Biochemical characterization of metabolism-based atrazine resistance in *Amaranthus tuberculatus* and identification of an expressed GST associated with resistance

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**Summary**

Rapid detoxification of atrazine in naturally tolerant crops such as maize (*Zea mays*) and grain sorghum (*Sorghum bicolor*) results from glutathione S-transferase (GST) activity. In previous research, two atrazine-resistant waterhemp (*Amaranthus tuberculatus*) populations from Illinois, U.S.A. (designated ACR and MCR), displayed rapid formation of atrazine-glutathione (GSH) conjugates, implicating elevated rates of metabolism as the resistance mechanism. Our main objective was to utilize protein purification combined with qualitative proteomics to investigate the hypothesis that enhanced atrazine detoxification, catalysed by distinct GSTs, confers resistance in ACR and MCR. Additionally, candidate *AtuGST* expression was analysed in an *F*$_2$ population segregating for atrazine resistance. ACR and MCR showed higher specific activities towards atrazine in partially purified ammonium sulphate and GSH affinity-purified fractions compared to an atrazine-sensitive population (WCS). One-dimensional electrophoresis of these fractions displayed an approximate 26-kDa band, typical of GST subunits. Several phi- and tau-class GSTs were identified by LC-MS/MS from each population, based on peptide similarity with GSTs from *Arabidopsis*. Elevated constitutive expression of one phi-class GST, named *AtuGSTF2*, correlated strongly with atrazine resistance in ACR and MCR and segregating *F*$_2$ population. These results indicate that *AtuGSTF2* may be linked to a metabolic mechanism that confers atrazine resistance in ACR and MCR.

**Introduction**

There are over 75 species in the genus *Amaranthus* found worldwide, including both monoecious and dioecious species (Mosyakin and Robertson, 2003). A dioecious species called waterhemp (*Amaranthus tuberculatus* (Moq.) Sauer var. *rudis* (Sauer) Costea & Tardif or syn. *A. rudis* Sauer) (Costea et al., 2005; Pratt and Clark, 2001) has become a major problem in the United States due to several ecological, biological, and genetic factors (Steckel, 2007). For example, waterhemp is difficult to selectively manage in maize and soybean (*Glycine max*) production systems because it is a summer annual with a prolonged germination period (Costea et al., 2005; Hartzler et al., 1999). In addition, the obligate outcrossing nature and dioecious biology of waterhemp facilitates the spread of genes conferring herbicide resistance via pollen flow throughout natural populations (Costea et al., 2005; Tranel et al., 2011). Multiple genes or alleles conferring resistance can occur within single waterhemp populations or individual plants due to strong herbicide selection pressures, resulting in multiple-resistant phenotypes (Heap, 2016). For example, resistance to herbicides that inhibit 4-hydroxyphenyl-pyruvate dioxygenase (HPPD), protoporphyrinogen oxidase, acetalactate synthase (ALS), EPSP synthase, photosystem II (PS II) and the auxin herbicide 2,4-D has been reported in waterhemp populations (Hausman et al., 2011; Heap, 2016; Patzoldt et al., 2005).

Atrazine is a commonly used herbicide for weed management in maize (LeBaron et al., 2011). PS II-inhibiting herbicides such as atrazine inhibit the light reactions of photosynthesis by competing with plastoquinone for the Q$_q$ binding site of the D1 protein (Fuerst and Norman, 1991; Hess, 2000), thus blocking the flow of electrons to cytochrome b$_6$f and subsequently triggering the rapid formation of triplet chlorophyll followed by singlet oxygen in the presence of light (Krieger-Liszkay, 2005; Triantaphyllides and Havaux, 2009) in sensitive dicots. In contrast, natural tolerance in maize and grain sorghum is due to the high constitutive activity of glutathione S-transferase (GSTs) that can use atrazine as a substrate, leading to rapid metabolic detoxification in these crops (Timmerman, 1989). The most common mechanism conferring atrazine resistance in dicot weeds is an insensitive target-site protein. A point mutation in the *psbA* gene (which encodes the D1 protein) frequently identified in atrazine-resistant weeds results in a SER to GLY mutation at amino acid 264, which confers an approximate 1000-fold level of resistance compared with sensitive biotypes (Devine and Preston, 2000; Hirschberg and McIntosh, 1983). By contrast, evolved resistance to atrazine in velvetleaf (*Abutilon theophrasti*) has been linked to elevated GST binding site of the D1 protein.
activity (Anderson and Gronwald, 1991; Gray et al., 1996). Similarly, GST-based detoxification mechanisms have also been documented in several resistant grass weeds (Cummins et al., 2013; Reade et al., 2004; Yu and Powles, 2014). Rapid metabolism of atrazine in multiple-resistant waterhemp resulted in a several hundred-fold resistance level compared to atrazine-sensitive plants (Evans, 2016).

GSTs are found in both plant and animals and are a widely studied class of primarily cytosolic (Mashiyama et al., 2014), dimeric enzymes mainly due to their detoxification abilities (Dixon et al., 2010; McGonigle et al., 2000; Wagner et al., 2002). Plant GST subunits belong to several different classes, including theta, zeta, lambda, phi, tau and glutathione-dependent dehydroascorbate reductases (DHARs), based on sequence similarity, essential catalytic residues and immunological cross-reactivity (Edwards and Dixon, 2005; Frova, 2006; Mashiyama et al., 2014). The most common subclasses of plant GSTs are the phi and tau classes (Labrow et al., 2015), although the relative proportions differ depending on species (Chi et al., 2011). Phi-class GSTs were among the first GSTs shown to catalyse herbicide detoxification reactions in maize (Fuerst et al., 1993; Holt et al., 1995; Izyk and Fuerst, 1993; Jeppson et al., 1994).

