Several grass and broadleaf weed species around the world have evolved multiple herbicide resistance at alarmingly increasing rates, which is becoming more of a norm than an exception. Research on the biochemical and molecular resistance mechanisms of multiple resistant weed populations indicate a prevalence of herbicide metabolism brought about by enzyme systems such as cytochrome P450 monooxygenases and glutathione-S-transferases, and to a lesser extent, by glucosyl transferases. A symposium was conducted to gain an understanding of the current
state of research affairs of metabolic resistance mechanisms in weed species that pose major
management problems around the world. These topics as well as future directions of
investigations that were identified in the symposium are summarized herein. In addition, the
latest information on selected topics such as the role of safeners in crop tolerance to herbicides,
selectivity to clomazone, glyphosate metabolism in crops and weeds, and bioactivation of natural
molecules is reviewed.

Keywords: Crop tolerance, cytochrome P450, glutathione, glutathione-S-transferase, natural
phytotoxin, herbicide safener, oxylipin

A common mode of tolerance to herbicides in agronomic crops is by metabolism brought
about by enzyme systems such as cytochrome P450 monoxygenases (CYPs), glutathione S-
transferases (GSTs), and glucosyl transferases (GTs). These enzymes as well as cofactors such as
reduced glutathione (GSH) are activated by certain chemicals called safeners. Safeners are
applied in combination with herbicides to provide tolerance to grass crops such as wheat
(Triticum aestivum L.), rice (Oryza sativa L.), corn (Zea mays L.), and grain sorghum [(Sorghum
bicolor (L.) Moench.] against certain thiocarbamate, chloroacetamide, sulfonylurea (SU), and
aryloxyphenoxypropionate (AOPP) herbicides applied preemergence (PRE) or postemergence
(POST). Metabolism of herbicides usually occurs in three phases: a conversion of the herbicide
molecule into a more hydrophilic metabolite (Phase 1), followed by conjugation to biomolecules
such as glutathione/sugar (Phase 2), and further conjugation/breakup/oxidation reactions
followed by transport to vacuoles or cell walls where additional breakdown or sequestration
occurs (Phase 3).
The next and most important phase after the confirmation of herbicide resistance in a weed population is the deciphering of the underlying resistance mechanism(s), which can greatly determine the effectiveness of resistance management strategies. One of the common mechanisms of resistance is metabolic deactivation, whereby the herbicide active ingredient is transformed to nonphytotoxic metabolites (Yu and Powles 2014).

An immediate and urgent challenge for weed scientists is to understand and characterize the basis of metabolic resistance in order to sustain the limited herbicide portfolio and develop integrated weed management strategies. Metabolic resistance research in weeds has mostly been limited to grass species such as rigid ryegrass (Lolium rigidum Gaudin), blackgrass (Alopecurus myurosoides Huds.), and Echinochloa spp. However, dicot species such as tall waterhemp [Amaranthus tuberculatus (Moq.) Sauer] and Palmer amaranth [Amaranthus palmeri (S.) Wats.] have evolved resistance to herbicides with different mechanisms of action by enhanced metabolic degradation. Thus, both grass and dicot weed species that develop metabolic herbicide resistance can pose a severe management challenge.

The main objective of this symposium was to gain an understanding of current research on metabolic resistance in weeds by revisiting the history of related research including crop tolerance, reporting recent advances, and identifying future research opportunities.

Complex Signaling, Defense, and Detoxification Pathways in Safener-Treated Grain Sorghum Shoots

Induction of herbicide-detoxification enzymes catalyzing Phases I-III metabolic reactions by safeners is well documented (Cummins et al. 2011; Theodoulou et al. 2003; Zhang et al. 2007).
and has been reviewed extensively in recent years (Kraehmer et al. 2014; Riechers et al. 2010; Riechers and Green 2017). However, signaling genes and pathways leading to safener-induced herbicide metabolism have remained mostly elusive to identify. Recent research findings have indicated oxidized lipids, or oxylipins, play an important role in plant defense responses to abiotic and biotic stresses (Hou et al. 2016; Mueller and Berger 2009) and may also play a key role in safener-mediating signaling (Brazier-Hicks et al. 2018; Matsumoto et al. 2015; Riechers et al. 2010; Skipsey et al. 2011). In addition to oxylipins, plant hormones such as salicylic acid (SA) and jasmonic acid (JA) regulate many plant responses to pathogen attack or herbivore injury (Gao et al. 2015; Koo 2018; Larrieu and Vernoux 2016) and may also function in safener-regulated responses (Behringer et al. 2011). Although a precise signaling cascade has yet to be established for safener-regulated induction of herbicide detoxification in cereal crops, several new areas have emerged that will likely be the subject of future research, as described below.

**Oxylipins and their Involvement in Safener-Mediated Signaling.** A key finding from research in the mid-2000s was that several classes of oxylipins (Mosblech et al. 2009; Mueller 2004) are detected in plants following exposure to stresses, and subsequent work demonstrated that oxylipins induce the expression of plant defense and detoxification genes that mimic safener-induced genes and proteins (Loeffler et al. 2005; Mueller et al. 2008; Riechers et al. 2010; Zhang et al. 2007). Two major categories of oxylipins have been detected in plants (Cuyamendous et al. 2015; Durand et al. 2011; Mosblech et al. 2009; Mueller and Berger, 2009): (1) phytoprostanes and phytofurans, which are categorized based on their non-enzymatic formation via interaction of reactive oxygen species (ROS) with α-linolenic acid, (ALA), or (2) the enzymatic conversion of ALA to 12-oxo-phytodienoic acid (OPDA) and subsequent β-
oxidation to yield JA (Figure 1). Interestingly, the enzyme catalyzing conversion of OPDA to
OPC-8:0 (the precursor of JA), OPDA reductase (OPR), has been frequently identified in
transcript or protein profiling studies of plant responses to stress (Okazaki and Saito 2014; Taki
et al. 2005; Yan et al. 2012), including safener-treated plants and tissues (Riechers et al. 2010;
Rishi et al. 2004; Zhang et al. 2007).
Recent research has investigated possible links between oxylipin-mediated defense signaling
and safener mechanism of action. The tau-class AtGSTU19 enzyme catalyzed the conjugation of
GSH to OPDA (Dixon and Edwards 2010), leading to a reduction in its reactivity. As mentioned
above, OPR enzymes reduce the double bond in the cyclopentenone ring of OPDA, resulting in
an analogous reduction in reactivity (i.e., electrophilicity) but also leading towards biosynthesis
of JA (Mueller and Berger 2009). Root cultures from *Arabidopsis* mutants defective in fatty acid
desaturation (*fad*3-2/*fad*7-2/*fad*8), which are impaired in forming the oxylipin precursor ALA,
demonstrated a decreased ability to respond to safener treatment when measuring *AtGSTU24*
expression compared with wild type *Arabidopsis* (Skipsey et al. 2011). Since these *fad* mutants
accumulate linoleic acid (18:2) instead of ALA (18:3), they are unable to synthesize OPDA or
phytoprostanes from ALA substrate released via lipase activities (Christeller and Galis 2014).
The decreased ability of these mutant lines to respond to safener treatment via induction of *GST*
expression is consistent with a link between safener-regulated responses and endogenous
oxylipin signaling.
Based on the literature regarding oxylipin-regulated gene expression (Mueller and Berger
2009) and recent results with *fad* mutants in *Arabidopsis* (Skipsey et al. 2011), it was postulated
that certain oxylipins may not only rapidly induce genes involved in herbicide detoxification
pathways but may also confer safener activity in cereals (Brazier-Hicks et al. 2018; Riechers et
al. 2010). To directly test this hypothesis, a series of compounds modeled on oxylipin structures was chemically synthesized and tested for biological activity as herbicide safeners in rice (Brazier-Hicks et al. 2018), in comparison with the commercial rice safener fenclorim. Three of the 21 compounds tested rapidly induced GST expression in Arabidopsis, but only showed minor whole-plant safening activity against pretilachlor herbicide in rice seedlings. In addition to possible species-specific differences in responses to these potential crop safening compounds (Brazier-Hicks et al. 2018), metabolic pathways and turnover rates of oxylipins (Dueckershoff et al. 2008) may differ significantly from those of commercial safeners in cereal crop seedlings tissues (Miller et al. 1996; Riechers et al. 2010), therefore requiring further investigations.

