glutathione thioesters derived from the detoxification of formaldehyde (25) and methylglyoxal (26) are well known metabolites in both eukaryotes and prokaryotes. However, the long chain thioesters observed after the expression of the GSTUs in bacteria have not to our knowledge been identified as metabolites in vivo, although they have been chemically synthesized from acyl chlorides (13) and by transacylation from the respective CoA derivatives (27). The latter reaction suggests that the most likely origin of GSH thioesters in the E. coli screens was through acyl transfer, either from acyl-CoA or from acyl-carrier protein, to the acceptor GSH. Our results suggest that such transacylation in E. coli proceeds nonenzymically (27), with GSTU7 unable to enhance the rate of transfer of the fatty acid from myristoyl-CoA to GSH. As such, acyl-glutathione derivatives are likely to be formed under normal conditions in E. coli, albeit as unstable transient intermediates which undergo transacylation to CoA.
or acyl carrier protein acceptors or are acted on by thioesterases. In this instance, the ectopic expression of the GSTUs leads to the selective binding, stabilization, and accumulation of these unusual acyl derivatives.

In plants, mainstream fatty acid synthesis proceeds in the plastid, so it is not unexpected that S-acyl-glutathione thioesters were not identified in the expression studies in *N. benthamiana* and *Arabidopsis*, where the GSTUs are localized to the cytosol. Furthermore, plants contain a range of high affinity acyl CoA binding proteins (28), which would further limit the potential for the accumulation of significant amounts of the acyl-GSH intermediates. It, therefore, seems extremely unlikely that plant GSTUs play a major role in specifically binding S-acylated-glutathione derivatives in vivo. Instead, the discrimination of the GSTUs for acyl chain length, desaturation, and the presence of hydroxy groups appeared to more likely reflect their selective binding of glutathionylated oxidized fatty acid derivatives formed as both antimicrobial agents and signal compounds in plants during abiotic and biotic stress (29). Analogous to the S-acyl glutathione thioesters, these oxidation products exist in a diverse range of forms differing in chain length, degree of desaturation, and oxidation state. Using high resolution MS, the S-C18-glutathionylated derivatives were the most easily characterized metabolites, corresponding to conjugates of the oxylipins OPDA, oxo-octadecadienoic acid, and oxo-octadecatrienoic acid. In each case the most chemically likely conjugation of GSH was through Michael addition to the α,β-unsaturated carbonyl group (Fig. 3). Such GSH conjugates accumulate in plants fed with oxylipins or exposed to infection (30, 31). Although it is possible that GSTUs bind these conjugates after their spontaneous glutathionylation, our studies demonstrated that GSTU19 and other GSTUs actively catalyzed the formation of the OPDA conjugate. Similarly, the Phi class *Arabidopsis* GSTF8 also catalyzes the glutathionylation of OPDA (29). The physiological consequence of oxylipin conjugation in plants is unknown, although similar derivatives of the chemically related leukotrienes are known to be bioactive in mammals (32). It is also of interest that OPDA is an intermediate in jasmonic acid synthesis and that GSTU19 may have a role in regulating the passage of this compound from the chloroplast to the peroxisome (31).

In addition to the oxylipins, a number of other glutathionylated natural products were identified as binding to GSTUs in *N. benthamiana*. As defined by MS analysis, GSTU25 bound to a conjugate that was tentatively identified as S-hexanol-glutathione. Based on the literature (31), we can speculate that this conjugate has arisen from the glutathionylation of trans-2-hexenal, a compound released from membranes during oxidative damage (29). Presumably the conjugated aldehyde was then reduced to the alcohol, with related intermediates having been identified in wine must (33). Intriguingly, these compounds serve as precursors of the volatile organosulfur compound 3-mercaptotrihexanol, which is a high impact aroma chemical in passion fruit (34). In addition, GSH conjugates of metabolites, which were unrelated to fatty acid oxidation, were determined, notably, 2-S-glutathionyl-chlorogenic acid. Similar derivatives are produced when GSH is incubated with chlorogenic acid and peroxidases (14). In this case it, therefore, seems likely that such conjugates arise as a consequence of oxidative phenolic metabolism, with the GSTs binding the end products rather than playing an active role in their synthesis. In addition to glutathionylated ligands, GSTU9 and GSTU10 differed from the other GSTs in that they preferentially bound non-GSH conjugated fatty acid derivatives. The properties of these compounds matched the divinyl ether fatty acids colneleic acid and colnelenic acid, which are stress metabolites derived from the hydroperoxides of linoleic acid and linolenic acid, respectively, through the action of the cytochrome P450 enzyme divinyl ether synthase (24).