Previous research demonstrated that atrazine resistance in two populations of waterhemp from Illinois (designated ACR and MCR; Hausman et al., 2011) results from non-target-site resistance (NTSR) mechanism(s), as indicated by the lack of a mutation in the psbA gene and rapid accumulation of a polar metabolite with the same retention time (via reverse-phase HPLC) as a synthetic GSH-atrazine standard in resistant populations (Ma et al., 2013). Therefore, we hypothesize that rapid formation of this metabolite results from increased GST activity in ACR and MCR compared to an atrazine-sensitive population (WCS; Hausman et al., 2011) and that this increased activity results from either higher constitutive expression of GST(s) or the presence of novel GST isoforms with greater affinity towards atrazine. As a result, the objectives of this study were to (i) determine whether differences in GST activity exist between atrazine-resistant and atrazine-sensitive waterhemp populations, (ii) utilize ammonium sulphate (AMS) fractionation combined with GSH affinity chromatography to partially purify GSTs from each population and obtain peptide sequences, (iii) search a waterhemp transcriptome database to identify partial cDNA sequences encoding GSTs and (iv) determine whether expression of candidate GST(s) correlates with whole-plant phenotypic responses to atrazine in the greenhouse, using an F2 population segregating for atrazine resistance (Huffman et al., 2015). Our results demonstrate that basal expression levels of a single candidate gene, named AtuGSTF2, correlate strongly with the atrazine-resistant phenotype in ACR and MCR and segregating F2 population.

### Results

**GST activity towards atrazine in atrazine-sensitive and atrazine-resistant waterhemp populations**

GST activity using atrazine as a substrate was measured in each waterhemp population (MCR, ACR, and WCS). The McLean County, Illinois (MCR), and Adams County, Illinois (ACR), populations are both resistant to atrazine due to rapid GST-catalysed metabolism (Ma et al., 2013), while the Wayne County, Illinois (WCS), population is atrazine-sensitive. Specific activities were compared among crude plant extracts (prepared from leaves and petioles), AMS-precipitated fractions and GSH affinity-purified fractions (Table 1). Differences in total or specific activity were not detected between crude protein extracts prepared from the resistant populations (ACR and MCR) compared to WCS. However, GST-specific activities towards atrazine among the AMS fractions showed higher activity in ACR and MCR when compared to WCS, but a significant difference was not observed between ACR and MCR (Table 1). In order to further enrich and purify these fractions and attempt to isolate and identify unique or over-expressed GST isozymes in MCR and ACR, a GST affinity purification method was incorporated following AMS purification (Figure 1). This purification scheme was based on previous methods used to investigate the role of specific GST isozymes in herbicide tolerance in etiolated maize, grain sorghum and wheat shoots (Gronwald and Plaisance, 1998; Izyk and Fuerst, 1993; Riechers et al., 1997).

Specific activities increased significantly following each purification step. The largest fold-increase in specific activity occurred during affinity purification with MCR, resulting in a c.a. 9-fold purification relative to the crude extract (Table 1). Significant differences in specific activity between ACR or MCR and WCS were only determined in partially purified fractions (AMS and GSH affinity; Table 1). In addition, the specific activity yields measured in AMS fractions from ACR and MCR were greater than 100% (Table 1), consistent with the presence of GST inhibitors present in crude extract preparations. For example, this may occur when crude extracts contain many non-GST proteins, pigments, metabolites or other cell debris that inhibit the GSH-atrazine conjugation reaction in vitro, which are removed by AMS fractionation. After AMS precipitation, the 40%–80% fraction contained concentrated proteins that were readily soluble in buffer, with a significant removal of plant pigments, lipids and cell debris clearly visible. Partial protein purification was particularly evident when comparing the crude extract (CE) and AMS samples in Coomassie-stained gels, where the dominant band in the CE fraction (presumably the large subunit of Rubisco; Figure 2a) at approximately 53 kDa is absent in the AMS fraction. GST-specific activities measured in these fractions displayed a trend that was

| Table 1 GST activities partially purified from three different waterhemp (*Amaranthus tuberculatus*) populations, measured using atrazine as a substrate. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Purification step | Total activity (pmol) | Specific activity (pmol/min/mg) | Activity yield | Fold purification |
| WCS | ACR | MCR | WCS | ACR | MCR | WCS | ACR | MCR |
| Crude | 220.5 | 199.1 | 249.8 | 32.7 ± 1.4 | 36.0 ± 10.6 | 27.9 ± 0.9 | 100.0% | 100.0% | 100.0% | 1.0 | 1.0 | 1.0 |
| AMS | 87.0 | 353.8 | 263.9 | 33.5 ± 5.5 | 86.9 ± 7.3 | 101.9 ± 8.6 | 39.5% | 177.8% | 105.6% | 1.0 | 2.4 | 3.7 |
| GSH | 63.8 | 91.3 | 142.8 | 39.4 ± 9.1 | 163.1 ± 5.2 | 250.6 ± 8.0 | 28.9% | 45.9% | 57.2% | 1.2 | 4.5 | 9.0 |

Crude, crude extract; AMS, 40%–80% ammonium sulphate cut; GSH, glutathione-affinity column eluate.
expected based on prior atrazine metabolism assays in these populations (Ma et al., 2013), with each resistant population having higher specific activity than WCS (Table 1).