Organ- and Tissue-Specific Expression of Safener-Induced Herbicide Detoxification Enzymes. As described previously, although the precise signaling pathway(s) that regulate gene expression within herbicide detoxification pathways have not been elucidated, previous research demonstrated that tau-class GST proteins and GST enzyme activities involved in herbicide detoxification are highly expressed in the outermost cells of wheat seedling coleoptiles after safener treatment (Riechers et al. 2003). Interestingly, similar results were found in safener-treated sorghum coleoptiles using the same tau-class wheat GST antiserum (Figure 2). Additional research examining stress-responsive gene expression in Arabidopsis cell cultures (Mueller et al 2008) and protein abundance in leaves (Dueckershoff et al. 2008) showed that oxylipins (such as phytoprostanes or OPDA) trigger detoxification and defense responses in a manner similar to safener treatments. As a result, current experiments were designed to test the hypothesis that safeners and phytoprostanes induce GST activity and the expression of genes related to plant defense and detoxification in sorghum shoot coleoptiles in an analogous manner.
A novel cryostat-microtome sectioning method was developed to extract high-quality RNA from the outermost cells of frozen coleoptiles (excluding leaf tissues) for transcript profiling to enrich for safener- and phytoprostane-responsive mRNAs at different time points after treatment (Riechers et al. 2018). Current localization experiments are utilizing an antiserum raised against a specific phi-class sorghum GST isozyme (SbGSTF1) to further investigate tissue-specific expression of different GST subclasses (Labrou et al. 2015) in safener-treated sorghum seedling tissues (as shown in Figure 2).

Initial RNAseq results have identified >10-fold increases in transcripts of several detoxification genes, including multiple GSTs, P450s, and GTs, in safener- or phytoprostane-treated seedlings compared with untreated controls (Riechers et al. 2018). Moreover, transcripts encoding proteins related to plant development and defense were highly upregulated by safener, such as enzymes involved in lipid signaling (including OPRs), hormone-related processes (i.e., synthesis of benzoic acid and salicylic acid), or auxin metabolism and homeostasis. Transcripts encoding biosynthetic enzymes possibly involved in chemical defense mechanisms in roots (Cook et al. 2010) and shoots (Busk and Möller 2002; Halkier and Möller 1989) of sorghum seedlings were also strongly induced by safener treatment in coleoptile tissues (Riechers et al. 2018). These results indicate that safeners may be utilizing signaling pathways and enzymatic mechanisms related to generating allelochemicals (Baerson et al. 2005) or other defense chemicals against abiotic or biotic stresses, as well as up-regulating enzymes with the putative function of preventing auto-toxicity from these chemicals in sorghum seedlings (Bjarnholt et al. 2018).
Future Research Directions. Ongoing analyses using bioinformatics and comparative gene expression approaches are aimed at further mining these RNAseq data to provide additional insight into how transcriptional responses are reprogrammed in sorghum coleoptiles following safener or phytoprostane treatment (Baek, Ma, and Riechers, unpublished results). An emerging hypothesis resulting from this research is that safeners regulate a specific, coordinated, and rapid defense and detoxification response following safener treatment in cereal crop seedlings, which includes both up- and down-regulation of gene expression in response to safener treatment. This research is helping to elucidate the yet-to-be discovered mechanisms that trigger specific detoxification responses related to safener-regulated protection of cereal crops.

In summary, herbicide safeners are unique organic molecules that increase the probability of successfully discovering new herbicides by offering scientists a tool for achieving sufficient crop tolerance and/or crop-weed selectivity, therefore providing an alternative to creating genetically-modified crops (Kraehmer et al. 2014). Furthermore, safeners may expand the utility of existing herbicides that do not exhibit adequate crop tolerance without a safener (Riechers and Green 2017) as well as expand our knowledge of plant responses to abiotic stresses.

Metabolism Contributions to Clomazone Activity and Selectivity

Clomazone (Figure 3), a 3-isoaxazolidinone, was initially introduced by FMC Corporation in the 1980’s for weed management in soybean (Chang et al. 1987). Since that time, use of clomazone (also known in the literature as FMC 57020 and dimethazone) expanded to several additional crops (Anonymous 2018). Clomazone injury manifests itself as bleaching of new leaves (Duke and Kenyon 1986). However, attempts to tie the clomazone mechanism of action
to inhibition of phytoene desaturase or steps in the cytoplasmic isoprenoid biosynthesis pathway were unsuccessful (Croteau 1992; Lutzov et al. 1990; Weimer et al. 1992).

Seeking to expand the uses of clomazone shortly after its commercialization, FMC explored the use of safeners. Naphthalic anhydride seed treatment was found to afford some protection from clomazone injury to corn, but this system was never commercially developed. However, the organophosphate insecticides phorate and disulfoton were found to protect cotton from clomazone injury (Culpepper et al. 2001). This is still a commercial practice and the clomazone label (Anonymous 2018) contains specific language regarding use of the insecticides to protect cotton from clomazone damage: “Do not apply Command 3ME Herbicide to cotton unless disulfoton or phorate organophosphate insecticide is applied in-furrow with the seed at planting time” and “Failure to apply either disulfoton or phorate insecticides with Command in accordance with in-furrow label use directions can result in crop phytotoxicity (bleaching) and/or stand reduction.”