It is clear from this work that many of the GSTUs tested have a strong affinity for glutathione-derivatized fatty acids. Furthermore, the conserved selectivity in binding based on acyl chain length, degree of desaturation, and oxidation in the ligands identified in plants and bacteria strongly suggests that this role is physiologically relevant. For example, GSTU25 uniquely bound hydroxy-C6 conjugates in both plants and bacteria, whereas GSTU9 and GSTU10 both showed strong preferences for longer chain length ligands in both hosts. This further suggests that the role of these GSTs is to bind such acylated glutathione conjugates (catalyzing their formation if necessary or binding spontaneously formed conjugates) and presumably transport them to an appropriate compartment, perhaps through interaction with GSH conjugate transporters. Importantly, the reversible nature of Michael addition to oxo-fatty acids (35) means that substrates can subsequently be readily regenerated by removal of the GSH. Similarly, non-electrophilic ligands such as colneleic and colnelenic acids may also bind GSTs but without subsequent addition of GSH; however, GSH may be required for co-transport. Such findings have marked similarities to earlier studies showing selective roles for plant GSTs in transporting flavonoid metabolites in *planta* (36), suggesting that GSTs have broad-ranging activities as selective transporter proteins for unstable ligands derived from both primary and secondary metabolism.

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REFERENCES

1. McGonigle, B., Keeler, S. J., Lau, S. M., Koepp, M. K., and O’Keefe, D. P. (2000) *Plant Physiol.* **124**, 1105–1120

2. Edwards, R., and Dixon, D. P. (2000) in *Herbicides and Their Mechanisms of Action* (Cobb, A. H., and Kirkwood, R. C., eds) pp. 38–71, Sheffield Academic Press, Sheffield, UK

3. Holt, D. C., Lay, V. J., Clarke, E. D., Dinsmore, A., Jepson, I., Bright, S. W., and Greenland, A. J. (1995) *Planta* **189**, 295–302

4. Andrews, C. J., Cummins, I., Skipsey, M., Grundy, N. M., Jepson, I., Townson, I., and Edwards, R. (2005) *Pestic. Biochem. Physiol.* **82**, 205–219