GSH affinity-purified fractions from each population showed a single, broad band at approximately 26 kDa when analysed by SDS-PAGE and silver staining (Figure 2b), which is within the typical range (23–32 kDa) of plant GST subunits (Edwards and Dixon, 2005; Li et al., 2013) with few extraneous bands. However, these affinity-purified fractions were presumed to include a heterogeneous mixture of GSTs and other cytosolic proteins (Figure 2b) with the ability to bind GSH (e.g. GSH reductase; Gill et al., 2013). Specific activity of these fractions followed the same pattern as with the AMS fractions (Table 1). However, fold-purification levels for ACR and MCR (4.5 and 9.0, respectively, relative to crude extracts) were lower than previously reported in cereal crops (Gronwald and Plaisance, 1998; Irzyk and Fuerst, 1993; Riechers et al., 1997; Timmerman, 1989) for affinity-purified GSTs.

Qualitative LC-MS analysis and identification of peptides from waterhemp GSTs

Excised SDS-PAGE gel slices containing the approximate 26-kDa protein bands from each population were subjected to protease digestion, LC-MS analysis and subsequent peptide identification via alignment with the *Arabidopsis* reference genome. From the 142 *Arabidopsis* sequence matches obtained from one representative experiment (Figure 3a), proteins were initially screened for reported molecular weights within the typical range of plant GST subunit masses (i.e. 23–32 kDa) and approximate size of the excised protein bands (Figure 2b). Based on protein identification probability scores and unique peptide sequences, several phi-class and tau-class GSTs were subsequently identified from each waterhemp population. The Venn diagram lists the distribution of matched proteins among the MCR, ACR and WCS samples, with proteins only identified in MCR and ACR highlighted and described further (Figure 3a).

The waterhemp peptide sequence KVLDDYEARL identified from MCR and ACR digests (Figure 3a) matched an *Arabidopsis* protein annotated in GenBank as AtGSTF2 (Gene ID: AT4G02520), a phi-class GST involved in various hormone and stress responses (Lieberherr et al., 2003; Smith et al., 2003, 2004) and binding of defence compounds (Dixon et al., 2011), and was the most probable match among all GSTs (Figure 3b). However, this diagnostic peptide is also found in AtGSTF3 and AtGSTF8 proteins (Table S1). The waterhemp peptide sequence RWADYDKKK provided a unique and second strongest protein match among GSTs (Figure 3b); it was annotated in GenBank as AtGSTU23 (accession D7KVA1_ARALL), a tau-class GST. Two additional tau-class GSTs were identified from peptides that were present only in ACR. The peptide SPILEMPHVKK uniquely matched AtGSTU25, and the peptides SSLLEMMPHVK and RIWAVKGEQAEAAK uniquely matched AtGSTU24.

Using these *Arabidopsis* GST protein sequences (identified using ACR and MCR peptides; Figure 1), best-matching cDNA sequences were then identified from a waterhemp transcriptome database.
(Riggins et al., 2010), which resulted in five contigs encoding partial phi- or tau-class waterhemp GSTs (Table S1). Five primer pairs (Table S2) were designed for RT-PCR to amplify the largest possible section of each contig (from 200–800 bp), which were named based on the putative GST subclass (described in Edwards et al., 2000). *Atu* (*A. tuberculatus*) was chosen to identify the species name for these five waterhemp GSTs to prevent confusion with *Arabidopsis thaliana*. Each of the five primer pairs generated amplicons of the predicted size from each waterhemp population except for AtuGSTF3, which was not investigated further.

RT-qPCR analysis of AtuGST expression in MCR, ACR and WCS populations

RT-qPCR was performed to determine whether the four remaining *Atu*GST genes differed in constitutive expression. Each of the four AtuGST cDNAs was amplified, and three of the four showed a single peak during melting-curve analysis, indicating that a single amplicon was formed. AtuGSTF1 was subsequently excluded from further RT-qPCR analysis due to the nonspecific formation of multiple PCR amplicons. Transcript levels of AtuGSTF2, AtuGSTU1 and AtuGSTU2 were then determined in ACR or MCR relative to their corresponding expression in WCS using a stably expressed β-tubulin reference gene (Table S1).

AtuGSTF2 transcript levels in ACR and MCR were approximately 1000-fold higher than in WCS, but a significant difference between transcript levels in ACR and MCR was not detected (Figure 4a). Significant differences were not detected in AtuGSTU1 transcript levels among populations (Figure 4b). In contrast, transcript levels of AtuGSTU2 were significantly higher in MCR than in ACR, but not between MCR and WCS or ACR and WCS.
(Figure 4c). Although not statistically different, a trend was observed where the abundance of AtuGSTU1 and AtuGSTU2 transcripts in MCR was higher than in WCS (Figure 4b–c). However, it is important to note that absolute expression levels of these two tau-class GSTs were much lower (about 30–300 fold lower for AtuGSTU1 and AtuGSTU2, respectively) when compared with AtuGSTF2 expression levels in ACR and MCR (Figure 4a).

Higher constitutive expression of AtuGSTF2 in ACR and MCR relative to WCS suggests that this gene (or allele) may contribute to higher levels of GST activity (Table 1) and rapid metabolism of atrazine (Ma et al., 2013) in these populations. As transcript abundance of AtuGSTF2 in ACR and MCR (relative to WCS) was the only case where a clear pattern or association existed between basal expression and phenotype (Figure 4), AtuGSTF2 was utilized as a candidate gene to further investigate its constitutive expression patterns in an F2 population of waterhemp (MCR x WCS) segregating for atrazine resistance as an incompletely dominant, qualitative trait (Huffman et al., 2015).