A series of experiments were initiated, in cooperation with FMC, to understand the mechanism of organophosphate safening of cotton from clomazone. Results of these experiments were originally published in two manuscripts (Ferhatoglu et al. 2005 and Ferhatoglu and Barrett 2006). Briefly, the experimental system employed was to place 7-d-old cotton seedlings into hydroponic solution with or without clomazone and with or without phorate. The chlorophyll and carotenoid content of the leaves emerging after the beginning of the treatment was measured 6 d after the start of the experiment. Because both clomazone and phorate are volatile, care was taken to keep the vessels for the hydroponic solution sealed. Complete experimental details can be found in Ferhatoglu et al. (2005) and Ferhatoglu and Barrett (2006).
Using this system, it was found that 100 nM clomazone reduced the levels of both chlorophyll and carotenoids in the new cotton leaves approximately 80% (Figure 4). Phorate at 50 µM partially reversed this reduction while 0.5 and 5 µM were ineffective. Overall, the hydroponic system with cotton seedlings was useful as an experimental system to further investigate the mechanism of clomazone safening by phorate.

Phorate, as well as other organophosphate insecticides, are known inhibitors of cytochrome P450 enzymes (Baerg et al. 1996; Diehl et al. 1995; Kreuz and Fonse-Pfister 1992; Mougin et al 1991). They can act as herbicide synergists blocking the P450 mediated detoxification of the active herbicide molecule (Ahrens 1990; Chample and Shaner 1982). In order to test whether phorate affected clomazone metabolism in cotton plants, the roots of cotton seedlings were incubated for 8 h in 0.45 µCi 14C-ring labelled clomazone plus 100 nM clomazone with or without 50 µM phorate. After the 8h-treatment period, the plants were moved to fresh hydroponic solution, without either clomazone or phorate present, for an additional 16 h. At the end of the 16 h period, clomazone and metabolites were extracted from the shoots and roots and separated using HPLC. Unextracted radioactivity was determined by oxidation of the residue following extraction and trapping plus quantifying the released 14CO2. The phorate reduced the conversion of the clomazone in the shoots, but not the roots (Table 1). There was no effect of the phorate treatment on the unextracted radioactivity. Because the phorate effect on clomazone metabolism was confined to the shoot tissue, the experimental system was simplified by directly feeding 14C-clomazone with or without phorate to excised cotton shoots. Like the results with the intact seedlings, phorate reduced the clomazone metabolism in the excised shoots (data not shown).
Isolated microsomes are an experimental system that can be used for *in vitro* studies of pesticide, including herbicide, metabolism by plant P450s. While cotton microsomes had been isolated with the capacity to metabolize herbicides (Frear 1968, Frear et al. 1969), microsomes isolated from etiolated corn shoots to study herbicide metabolism *in vitro* were used here. Therefore, the suitability of this system to examine interactions between phorate and clomazone metabolism was explored. It was first verified that phorate could act as a safener for clomazone in corn like with cotton. Using the same hydroponic system as for cotton and 7-d old corn seedlings, phorate (50 µM) reduced the effect of clomazone (10 and 100 nM) on chlorophyll and carotenoid levels in the new leaves of the corn seedlings (Table 2). Microsomes were prepared from etiolated 3-d old corn seedlings grown from naphthalic anhydride (NA, 0.5% w/w) treated or untreated seed in the presence or absence of 10% v/v ethanol applied 24 h before harvest of the seedlings for microsome preparation. Microsomes prepared from the corn seedlings produced three metabolites from 14C-clomazone eluting at 12.6, 15.4 and 23 minutes (Table 3). NA and ethanol alone induced the activity for the metabolite eluting at 15.4 but not 12.6 min. NA, but not ethanol, induced the activity for the 23 min metabolite. The combination of NA and ethanol did not induce the activity for the 15.4 min metabolite but did for the 23 min metabolite. Overall, NA was the more effective treatment, so this was used in subsequent experiments with corn microsomes. The metabolite eluting at 12.6 min was not NADPH dependent (Table 4) so it is not produced by P450 activity. In fact, in the presence of NADPH, the production of this metabolite was reduced. This is likely due to substrate competition as two other metabolites, the ones eluting at 15.4 and 23 min, were produced when NADPH was added. Production of the metabolite eluting at 23 min was totally inhibited by phorate while the production of the metabolite at 15.4 was unaffected. This showed that there were two NADPH dependent
clomazone metabolism activities present in the corn microsomes, presumably P450 mediated, and that one was sensitive to phorate inhibition while the other was not. The clomazone metabolite standards 2-chlorobenzyl alcohol and 5-OH clomazone, supplied by FMC, eluted at 15.4 and 23 min, respectively. Therefore, the phorate sensitive activity was presumed to be the production of 5-OH clomazone from clomazone.

It was further showed that 5-OH clomazone could cause bleaching in cotton seedlings, reducing both chlorophyll and carotenoid levels in the plants (Table 5). The 5-OH clomazone was approximately 10% as toxic as clomazone (data not shown) which is consistent with data presented by Chang et al. (1987). While the 5-OH clomazone was phytotoxic to the cotton plants, phorate was ineffective as a safener for this compound.

From this information, a working hypothesis was formed that phorate inhibited the P450 responsible for the conversion of clomazone to 5-OH clomazone (Figure 3) but the phorate was ineffective in preventing the formation of the actual toxicant, 5-keto clomazone (Figure 3). The underlying concept was that clomazone was bioactivated to 5-keto clomazone through 5-OH clomazone. The phytotoxicity and selectivity of clomazone was thought to be based on the balance between the rates of bioactivation through 5-OH clomazone, metabolism (detoxification) of the 5-keto clomazone after it was formed, and other clomazone metabolic pathways that did not involve 5-OH clomazone. Besides our data, this hypothesis was based on the metabolic pathway for clomazone in soybean (El-Naggar et al. 1992) which had multiple pathways for clomazone degradation, including one that included formation of 5-keto clomazone. In addition, 5-keto clomazone metabolite was also known to be phytotoxic (Chang et al. 1987). However, further demonstration of the key role that 5-OH clomazone plays in clomazone toxicity would
need to await the discovery of the plastidic isoprenoid pathway (Lichtenthaler et al. 1997, 
Lichtenthaler 1999).

Prior to this discovery, carotenoid biosynthesis in plants was thought to proceed in the cytoplasm from a starting point involving 3-acetyl CoA through mevalonate. This pathway was the focus of the early attempts to identify the site of clomazone action. What Lichtenthaler et al. (1997) showed was that there is a plastidic isoprenoid biosynthetic pathway beginning with glycerol-3-phosphate and pyruvate, through 1-deoxy-D-xylulose-5-phosphate (DXP) then 2-methyl-D-erythritol-4-phosphate (DXR) that leads to the formation of isopentenyl pyrophosphate (IPP) as a building block for terpenes, carotenoids and plastoquinone, among others. Armed with this information and the hypothesis that 5-keto clomazone was the actual clomazone toxicant, a series of experiments were undertook to test the hypothesis.