5. Cummins, I., Cole, D. J., and Edwards, R. (1997) *Pestic. Biochem. Physiol.* **59**, 35–49

6. Pascal, S., Debrauwer, L., Ferte, M. P., Anglate, P., Rouimi, P., and Scalla, R. (1998) *Plant Sci.* **134**, 217–226

7. Edwards, R., and Dixon, D. P. (2005) *Methods Enzymol.* **401**, 169–186

8. Mozer, T. J., Tiemeier, D. C., and Jaworski, E. G. (1983) *Biochemistry* **22**, 1068–1072

9. Reinemer, P., Prade, L., Hof, P., Neuveind, T., Huber, R., Zettl, R., Palme, K., Schell, J., Koelln, I., Bartunik, H. D., and Bieseler, B. (1996) *J. Mol. Biol.* **250**, 937–954
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255, 289–309
10. Thom, R., Cummins, I., Dixon, D. P., Edwards, R., Cole, D. J., and Lapthorn, A. J. (2002) Biochemistry 41, 7008–7020
11. Edwards, R., Dixon, D. P., and Walbot, V. (2000) Trends Plant Sci. 5, 193–198
12. Dixon, D. P., Lapthorn, A., Madesis, P., Mudd, E. A., Day, A., and Edwards, R. (2008) J. Biol. Chem. 283, 20268–20276
13. Falck, J. R., Sangras, B., and Capdevila, J. H. (2007) Bioorg. Med. Chem. 15, 1062–1066
14. Panzella, L., Napolitano, A., and d’Ischia, M. (2003) Bioorg. Med. Chem. 11, 4797–4805
15. Dixon, D. P., Hawkins, T., Hussey, P. J., and Edwards, R. (2009) J. Exp. Bot. 60, 1207–1218
16. Clough, S. J., and Bent, A. F. (1998) Plant J. 16, 735–743
17. Dixon, D. P., Cole, D. J., and Edwards, R. (1998) Plant Mol. Biol. 36, 75–87
18. DeRidder, B. P., Dixon, D. P., Beussman, D. J., Edwards, R., and Goldsborough, P. B. (2002) Plant Physiol. 130, 1497–1505
19. Loutré, C., Dixon, D. P., Brazier, M., Slater, M., Cole, D. J., and Edwards, R. (2003) Plant J. 34, 485–493
20. Smith, A. P., DeRidder, B. P., Guo, W. J., Seeley, E. H., Regnier, F. E., and Goldsborough, P. B. (2004) J. Biol. Chem. 279, 26098–26104
21. Edwards, R., Del Buono, D., Fordham, M., Skipsey, M., Brazier, M., Dixon, D. P., and Cummins, I. (2005) Z. Naturforsch. 60, 307–316
22. Sappl, P. G., Oñate-Sánchez, L., Singh, K. B., and Millar, A. H. (2004) Plant Mol. Biol. 54, 205–219
23. Takahashi, Y., and Nagata, T. (1992) Plant Cell Physiol. 33, 779–787
24. Grechkin, A. N. (2002) Prostaglandins Other Lipid Mediat. 68–69, 457–470
25. Kordic, S., Cummins, I., and Edwards, R. (2002) Arch. Biochem. Biophys. 399, 232–238
26. Dixon, D. P., Cummins, L., Cole, D. J., and Edwards, R. (1998) Curr. Opin. Plant Biol. 1, 258–266
27. Pensalfini, A., Cecchi, C., Zampagni, M., Becatti, M., Favilli, F., Paoli, P., Catarzi, S., Bagnoli, S., Nacmias, B., Sorbi, S., and Liguri, G. (2008) Free Radic. Biol. Med. 44, 1624–1636
28. Leventis, R., Juel, G., Knudsen, J. K., and Silvius, J. R. (1997) Biochemistry 36, 5546–5553
29. Feussner, I., and Wasternack, C. (2002) Annu. Rev. Plant Biol. 53, 275–297
30. Davoine, C., Douki, T., Iacazio, G., Montillet, J. L., and Triantaphylides, C. (2005) Anal. Chem. 77, 7366–7372
31. Davoine, C., Falletti, O., Douki, T., Iacazio, G., Ennar, N., Montillet, J. L., and Triantaphylides, C. (2006) Plant Physiol. 140, 1484–1493
32. Wang, W., and Ballatori, N. (1998) Pharmacol. Rev. 50, 335–356
33. Peyrot, Des Gachons, C., Tominaga, T., and Dubourdieu, D. (2002) J. Agric. Food Chem. 50, 4076–4079
34. Tapp, E. J., Cummins, I., Brassington, D., and Edwards, R. (2008) J. Agric. Food Chem. 56, 6623–6630
35. Blackburn, M. L., Ketterer, B., Meyer, D. J., Juett, A. M., and Bull, A. W. (1997) Chem. Res. Toxicol. 10, 1364–1371
36. Alfenito, M. R., Souer, E., Goodman, C. D., Buell, R., Mol, J., Koes, R., and Walbot, V. (1998) Plant Cell 10, 1135–1149