Phenotyping atrazine responses, constitutive AtuGSTF2 expression and genotyping in a segregating F2 population

Due to the large degree of variability at the highest atrazine rate tested (28.8 kg/ha), a discriminatory rate (14.4 kg/ha) was determined as optimal for distinguishing between resistant genotypes. In order to determine whether constitutive expression of AtuGSTF2 also correlated with phenotypic responses in the F2 population (Huffman et al., 2015), 10- to 12-cm plants were treated with foliar-applied atrazine at this rate. Treated plants revealed significant phenotypic differences among segregating F2 lines, as shown in Figure 5a and further described below. Plants from several F2 lines that rapidly developed healthy, new green tissue following atrazine treatment and were as tall as non-treated controls were tentatively assigned a homozygous (RR) atrazine-resistant genotype (Figure 5b). By comparison, plants from numerous F2 lines that developed less green meristematic tissue than RR lines following atrazine treatment, were stunted, and did not grow significantly taller after application were tentatively assigned a heterozygous (Rr) atrazine-resistant genotype (Figure 5c). Plants from several F2 lines died at this discriminatory rate within 7 days after treatment and were assigned an atrazine-sensitive (rr) genotype (Figure 5d).

Dry weight reductions and constitutive AtuGSTF2 expression (relative to WCS) in 10 representative lines from the F2 population are summarized in Table 2. Phenotypic responses resulting from atrazine treatment at 14.4 kg/ha correlated strongly with basal expression of AtuGSTF2 (Table 2). In general, dry weight (biomass accumulation) and expression data followed the same trend, where dry weights were much higher in putative RR plants (lines 11 and 22), mainly because these plants developed a significant amount of green tissue following atrazine treatment (Figure 5b). Furthermore, lines 10, 21, 23, 31 and 32 displayed intermediate dry weights and AtuGSTF2 expression values (Table 2), consistent with a putative Rr genotype. By comparison, rr lines did not accumulate biomass following atrazine treatment and eventually died at this rate (Figure 5d; Table 2).

These whole-plant results are consistent with the corresponding AtuGSTF2 expression levels in each line, which were extremely high in RR and Rr lines by comparison with rr lines (Table 2), ranging from approximately 200-fold (line 10) to 1140-fold (line 22) higher. When considering all RR and Rr lines together, the mean AtuGSTF2 expression value of 3304 units is 661-fold greater than the mean expression of 5 units for all rr lines. The large difference in AtuGSTF2 expression between resistant and sensitive genotypes indicates the robustness of utilizing this constitutively expressed gene as a marker for identifying metabolic-based atrazine-resistant genotypes in this F2 population, as well as in MCR, ACR and possibly other NTSR waterhemp populations yet to be analysed.

Exceptions to the overall strong association were noted, as evidenced by a weak fit within a discrete statistical category when considering both dry weight reductions and their AtuGSTF2 expression (e.g. lines 23 and 32; Table 2). Based on the statistical groupings and categorization of phenotypic responses displayed in the glasshouse (Table 2), however, genotypes assigned for the atrazine-resistance trait in these lines are consistent with the F2 population segregating for resistance in a 3 : 1 ratio (Huffman et al., 2015); viz. more Rr lines were identified than either homozygous genotype (RR or rr) from the original 32 F2 lines investigated.

Figure 4 RT-qPCR assays conducted for each target AtuGST gene using gene-specific primers (Table S3). Dissociation curves for each reaction were analysed to ensure only one replicon was amplified. Constitutive expression of (a) AtuGSTF2, (b) AtuGSTU1 and (c) AtuGSTU2 in MCR and ACR quantified relative to expression of the corresponding AtuGST in WCS using the 2-ΔΔCt method (Livak and Schmittgen, 2001) and β-tubulin (Tables S1, S3) as a constitutively expressed control. Bars represent the standard error of the mean.
Sequence analysis of individual AtuGSTF2 amplicons from parent waterhemp populations and individual F2 lines

Sequencing results from a total of 11 individual RT-PCR products (185-bp) indicated the presence of identical transcripts (AtuGSTF2.2) in each Rr or RR F2 line tested (5 total; lines 10, 11, 22, 31 and 32). In addition, four individual amplicons derived from the MCR population (four different plants) possessed this same sequence, which differed from the original waterhemp transcriptome sequence (AtuGSTF2.1) by one conservative amino acid change within this region (Figure S1a). In contrast, analysis of two different amplicons from two rr genotypes (lines 7 and 26) and four individual amplicons from the WCS population (two different plants) revealed three sequences; the AtuGSTF2.2 allele (from all resistant plants tested) and the AtuGSTF2.1 allele, plus an additional allele (AtuGSTF2.3) found only in line 26 (Figure S1a).

Sequence variants of AtuGSTF2 identified from the limited amount of amplicons are consistent with the dioecious, outcrossing nature of waterhemp. Sequence analysis of additional F2 lines and individual clones may yield more allelic variants of the genes listed in Figure S1a–b. However, the lack of polymorphisms among all AtuGSTF2 amplicons sequenced from atrazine-resistant plants thus far (15 total) suggests a single haplotype containing the R allele (AtuGSTF2.2) in MCR and the F2 population. This haplotype might occur if higher constitutive expression in resistant genotypes, as compared to sensitive genotypes, results from genetic variability that exists within the promoter (or untranslated regions) several kb upstream of the AtuGSTF2 gene (Mahmood et al., 2016). Further analysis is required, however, because the 185-bp AtuGSTF2 sequence represents c.a. 30% of the coding region (Figure S1c).