It was found, using intact chloroplasts isolated from spinach (*Spinacia oleracea* L.), a clomazone sensitive species, that neither clomazone, 5-OH clomazone or 5-keto clomazone (all at 20 µM) affected the incorporation of 1-14C IPP into products that partitioned into the organic phase following incubation (data not shown). The major product found in the organic phase eluted at the same time as an authentic standard of lutein. Norflurazone (20 µM) caused an accumulation in the chloroplasts of a product that eluted at the same time as an authentic standard of phytoene indicating active carotenoid synthesis in the preparations. When the chloroplasts were fed 2-14C-pyruvate, the 5-keto metabolite of clomazone and fosmidomycin, which inhibits the second step in the chloroplastic isoprenoid pathway (DXP reductoisomerase, Fellermeier et al. 1999), increased the formation of a product eluting at 5-8 min and inhibited the formation of one eluting at 12.4 min, the same elution time as an authentic standard of lutein (Table 6). The 5-keto clomazone inhibition of the 12.4 min product was found to be dose-
dependent with an \( I_{50} \) of approximately 150 nM (data not shown). Nine \(^{14}\)C products were found in the aqueous phase from the chloroplasts fed \(^{2}\)\(^{14}\)C-pyruvate. The 5-keto clomazone prevented production of the products eluting at 66.3, 67.9 and 69.9 min while neither clomazone or 5-OH clomazone had any effect on these peaks (data not shown). Fosmidomycin inhibited formation of the products eluting at 66.3 and 69.9 min but not 67.9 min. Because fosmidomycin inhibits DXP reductoisomerase, the second step in the chloroplastic isoprenoid pathway (Fellermeier et al. 1999), and 5-keto clomazone inhibited formation of an additional product than fosmidomycin, it was hypothesized that the 5-keto clomazone was inhibiting DXP synthase, the first step in the pathway.

To test this hypothesis, an \textit{in vitro} assay of DXP synthase was performed using a crude extract prepared from \textit{E. coli} expressing \textit{Catharanthus roseus} (L.) DXP synthase (Fellermeier et al. 1999) as described by Chahed et al. (2000). The DXP synthase activity in the preparations from \textit{E. coli} expressing the plant enzyme was inhibited 47% by 20 \( \mu \)M 5-keto clomazone (Table 7). There was no inhibition of the plant DXP synthase by either clomazone or 5-OH clomazone (data not shown). The bacterial DXP synthase enzyme activity did not appear sensitive to 5-keto clomazone.

Taken together, our studies show that clomazone must be bioactivated to its 5-keto clomazone metabolite to be phytotoxic at its site of action, DXP synthase, the first step in the chloroplastic isoprenoid biosynthesis pathway. The first step in the conversion of clomazone to 5-keto clomazone is the P450 catalyzed formation of 5-OH clomazone. The process can be inhibited by organophosphate insecticides such as phorate which can serve as safeners for clomazone in a crop like cotton. While the formation of 5-keto clomazone from 5-OH clomazone is also likely P450 catalyzed, additional studies would be required to establish this. It
would also be interesting to test whether this conversion could be prevented by P450 inhibitors.

It was apparent from our work that phorate, and presumably other organophosphate insecticides, did not prevent the conversion of 5-OH clomazone to 5-keto clomazone as the insecticide was ineffective as a safener for 5-OH clomazone.

This all means that clomazone selectivity is complicated. The rates of conversion of clomazone conversion to 5-OH clomazone, 5-OH clomazone to 5-keto clomazone, and the conversion of all three of these compounds to their own metabolites will combine to determine how much 5-keto clomazone will be present in a plant and for how long. The clomazone metabolic pathway in tolerant soybean demonstrates this (El-Naggar et al. 1992). More recently, Yasuor et al. (2010) proposed that changes in the rates of the conversion of 5-OH clomazone to dihydroxy-clomazone and clomazone to hydroxymethylclomazone plus 3’-hydroxycyclomazone all contribute to clomazone resistance in *Echinochloa phyllopogon* (Stapf.) Koss.

**Glyphosate Metabolism in Crops and Weeds**

Glyphosate (N-phosphonomethyl glycine) was introduced to the herbicide market in 1974. It has become the most heavily used herbicide in the world, in large part because of the huge success of glyphosate-resistant (GR) crops (Duke 2018). Duke (1988) summarized the literature on glyphosate through the mid-1980s, including its metabolic degradation and concluded that the evidence for plant metabolism of glyphosate was not conclusive because of the low or no levels of degradation reported, sometimes over long time periods. Evidence of its degradation by microbes was clear, and it was speculated that reported plant metabolism might have actually been microbial metabolism due to microbial contamination. Early work was also limited by difficult analytical methods that have improved with time (Koskinen et al. 2016). It
later became unequivocal that many plant species can metabolize glyphosate, especially to aminomethylphosponic acid (AMPA) and glyoxylate (Duke 2011). Indeed, metabolism of glyphosate to AMPA found in microbe-free cell cultures of soybean \textit{(Glycine max (L.) Merr.)}, wheat, and corn proved that plant cells can metabolize glyphosate (Komoßa et al. 1992). In this system, soybean metabolized glyphosate much better than wheat or corn.

Glyphosate’s two most commonly reported routes of metabolism are to AMPA and glyoxylate by a glyphosate oxidoreductase (GOX) and to sarcosine via a glyphosate C-P lyase. In most cases of plant or microbe metabolism of glyphosate, the only metabolite reported is AMPA. This may be because sarcosine is rarely looked for; however, when it has been, it is almost always not found (e.g., Riberio et al. 2015). Metabolism of glyphosate to AMPA has been verified in many plant species now, but the capability for metabolism varies dramatically. For example, Reddy et al. (2008) found a broad range of glyphosate to AMPA metabolism in eleven plant species, ranging from AMPA levels being half, one fourth, or one sixth the concentration of glyphosate measured in treated tissues 7 days after application in Illinois bundleflower \textit{(Desmanthus illinoensis (Michx.) MacMill. ex B. L. Robins and Fern)}, sicklepod \textit{(Senna obtusifolia (L.) H. S. Irwin and Barneby)}, and cowpea \textit{(Vigna unguiculata (L.) Walp.)}, respectively, to no detectable AMPA in Italian ryegrass \textit{(Lolium perenne L. ssp. multiflorum (Lam.) Husnot)}, corn, and hemp sesbania \textit{(Sesbania herbacea (P. Mill.) McVaugh)}. In that study, each species was treated with the glyphosate rate that inhibited growth 50%, so that differential phytotoxicity of glyphosate was unlikely to account for any differences in metabolism. Later work found that corn does metabolize glyphosate to AMPA when GR corn was treated with full recommended rates, and the AMPA as a proportion of glyphosate was very low at most times after application (Bernal et al. 2012; Reddy et al. 2018). So, species that are
not reported to metabolize glyphosate may metabolize it very slowly with levels of AMPA too low to detect at specific times after application. GR crops are ideal for studying glyphosate metabolism, because high rates of glyphosate are not phytotoxic, allowing enzymatic degradation to occur unhindered with ample substrate. Glyphosate metabolism in GR soybeans, maize and canola (Brassica napus L.) has been studied.