Sequence alignment of AtuGSTF2 with the partial amino acid sequence of AtuGSTF2.1 (Figure S1c) revealed 67% identity, although this is a preliminary comparison as the AtuGSTF2.1 sequence only represents a portion of the full-length protein. The sequence of maize ZmGSTF2, a phi-class GST (previously called maize GST II, GST IV, or GST-27), was included for comparison because its involvement in herbicide detoxification and stress responses has been well documented (Edwards et al., 2000; Holt et al., 1995; Irzyk and Fuerst, 1993; Jepson et al., 1994). Comparison of the full-length sequences of ZmGSTF2 and AtuGSTF2 showed 39% identity and comparison of ZmGSTF2 with the partial AtuGSTF2.1 sequence revealed 44% identity, which is within the expected range for interspecific comparisons of GSTs within a subclass (Labrou et al., 2015; Yang et al., 2009). It is important to note, however, that the c.a. 30% of AtuGSTF2.1 aligns closely with the N-terminus of AtuGSTF2 (Figure S1c). Plant GST sequences from the same subclass are strongly conserved in the N-terminal domain of the protein, which typically corresponds with Exon 1 of the genomic sequence (Frova, 2006; Labrou et al., 2015), relative to the C-terminus. Interestingly, the diagnostic phi-class waterhemp GST peptide KVLDVVYEARL is present in AtuGSTF2 and ZmGSTF2, although the ZmGSTF2 sequence contains one conservative amino acid change (Figure S1c).
Discussion

NTSR mechanisms to herbicides in weeds (such as enhanced herbicide detoxification) have drawn great interest in recent years, particularly in grass weed species (Cummins et al., 2013; Gaines et al., 2014; Reade et al., 2004; Yu and Powles, 2014). However, metabolic resistance in dicots is not well characterized and remains markedly under-explored, particularly regarding the underlying biochemical mechanisms, enzymes and specific genes in these species (Anderson and Gronwald, 1991; Gray et al., 1996; Ma et al., 2013). Atrazine resistance in dicots is typically conferred by a point mutation in the plastidic target-site gene psbA (encoding the D1 protein in PS II), leading to decreased atrazine binding (reviewed by Devine and Preston, 2000). In contrast to previous research aimed at sequencing psbA, our primary goal was to characterize total and specific GST activities from atrazine-resistant MCR and ACR populations and compare with activities in the atrazine-sensitive population WCS, thereby following up on previous atrazine metabolism findings (Ma et al., 2013).

In spite of a significant enrichment in specific activity in MCR and ACR (and WCS to a lesser extent) protein extracts throughout the purification scheme, fold-purification levels were much lower than previously reported in cereal crops (Gronwald and Plaisance, 1998; Izyk and Fuerst, 1993; Riechers et al., 1997; Timmerman, 1989) for affinity-purified GSTs. These lower fold-purification levels in our research may have resulted from use of photosynthetic tissues instead of etiolated seedling, shoot or coleoptile tissues from cereals (Izyk and Fuerst, 1993; Riechers et al., 1997) or loss of activity during sample processing following initial extract preparation through GSH affinity purification as protein fractions become more dilute. However, these results establish a framework for continued mechanistic investigations of evolved resistance to atrazine, other pesticides or metabolism of environmental/endogenous toxins by GSTs in weedy Amaranthus. In addition, these findings pave the way for new biotechnology applications aimed towards overcoming metabolic resistance in weedy plants, as described in detail below.

Possible underlying mechanisms for elevated basal AtuGSTF2 expression in waterhemp

AtuGSTF2 displayed higher constitutive expression in both atrazine-resistant waterhemp populations as well as in resistant F2 lines segregating as a single-gene trait. Greater transcript abundance of the AtuGSTF2 gene may contribute to elevated GST activity (Table 1) and higher levels of GSH-atazine metabolites formed in ACR and MCR compared with WCS (Ma et al., 2013). Thus far, higher GST-specific activities with atrazine quantified in partially purified ACR and MCR protein extracts can only be associated with higher constitutive expression of AtuGSTF2. Further experiments are required, however, to obtain the entire open reading frame for expression and biochemical analyses of the recombinant AtuGSTF2 enzyme (with atrazine as substrate) because plant genomes contain dozens of GST genes and isozymes (Chi et al., 2011; McGonigle et al., 2000; Riechers et al., 2010) that contribute to total activity. From the standpoint of gene regulation, the potential for induction of AtuGSTF2 expression by atrazine pretreatment in waterhemp should be examined in future research.

Higher basal expression levels of AtuGSTF2 could be due to a mutation, insertion or varying degrees of methylation in the AtuGSTF2 promoter or untranslated regions (Mahmood et al., 2016), an alteration in a DNA-binding protein, or a protein regulating mRNA stability. GSTs are unevenly dispersed throughout plant genomes (Gronwald and Plaisance, 1998), which was confirmed to confer resistance to DDT and cross-resistance to pyrethroid insecticides in the mosquito Anopheles funestus (Riveron et al., 2014). A genomewide transcriptional analysis was conducted in which the most highly upregulated gene was identified as a GST (termed GST62), which was confirmed to confer resistance to DDT and cross-resistance to pyrethroid insecticides through transgenic expression in sensitive Drosophila. The molecular basis for resistance resulted from both quantitative and qualitative mechanisms; increased expression in resistant

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mosquitos combined with a point mutation in the wild-type GSTe2 gene in which LEU was substituted for PHE (Riveron et al., 2014).