Metabolism of glyphosate to AMPA is significant in GR soybean, with levels of ca. 2 to 3 and 7 to 25 μg of glyphosate and AMPA g⁻¹ of dry harvested seed, respectively, from plants treated with 1.26 kg ha⁻¹ of glyphosate at full bloom or 8 weeks after planting (Duke et al. 2003). This is not surprising, as glyphosate is phloem mobile, translocating to metabolic sinks such as meristems and developing seeds (Duke 1988). Others have found similar levels of glyphosate and AMPA in seed for GR soybean (Bohm et al. 2014; Bøhn et al. 2014). In another study in which GR soybean was treated with 0.87 kg ha⁻¹ glyphosate at both 5 and 7 weeks after planting, very high levels of glyphosate (8-15 μg g⁻¹) but very little AMPA (ca. 0.1 μg g⁻¹) was detected in leaf tissues (Duke et al. 2018). Harvested seed had much lower levels of glyphosate, but relatively more AMPA. Whether the AMPA in the seed was translocated there or was formed by metabolism of glyphosate in the seed is unknown.

GR crops are essentially unharmed by glyphosate at rates recommended for weed management (Nandula et al. 2007). But, AMPA is moderately phytotoxic (Hoagland 1980), and GR crops are not resistant to AMPA (Reddy et al. 2004). Thus, if enough AMPA were formed in a GR crop, it might cause phytotoxicity. Under some environmental conditions, farmers have observed moderate chlorosis after treatment of GR soybean with glyphosate (termed yellow flash by farmers). This is a transient effect that does not influence yield. In greenhouse studies, this effect was found with AMPA and glyphosate treatments that resulted in the same in vivo AMPA
concentrations, whether from AMPA or glyphosate treatment (Reddy et al. 2004). These results suggest that yellow flash is due to accumulation of sufficient AMPA for noticeable chlorosis.

GR corn metabolizes glyphosate to AMPA, but at a much lower rate than GR soybean does (Bernal et al. 2012: Reddy et al. 2018). The first GR canola commercially grown was the only GR crop that contained a transgene for a microbial GOX, in addition to a gene for a microbial GR 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the target of glyphosate (Green 2009). Very little glyphosate is converted to AMPA in non-GR canola, whereas virtually all of the glyphosate applied to GR canola is converted to AMPA within 7 days, when glyphosate is supplied in small amounts (Corrêa et al. 2016) (Figure 5). Only AMPA and no glyphosate was found in untreated leaves of treated plants. Whether the AMPA was translocated to untreated leaves or formed by glyphosate metabolism in those leaves was not determined. The relative contributions to resistance of the GOX and GR EPSPS genes are unknown in GR canola, but current GR canola contains only a GR EPSPS transgene, as do all other GR crops. Thus, whether the canola GOX gene alone would provide adequate resistance for a GR crop is unknown.

Several glyphosate metabolism transgenes have been proposed for generation of GR crops. These include transgenes for an engineered bacterial oxidase (Nicolia et al. 2014), a modified bacterial glyphosate acetyltransferase (GAT) (Siehl 2007), and a decarboxylase type enzyme that inactivates glyphosate (Hammer et al. 2007). None of these transgenes have been used in commercial GR crops, although the GAT genes came close to commercialization (Green 2009).

Two generalizations can be made from existing glyphosate metabolism literature regarding glyphosate metabolism in non-transgenic plants. Legumes tend to have higher rates of
 glyphosate metabolism than other species, and Poaceae species usually have very low rates of metabolism. However, there is no methodical, systematic survey, using a consistent experimental design and reliable assay methods, of the capacity for glyphosate metabolism in a wide range of taxonomically diverse plant species.

Glyphosate is non-selective, but some species are more tolerant than others. Since glyphosate is perhaps the slowest acting commercial herbicide, the plant has more time to metabolize it before severe toxicity occurs than with other herbicides. Thus, rapid metabolism could be a tolerance mechanism. However, there is no good evidence that this is the case. The ability to metabolize glyphosate did not correlate well with the glyphosate I_{50} values for ten species (Reddy et al. 2008), although it was speculated that it might contribute to tolerance in some species. A problem with this study is that glyphosate and AMPA were determined at only one point in time after glyphosate application. The dynamics of metabolism is highly likely to vary between species, so that determination of the proportion of glyphosate metabolized over a critical period for herbicide damage would be needed to better evaluate the role of metabolism in producing tolerance.

Morningglory species (*Ipomoea* spp.) are some of the more glyphosate-tolerant weeds, although there is no proof of evolved resistance, as evidenced by no morningglory species being listed as GR on the Heap website (Heap 2018), despite some claims of evolved resistance without conclusive evidence (e.g., Debban et al. 2015). Glyphosate tolerance of pitted morningglory (*Ipomoea lacunosa* L.) biotypes varied by as much as 2.6-fold, but this was not explained by differential metabolism (Ribeiro et al. 2015). The most and least susceptible biotypes both readily metabolized glyphosate to AMPA at about the same rate, but there was differential translocation that was consistent with the differences in susceptibility.
Clearly, many plant species have a means of metabolizing glyphosate to AMPA. Considering the tremendous selection pressure that has resulted in several mechanisms of evolved resistance to glyphosate (Sammons and Gaines 2014), one would expect that the enzyme(s) responsible for AMPA production to be selected for, either to produce a mutated enzyme with greater efficiency as a GOX or to generate greater amounts of GOX enzyme. In their review, Sammons and Gaines state that metabolism of glyphosate in plants is rare and are skeptical of reports that metabolism is involved in reported resistance of *Digitaria insularis* (L.) Fedde and *Conyza canadensis* (L.) Cronq. to glyphosate, partly by conversion to sarcosine (de Carvalho et al. 2012; Gonzalez-Torralva et al. 2012b). Similar results were reported by some of the same authors for glyphosate tolerance of velvetbean [*Mucuna pruriens* (L.) DC.] (Rojano-Delgado et al. 2012). These papers are unusual, both in reporting sarcosine and glyoxylate as plant metabolites of glyphosate and by invoking metabolism as a resistance mechanism. Both sarcosine and glyoxylate are plant metabolites that can be found in untreated plants, so without 14C-labeling of these compounds from 14C–glyphosate, the results are inconclusive. However, they detected AMPA, a compound not found in plants not treated with glyphosate, to support their claims. Using 14C glyphosate, others have found no metabolism of glyphosate in either susceptible or resistant *C. canadenensis* or *C. bonariensis* (L.) Cronq. (Feng et al. 2004; Dinelli et al. 2006, 2008). Sammons and Gaines (2014) did not include glyphosate metabolism as a proven mechanism of evolved resistance to glyphosate.

More rapid metabolism of glyphosate in GR weeds has been searched for without result in many papers looking for the mechanism of glyphosate resistance. For example, although there were no statistical differences in AMPA found in susceptible and two GR Palmer amaranth biotypes, the resistant biotypes tended to have more metabolism than the susceptible biotypes.
However, none of the biotypes accumulated much AMPA at 7 days after treatment, with AMPA not reaching 1% of the glyphosate levels found in the tissues, except in the susceptible biotype treated with a very low I$_{50}$ rate (90 g ha$^{-1}$).

There are still important, unanswered questions about glyphosate metabolism in plants. These include the nature of the enzyme(s) that metabolizes glyphosate to AMPA in plants. Glycine oxidases from microbes can act as a GOX enzyme (e.g., Pollegioni et al. 2011). At least some legumes can oxidize glycine in nodules via a leghaemoglobin-associated reaction (Lehtovaara 1978). But, non-nodulated species and leaf tissues of legumes can convert glyphosate to AMPA and glyoxylate. D-amino acid oxidase from *Bradyrhizobium japonicum* can also oxidize glyphosate to AMPA and glyoxylate, and the transgene for this enzyme can provide glyphosate resistance to *Arabidopsis thaliana* L. (Han et al. 2015). However, equivalent glycine and D-amino acid oxidases have not been identified in plants. The clear ability of some plant species to oxidize glyphosate to AMPA indicates that they have an enzyme similar to these microbial enzymes. The enzymatic or other potential mechanisms of glyphosate conversion to AMPA are still a mystery.

An alternative explanation to direct metabolism of glyphosate by plants is that some plants might have endophytes that can metabolize glyphosate. Evidence is growing that endophytes can contribute to metabolic degradation of herbicides (Tetard-Jones and Edwards 2016), and some plant-associated microbes can clearly metabolize glyphosate (e.g. Kryuchkova et al. 2014). The soybean endophyte *Burkholderia gladioli* is resistant to glyphosate (Kuklinsky-Sobral et al. 2005), but whether this is due to metabolism is unknown. There is no available proof of any endophyte-mediated glyphosate metabolism *in planta*. Endophyte infection increases with glyphosate resistance in Italian ryegrass (Handayani et al. 2017), but glyphosate
metabolism in the populations was not determined, and metabolism has been found to be very low and uninvolved in L. multiflorum resistance to glyphosate at other places in the world (Barroso et al. 2018; Gonzalez-Torralva et al. 2012). It is possible that there has been horizontal gene transfer of a gene for an enzyme with GOX activity. But, it does not appear that the extreme selection pressure caused by glyphosate in recent decades has caused this to occur, as metabolism is not a firmly established mechanism of weed resistance to glyphosate. This supports the view that horizontal gene transfer from microbes to plants is not a common occurrence.

Bioactivation of Natural Phytotoxins: The Exception or the Rule?

Evolution of Bioactive Natural Products. Within ecosystems, plants cohabit in association with a wide variety of microorganisms, insects and nematodes, and other plants. These constant multitrophic interactions has led to the coevolution of positive interactions such as mutualistic and symbiotic relationships and negative interactions such as competitive and parasitic relationships. Within this context, pathogens have adapted to their plant host by deploying virulence proteins that either suppress plant immune responses or increase plant susceptibility. Plant-pathogen interactions also involve a form of chemical warfare derived from novel metabolic pathways (Schueffler and Anke 2014) that aims to strategically utilize one’s opponent weakness to their benefits (Maor and Shirasu 2005; Verhoeven et al. 2009). There is great interest in utilizing these natural phytotoxins to develop new herbicide chemical classes or discovering novel mechanisms of action (Duke and Dayan 2015).

These secondary metabolic pathways evolve through gene duplication. This slow evolutionary process most often leads to loss of function of the duplicated genes
(pseudogenization), but it occasionally leads to a beneficial gain of a new function (neofunctionalization) (Moore and Purugganan 2005). Over time, this has resulted in a vast array of structurally diverse biologically active molecules (Ober 2005; Flagel and Wendel 2009).

This evolutionary process is similar to the high-throughput screens developed by all major Agrochemical companies searching for new herbicides. While conventional *in vitro* screens test a large number of compounds on a single enzyme target very rapidly, natural high-throughput processes allows for the identification and customization of molecules that optimize their *in vivo* activities. More than 200,000 secondary metabolites have been characterized and many more are expected to be discovered (Clevenger et al. 2017).

Natural phytotoxins are typically small molecules that explore chemical spaces not covered by herbicides obtained through the usual organic synthetic approaches. Many of these chemicals have novel mechanisms of action that target enzymes and/or pathways that also exist in the organisms producing them. Consequently, these molecules are often synthesized and/or stored as inactive protoxins that are bioactivated in the target organisms. Bioactivation may take different forms, such as removing protecting groups or by adding functional groups.

Alternatively, some organisms produce the phytotoxins in their active forms but protect themselves from autotoxicity by sequestering them in subcellular compartments or specialized cells (Figure 6). A few examples of the various mechanisms of bioactivation are examined in the following sections.

**Bioactivation by Removing Protecting Group.** Some organisms produce toxins as protoxins that possesses additional groups to protect themselves from autotoxicity. This is often necessary because these organisms have enzymes that are sensitive to the toxin they produce. A well-
known example is the conversion of the protoxin bialaphos to the herbicidal L-phosphinothricin, the active enantiomer of glufosinate, is perhaps the most relevant example of bioactivation via cleavage of protecting groups (Figure 7A). Bialaphos is a tripeptide L-alanyl-L-alanyl-phosphinothricin produced by the soil bacteria *Streptomyces hygroscopicus* and *S. viridochromogenes*. This metabolite is very rapidly bioactivated *in planta* by removing two alanine residues to release L-phosphinothricin. Phosphinothricin is a potent inhibitor of glutamine synthetase (Wild and Ziegler 1989), a key enzyme responsible for glutamine biosynthesis and for ammonia detoxification. Organisms producing bialaphos also have specific acetylases that rapidly deactivate L-phosphinothricin as another mechanism of protection against the toxic effect of this bioactive natural product. Another common bioactivation by cleavage involves the removal of glycosides by glucosidases to release herbicidal aglycones, such as that observed with podophyllotoxin produced by the plant belonging to the *Podophyllum* species.