In addition to improved resistance screening methods, the coding sequence of AtuGSTF2 could be utilized to engineer targeted gene knockout strategies such as RNAi directed to a specific Amaranthus GST (Yu and Powles, 2014) or to synthesize new chemical inhibitors of herbicide-detoxifying GSTs (Cummins et al., 2013; Lamoureux and Rusness, 1986; Ma et al., 2016). RNAi-based knockdown techniques have been used successfully in insect systems where insecticide-detoxifying P450s have been targeted (Bautista et al., 2009; Zhu et al., 2016), thus regaining activity of the insecticide in resistant populations. Polynucleotide-based gene knockdown systems are also being generated to overcome herbicide resistance in weeds (Sammons et al., 2015), which to date have been targeted primarily towards herbicide target-site proteins. However, additional knowledge of specific herbicide-detoxifying isozymes in weeds, such as those belonging to large, multigene GST and P450 families, provides a new opportunity to regain herbicide activity in multiple-resistant weeds. The findings presented herein support the conclusion that increased basal expression of a specific herbicide-detoxifying GST is associated with atrazine resistance in MCR and ACR, which may ultimately confer atrazine resistance, but might also lead to innovative and integrated weed management strategies.

**Experimental procedures**

**Plant populations**

Waterhemp populations used in these experiments were from McLean County (atrazine-resistant [MCR]), Adams County (atrazine-resistant [ACR]) or Wayne County (atrazine-sensitive [WCS]) Illinois, USA, as described previously (Hausman et al., 2011; Ma et al., 2015). Seeds were germinated in 12 × 12-cm containers with a commercial potting medium in a growth chamber using previously described methods (Ma et al., 2013). Waterhemp seedlings (2-cm) were transferred to 80-cm³ containers with a commercial potting medium in a growth chamber containing the same medium. When seedlings reached 4-cm, plants were transferred to 950-cm³ containers in the glasshouse containing the same medium. When seedlings reached 10- to 12-cm in height) for subsequent protein and enzyme extraction. Growth chamber and glasshouse conditions were maintained at 28 °C/22 °C day/night with 16/8-h photoperiod. Growth chamber light was provided by incandescent and fluorescent bulbs delivering 550 μmol/m²/s photon flux at plant canopy level. Natural glasshouse light was supplemented with mercury halide lamps, with a minimum output of 500 μmol/m²/s photon flux at plant canopy level.

**Tissue homogenization, crude protein extraction and enrichment of GST activity**

Plants from each population were harvested as described above and stems were removed due to interference with mechanical disruption. All leaves (including the attached petioles) were frozen in liquid nitrogen and pulverized. Polyvinylpolypyrrolidone (7.5% w/v) was added along with 3 mL of protein extraction buffer (100 mM Tris-HCl (pH 7.0), 1 mM DTT, 1 mM Na₂EDTA) per gram fresh weight. Samples were then filtered through three layers of cheesecloth and centrifuged at 3000 g for 30 min at 5 °C to obtain a clarified crude extract.

Clariﬁed extracts were used to prepare 40%-80% AMS fractions based on protein solubility in water at 5 °C. Initial experiments determined more than 90% of GST activity (with atrazine as substrate) precipitated in this range, which is similar to previously published reports of plant GST purification (Edwards and Dixon, 2005; Riechers et al., 1997; Smith et al., 2004). After slowly adding AMS during fractionation, samples were stirred for 20 min at 5 °C then centrifuged at 3000 g for 30 min at 5 °C. Pelleted material from the 40% cut was discarded as well as the soluble fraction following the 80% cut. The remaining pellet from the 80% cut was then desalted in protein storage buffer (100 mM Tris-HCl (pH 7.0), 1 mM DTT, 1 mM Na₂EDTA) using 7K MWCO Zeba™ Spin Desalting Columns (Thermo-Fisher Scientific, Waltham, MA, USA) and stored at −80 °C.

**GSH affinity purification and SDS-PAGE analysis**

Desalted and concentrated protein samples from each population obtained after AMS precipitation were further purified by immobilized GSH-Sepharose affinity chromatography (GSTrap™ FF columns, General Electric-Life Sciences, Pittsburgh, PA, USA) using a BioLogic DuoFlow fast-protein liquid chromatography (FPLC) system (Bio-Rad Laboratories, Hercules, CA, USA). A modification of the manufacturer’s recommended protocol for affinity chromatography was developed and utilized. The column was equilibrated with 5 volumes of binding buffer (140 mM NaCl, 10 mM NaHPO₄, 1.8 mM KH₂PO₄, and 2.7 mM KCI, pH 7.4). Protein samples (0.1–3.0 mg) were then loaded on the column and washed with 10 column volumes of binding buffer at a flow rate of 5 mL/min. Bound proteins were eluted with 10 volumes of elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM GSH, 1 mM DTT) at a flow rate of 5 mL/min and samples were then immediately desalted into protein storage buffer as described above. Protein concentration was measured with a Nanodrop spectrophotometer using bovine serum albumin (BSA) as standard. SDS-PAGE was conducted according to the method of Laemmli (1970) utilizing 12% resolving mini-gels (Bio-Rad, Hercules, CA, USA) to examine purity of each fraction. Proteins were visualized with either Coomassie Brilliant blue or silver stained according to the manufacturer’s protocol (FASTSilver™ kit, G-BioSciences, St. Louis, MO, USA).