Podophyllotoxin is an aryltetralin lignin that acts as a natural mitotic inhibitor. While this molecule is used as a precursor for the semi-synthesis of several anticancer pharmaceutical drugs because of its antimitotic activity, podophyllotoxin would cause great damage to the plants producing it. Consequently, Mayapple conjugates podophyllotoxin with a glucose and stores it within the vacuole, preventing it from interacting with the physiologically active cytosol and interfering with numerous microtubule-driven processes. Interestingly, there is a glucosidase present in the cytosol with high specificity for podophyllotoxin-O-glucoside that is mostly inactive at physiological pH of 7 but has its greatest activity at pH 4 (Dayan et al. 2003). These biochemical characteristics suggests that this glucosidase would be recruited for the hydrolysis of the glucoside to release the active aglycone upon injury to the leaf, which would cause the pH to drop as the large volume of the vacuole mixes with the cytoplasm.
Bioactivation by Adding Functional Groups. Other organisms produce toxins as protoxins that require the addition of functional groups to activate the structures. Some strains of *Fusarium solani* produce 2,5-anhydro-D-glucitol, a sugar analog that requires bioactivation to exert activity on its target site (Dayan et al. 2002). 2,5-Anhydro-D-glucitol has no known biological activity. However, up to millimolar concentrations of this molecule is released by *Fusarium solani* as it invades a plant. Through the process of coevolution this pathogen has hijacked the biochemical machinery of its host plant to bioactivate 2,5-anhydro-D-glucitol into a phytotoxin. 2,5-Anhydro-D-glucitol is a sugar analog that serves as substrate for two glycolytic kinases endogenous to the host plant (namely hexokinase and phosphofructokinase) (Figure 7B). The biochemical functions of these kinases have been hijacked to produce a diphosphate derivative of 2,5-anhydro-D-glucitol. This bioactivated toxin acts as a substrate analog that inhibits fructose-1,6-diphosphate aldolase, a key step in glycolysis (Dayan et al. 2002).

This type of protoxin bioactivation occurs by hijacking of native plant kinases to produce an active phosphorylated phytotoxins. For example, certain strains of *S. hygroscopicus* produce hydantocidin. Upon phosphorylation, hydantocidin becomes an analog of inosine monophosphate (IPM), which is a potent inhibitor of adenylosuccinate synthetase, an enzyme involved in purine biosynthesis (Fonne-Pfister et al. 1996). Similarly, carbocyclic coformycin is a protoxin produced by *Sacchavothvix* spp. whose mechanism of action requires phosphorylation of its 5'-hydroxy group to produce an irreversible inhibitor of adenosine mono phosphate (AMP) deaminase (Dancer et al. 1997).
Bioactivation by Altering the Shape of a Phytotoxin. Sometimes, protoxins can be bioactivated as the result of more subtle changes. For example, the gram-negative β-proteobacteria *Bukholderia* sp. A396 produces large amount of romidepsin, a 16-membered cyclic depsipeptide bridged by a 15-membered macrocyclic linked via a disulfide bridge. Reduction of the disulfide bridge is catalyzed *in planta* by native enzymes, and opens up the 15-membered macrocyclic structure to release a long side chain. This increases the potency of romidepsin on plant histone deacetylases.

Bioactivation by Releasing from Cellular Sequestration. Not all natural phytotoxins require bioactivation. In these cases, the metabolites are already highly toxic and these organisms have devised schemes to protect themselves from the lethal effect of these compounds by compartmentalizing or exuding them. For example, leptospermone is a metabolite produced by several plant species (e.g., *Callistemon* spp., *Leptospermum* spp. and *Eucalyptus* spp.). This molecule and several other analogues produced by these plants are potent inhibitor of p-hydroxyphenylpyruvate dioxygenase (HPPD) (Dayan et al. 2007). In fact, leptospermone served as a template for the development of HPPD-inhibiting herbicides (Beaudagnies et al. 2009). This β-triketone natural product is produced exclusively in schizogenous glands (Figure 8A). This allows the production of a potent toxin in a cellular compartment isolated from the rest of the physiologically active portion of the plant that possess endogenous HPPD enzyme sensitive to their own toxin.

As another example, sorgoleone is produced in most of the species in the *Sorghum* genus. This lipid benzoquinone is a potent photosystem II inhibitor and competes for the binding site of plastoquinone on the Qb binding site (just as atrazine) (Gonzalez et al. 1997). Isolated
chloroplasts of sorghum are just as sensitive to sorgoleone as other plant species. However, sorghum is able to produce large amount of this toxin by compartmentalizing its biosynthesis to specialized root hair cells that rapidly exude it from the root into the rhizosphere (Dayan et al. 2009) (Figure 8B). Since sorgoleone does not translocate from the root to the foliage, sorghum can grow without problem while repressing the growth of small seeded weeds germinating within its rhizosphere.

Bioactivation of Natural Phytotoxin - The Exception or the Rule? The short review above clearly shows that many phytotoxins are produced as protoxins or must be sequestered by the producing organism to avoid autotoxicity. This is particularly true if the pathogen producing the toxin possess the enzyme target site affected by the active form of the toxin. These complex systems are the results of coevolution between plant biotic interactions that cannot be achieved by the current approaches used by the agrochemical industry.

Glutathione Transferases Associated with Non-Target Site Resistance to Herbicides in Blackgrass (*Alopecurus myosuroides*)

Glutathione transferases (GSTs; EC 2.5.1.18) are a superfamily of detoxifying enzymes which have evolved into six discreet clades classified as the zeta, theta, phi, tau, lambda and dehydroascorbate reductase classes in plants (Dixon and Edwards 2010). GSTs from the tau (GSTU) and phi (GSTF) classes have long been associated with herbicide tolerance and selectivity in crops through their ability to rapidly conjugate and detoxify a range of chemistries (Cummins et al. 2011). Normally, the expression of GSTs is an order of magnitude lower in weeds than in crops leading to a corresponding reduced ability to detoxify herbicides (Dixon and
Repeated selection with herbicides can lead to enhanced GST expression in weeds, leading to resistance. Consistent with this, transcripts encoding GSTFs and GSTUs have been recently identified as being enhanced in populations exhibiting non-target site herbicide resistance (NTSR) in several weed species; notably wild oat (Avena fatua L.; Keith et al. 2017), American sloughgrass (Beckmannia syzigachne (Stud.) Fernald; Pan et al. 2016), shortawn foxtail (Alopecurus aequalis Sobol.; Zhao et al. 2017), annual ryegrass (Lolium sp.; Duhoux et al. 2017a) and blackgrass (Tétard-Jones et al. 2018). Recent studies have demonstrated that the enhanced expression of GSTs able to conjugate and detoxify atrazine, are responsible for the evolved resistance to this herbicide in populations of Palmer Amaranth (Amaranthus palmeri; Nakka et al. 2017a) and waterhemp (Amaranthus tuberculatus; Evans et al. 2017). However, in blackgrass and wild oat, the GSTs up-regulated in the NTSR populations have little activity toward herbicides, even though their enhanced expression is intimately linked to resistance (Burns et al. 2017; Cummins et al. 1999). Recent studies in blackgrass demonstrated that the phi AmGSTF1, which has little conjugating activity toward herbicides, was uniquely associated with all NTSR populations identified to date (Tétard-Jones et al., 2018). Overexpression of AmGSTF1 in Arabidopsis (A. thaliana ecotype Columbia-0), enhanced tolerance to several herbicides, notably to those that did not undergo glutathionylation in the course of their detoxification (Cummins et al. 2013; Tétard-Jones et al. 2018). This suggested that GSTs must possess alternative protective activities in NTSR that do not directly involve herbicide detoxification. However, it is known that the complex NTSR trait in different weed species is multi-genic (Duhoux et al. 2017b; Keith et al. 2017; Tétard-Jones et al, 2018), suggesting that a single GST cannot confer multiple resistance alone. In further investigating the roles of these proteins in NTSR, we now report the detailed characterization of the all detectable GSTs (the
Analysis of \textit{Am}GSTF1 Variants in Blackgrass. Recent proteomic studies confirmed the presence of elevated levels of the phi blackgrass \textit{Am}GSTF1 polypeptides in multiple NTSR populations, as compared with herbicide-sensitive (HS) populations (Tétard-Jones et al. 2018). Closer examination of the \textit{Am}GSTF1 polypeptides revealed they were derived from two of the four known isoforms (Supplemental Figure 1), namely the c and d variants, previously identified in the screening of a cDNA expression library prepared from the NTSR blackgrass population, ‘Peldon’ (Cummins et al. 1999). Whereas only the c and d variants were expressed as polypeptides, analysis of the transcriptomic data showed that all four isoforms were present as mRNAs in NTSR blackgrass (Tétard-Jones et al. 2018). Differences were observed in the relative abundance of the \textit{Am}GSTF1 transcripts, with the c form generally more abundant in leaves and the dominant in the stems. Previous enzymatic and transgenesis studies have exclusively concentrated on the characterization of the \textit{Am}GSTF1c isoform (Cummins et al. 2013). As the proteomic studies now showed that alternative isoforms were also being expressed (Tétard-Jones et al. 2018), it was clearly important to determine whether the variants were all functionally equivalent. \textit{Am}GSTF1a was selected for comparative enzymatic analysis with \textit{Am}GSTF1c, as the most variant isoform (Supplemental Figure S1). The GST sequences were then sub-cloned into the pET-STRP3 vector and expressed as Strep-tag II fusion proteins in \textit{E. coli} (Dixon et al. 2009). The affinity-purified enzymes were then assayed for glutathione transferase and glutathione peroxidase (GPOX) activity, with the enzymes showing similar activities and kinetic constants (Supplemental Table S1). It was concluded that while \textit{Am}GSTF1
is present as multiple isoenzymes in blackgrass, they are functionally identical and as such, all further references to \textit{Amgstf1} refer to the c isoenzyme.