**GST-atrazine activity assay**

A modified version of a previously published protocol was used to measure GST activity towards atrazine (Gronwald and Plaisance, 1998). Reactions (300 μL) contained 50 μM 14C-atrazine (2 mCi/mmol specific activity), 2 μM GSH, 100 μM Na-citrate (pH 6.5), 1 μM 2-mercaptoethanol, 0.2 mg/mL BSA and protein with a final concentration ranging from 0.05–1 mg/mL. Negative controls to determine nonenzymatic conjugation of GSH with atrazine were assayed for each replicate by replacing the protein fraction with extraction buffer. After incubation for 5 min at 30°C, reactions were initiated by addition of 14C-atrazine and incubated at 30°C for 30 min. Initial experiments under these conditions determined that product formation was linear until this time point. Reactions were terminated with 50 μL acetic acid and then partitioned against 900 μL methane chloride to separate parent atrazine from the atrazine-GSH conjugate. Radioactivity in a 200-μL aliquot of the aqueous phase was quantified by liquid scintillation spectrometry (LSS). Enzymatic conjugation rates were determined by averaging radioactivity found in the aqueous phase of the negative control reactions (i.e. no protein added) and subtracting this amount from the total activity measured in
the experimental reactions. Specific activity was determined based on protein concentration and nonenzymatically corrected radioactivity quantified in the aqueous phase by LSS. Units of GST activity are reported as pmol of GSH-conjugated atrazine per minute per mg protein. GST activity results represent the combined data from two independent experiments with three technical replications per assay.

Sample preparation, LC-MS/MS and bioinformatic analysis of peptide sequences
Coomassie-stained bands, representing proteins from each population eluting from GSH affinity columns (∼100 ng), were manually excised from one-dimensional SDS-PAGE gels. Protein fractions were analysed using nanoLC-MS/MS by the Stanford University Mass Spectrometry Laboratory (https://mass-spec.stanford.edu/proteomics) as described below. Gel slices were diced into 1-mm² sections, rinsed multiple times with 50 mM ammonium bicarbonate at 55 °C for 30 min. Residual solvent was removed and alkylation performed using 10 mM propionamide in 50 mM ammonium bicarbonate for 30 min at 23 °C. Gel pieces were rinsed with 50% acetonitrile and 50 mM ammonium bicarbonate and dried under vacuum for 5 min. Protein digestion was performed with trypsin/LysC (Promega Corporation, Madison, WI, USA) overnight at 37 °C. Tubes were centrifuged, and the supernatant including peptides was collected. Further peptide extraction was performed by the addition of 60% acetonitrile, 39.9% water, 0.1% formic acid and incubation for 10–15 min. Peptide pools were dried, concentrated and reconstituted for further analysis.

Digested peptides were injected onto a C18 reversed-phase analytical column (20 cm in length; 100 μm internal diameter). HPLC was performed with an Eksigent nanoLC at a flow rate of 600 nL/min using a linear gradient from 4% (mobile phase B) to 35% (mobile phase B). Mobile phase A consisted of 0.1% formic acid/water and mobile phase B was 0.1% formic acid/acetonitrile. All data were collected using an LTQ Orbitrap Velos mass spectrometer set to acquire data in a data-dependent fashion, selecting and fragmenting by CID the most intense precursor ions optimized to maximize duty cycle. An exclusion window of 60 s was used to improve proteomic depth and multiply charged ions were excluded based on protein concentration and nonenzymatically corrected radioactivity quantified in the aqueous phase by LSS. Units of GST activity are reported as pmol of GSH-conjugated atrazine per minute per mg protein. GST activity results represent the combined data from two independent experiments with three technical replications per assay.

Total RNA extraction, RT-PCR and initial contig analysis
Total RNA was extracted from waterhemp tissues and prepared using previously described methods (Riechers et al., 2003). Total RNA concentrations were determined using a NanoDrop spectrophotometer and rRNA quality was confirmed by visual analysis in agarose-formaldehyde gels. First-strand cDNA synthesis was performed using the Maxima H-Minus cDNA synthesis kit (Thermo-Fisher Scientific, Waltham, MA, USA) following the manufacturer’s protocol using 500 ng total RNA. The following parameters were then used for RT-PCR, with 1 μL of first-strand cDNA reaction: initial denaturing at 95 °C for 4.5 min, then 30 amplification cycles consisting of 95 °C for 5 s, 56 °C for 5 s and 72 °C for 1 min, followed by final extension at 72 °C for 8.5 min. RT-PCR products were visualized with 1% agarose gels stained with ethidium bromide.

Semi-quantitative RT-PCR (Riechers et al., 2003) was utilized first to determine whether the five matched AtuGST contigs identified in the waterhemp transcriptome (Riggins et al., 2010) were expressed in each waterhemp population. Five primer pairs (Table S2) were designed to amplify the largest possible section of each GST contig (ranging from 200–800 bp). A β-tubulin reference gene (AtuBTUB1; Table S1) with highest similarity to an Arabidopsis tubulin beta-7 chain (AtuBT7; Gene ID: AT2G29550) was selected from among several annotated tubulin contigs from waterhemp (Lee et al., 2009; Riggins et al., 2010) based on initial RT-PCR screening. Additionally, crosses between primers matching the same contig (i.e. forward primer for AtuGSTF1 with reverse primer for AtuGSTF2; Table S2) were used to confirm that individual waterhemp contigs originated from the same mRNA template.

RT-qPCR analysis of AtuGST expression in MCR, ACR and WCS populations
RT-qPCR was performed with total RNA isolated from each waterhemp population using the same tissues and growth stage as described previously. Gene-specific primers were redesigned to specifically amplify the AtuGSTs identified (based on the SYBR Green protocol for RT-qPCR; Table S3), although original primers for specifically amplifying AtuGSTF2 were retained. Stable, constitutive expression of AtuBTUB1 was demonstrated under the experimental conditions and waterhemp growth stage used in these studies as determined by <1-fold magnitude of differences in CT values. Primer efficiencies for RT-qPCR ranged from 95% to 99% for AtuGSTF2 and AtuBTUB1 amplifications from cDNA (Table S3). RT-qPCR was conducted using the 7900 HT Sequence Detection System (PerkinElmer, Applied Biosystems, Waltham, MA, USA) and reactions performed in 20 μL volumes following the manufacturer’s protocol (Syber® Green RNA-to-C™ 1-Step Kit; Applied Biosystems, Waltham, MA, USA). The protocol was as follows: 48 °C for 30 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min and a melting curve at 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s. Dissociation curves for each reaction were analysed to ensure only one replicon was amplified. Gene expression in each sample was calculated relative...
to transcript levels in WCS and the AtuBTUB1 reference gene using the 2−ΔΔCt method (Livak and Schmittgen, 2001).

For analysis of AtuGSTF1, AtuGSTU2 and AtuGSTF2 expression in the MCR, ACR and WCS populations, expression data represent the combined results from three independent experiments (i.e. biological replicates) with three technical replications for each RNA sample. ANOVA followed by Tukey’s multiple comparison test (P = 0.05) was conducted to determine significant differences in gene expression. For analysis of AtuGSTF2 expression in segregating F2 lines, experiments were independently conducted twice with three technical replications per RNA sample. Data from each experiment were combined and AtuGSTF2 relative expression values were analysed by LSD (P = 0.1) using PROC GLM in SAS (Release 9.2) to determine significant differences among F2 lines.

**Phenotyping of whole-plant responses to atrazine in an F2 population**

A total of 32 F2 plants were randomly selected from the original MCR x WCS cross (Huffman et al., 2015). Vegetative clones derived from these original F2 ‘parent’ plants were used to study segregation of whole-plant responses and gene expression due to the large amount of genetic variability in waterhemp (Ma et al., 2013; Steckel, 2007). When sufficient clones had been generated to represent each F2 line (Ma et al., 2015), a dose–response study was conducted to compare the response of the 32 F2 lines to foliar-applied atrazine. When plants reached 10–12 cm in height, they were treated with atrazine at rates evenly spaced along a 3.16 log scale (Hausman et al., 2011), ranging from 3.2 g/ha to 10 000 g/ha, and included 1% crop oil concentrate (COC) as a spray adjuvant. Control plants were treated with water plus COC only.

This initial study broadly determined which F2 lines were sensitive or resistant to atrazine. GR50 values for the sensitive lines ranged from 25 to 69 g/ha, significantly lower than the maximum field-use rate of 2.2 kg/ha (Ma et al., 2016). However, complete death was never achieved in atrazine-resistant lines, and estimated GR50 values for all resistant lines were greater than the field-use rate. A discriminatory rate was then determined to distinguish between RR and Rr plants. Atrazine rates ranged from 1.2 kg/ha to 28.8 kg/ha and included 1% COC and 2.5% liquid AMS as spray adjuvants. Due to the large degree of variability at the highest rate tested, the discriminatory rate of 14.4 kg/ha was determined for distinguishing between resistant genotypes. By comparison, a much lower rate of atrazine (985 g/ha) had been used previously to distinguish between resistant (RR and Rr) and sensitive genotypes in this F2 population (Huffman et al., 2015), but a different growth medium and nutrient system was utilized compared with the methods described herein. Experiments were independently conducted at least twice with five replications per treatment. Aboveground biomass was harvested at 12 DAT, dried in an oven at 65 °C, and dry weight data were combined and analysed by LSD (P = 0.1) using PROC GLM in SAS (Release 9.2) to determine significant differences among F2 lines.

**Sequence analysis of individual AtuGSTF2 and AtuBTUB amplicons from cDNA**

Total RNA was extracted from nontreated waterhemp tissues (using methods described earlier) from different F2 lines, with at least three representative lines from each putative genotype. Gene-specific primers (Table S3) were used to amplify AtuGSTF2 and AtuBTUB alleles using methods described earlier for RT-qPCR. RT-PCR products were purified directly from each reaction using the QIAquick™ PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Purified amplicons were then ligated into a pcRX™-4-TOPO cloning vector and transformed into competent E. coli cells (TOPO TA™ Cloning Kit, Invitrogen, Waltham, MA, USA). Plasmids were purified using the I-Basi Mini Plasmid Kit (IBI Scientific, Peosta, IA, USA) and submitted for sequencing. Amplicons were sequenced from a total of seven different F2 lines (two RR, three Rr and two rr), plus the original MCR and WCS populations, originating from at least two different colonies per transformation reaction.

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**Conflict of interest**

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Partial cDNA sequences of AtuGSTF2 and AtuBTUB1 alleles expressed in several F2 lines from the segregating population described by Huffman et al. (2015).

Table S1. Proposed gene nomenclature and GenBank accession numbers (for sequenced allelic variants) for waterhemp cDNAs (AtuGSTS and AtuBTUB1), best-matched GSTs from Arabidopsis, and waterhemp contig information. Contigs are derived from a waterhemp transcriptome database (Riggins et al., 2010; https://www.ncbi.nlm.nih.gov/sra/5RX018843).

Table S2. Primers used for waterhemp contig analysis and initial screening approach via semi-quantitative RT-PCR.

Table S3. Primers used for candidate gene expression analysis via RT-qPCR.