**GST Genes Expression in NTSR Blackgrass.** To identify the full range of GST genes associated with NTSR in blackgrass, the respective transcriptome contig sequences of NTSR (‘Peldon’) as compared to the HS (‘Rothamsted’) populations reported previously were examined (Tétard-Jones et al. 2018). Using this approach, a total of 53 contigs corresponding to GST genes were identified and analyzed by Blast (BlastX) searches against the respective translated proteome database (Tétard-Jones et al. 2018). This approach clustered the contigs into 15 distinct GSTs, which on online protein blast (BlastP) analysis, yielded eight GSTUs, six GSTFs and one lambda (L) GSTL gene (Supplemental Table S2). Of the phi GSTs, isoforms of the previously undescribed \textit{Amgstf2} and \textit{Amgstf3} genes were identified in addition to \textit{Amgstf1}. Of the eight GSTU genes, one sequence corresponded to \textit{Amgstu1} (Cummins et al. 2009). The remaining new unigenes were named \textit{Amgstu2} to \textit{Amgstu7}, of which \textit{Amgstu2} was the most highly represented in the NTSR populations (Tétard-Jones et al. 2018).

\textit{Amgstu2} was selected for further characterization, with multiple sequence variants, termed \textit{Amgstu2a-f}, recovered from the ‘Peldon’ cDNA library (Cummins et al. 1999).

Phylogenetic analysis identified orthologs in wheat, maize and barley. The phylogenetic analysis showed that \textit{Amgstu2a} and \textit{Amgstu2b} (95% similarity) were most likely derived from a lineage specific duplication (Figure 9). Both were members of a clade, also containing \textit{Amgstu1}, which is unique to monocots (Brazier-Hicks et al. 2018). In contrast, \textit{Amgstu3} to \textit{Amgstu7} were more evolutionarily diverse, sharing protein sequence identities ranging from 36% to 76% and aligning to several distinct tau class clades (Figure 9). Of the 15 up-regulated GST genes, \textit{Amgstu2a} was the most abundant at both transcript and expressed protein levels.
coding sequence of *Am*GSTU2a was isolated by PCR from the ‘Peldon’ cDNA library and expressed as the respective Strep-tag II fusion protein in *E. coli*. Enzyme activity assays of the recombinant protein showed that while *Am*GSTU2a conjugated CDNB and the herbicides fenoxaprop-ethyl and metolachlor, it was inactive as a GPOX (Table 9). Similarly, the related *Am*GSTU1 from blackgrass and an ortholog *Ta*GSTU4 (69% identity) in wheat (Thom et al. 2002), also showed activity toward multiple herbicides, suggesting this clade of tau GSTs is important in detoxification.

**GST Protein Expression in Blackgrass.** Proteomics was used to monitor changes in the GSTome in NTSR populations either derived from different geographical locations in the UK (‘Peldon’, ‘Essex’ and ‘Oxford’), or by repeated selection with the herbicides pendimethalin or fenoxaprop as compared with HS plants (Tétard-Jones et al. 2018). While 15 distinct GSTs showed induced transcriptional expression, only four of these genes were accompanied by increased protein expression as determined by differential two-dimensional proteomics of stem and leaf tissue (Table 8). In the leaves, the c and d isoforms of *Am*GSTF1 were the most highly abundant GSTs detected by proteomics (Table 8). In the case of *Am*GSTU2, the 140-fold enhancement in transcripts in the stems of ‘Peldon’ versus. HS plants was associated with only a 6-fold increase in the respective protein abundance. In the stem tissue, the enhancement of *Am*GSTF2, *Am*GSTU2, and the *Am*GSTF1c/d isoforms in the NTSR ‘Peldon’, ‘Oxford’ and ‘pendimethalin’ populations, was clearly distinct from that determined in the plants selected on fenoxaprop (Table 8). This suggested that the GSTome associated with NTSR in blackgrass could vary depending on the history of herbicide selection.
As GSTUs and GSTFs are associated with a wide range of plant stress responses (Dixon and Edwards 2010), it was then of interest to determine if the NTSR-associated GSTs were perturbed by biotic and abiotic stress in blackgrass. As described previously (Tétard-Jones et al. 2018), HS plants were exposed to a range of stress treatments including wounding, heat and drought, along with exposure to the herbicide safener, cloquintocet mexyl. The results demonstrated that the changes in the GSTome associated with NTSR could not be replicated by any of the stresses, with the abundance of *AmGSTU2* were actually suppressed by these treatments (Table 8).

**Conclusions on the Roles of GSTs in NTSR Blackgrass.** A characteristic feature of the GSTs induced by NTSR were their relative abundance and multiplicity in isoforms identified in the transcriptome studies, as compared with the proteome experiments. While the major phi *AmGSTF1* was encoded by at least four sequences (a-d isoforms) and the tau *AmGSTU2* by six open reading frames (a-f isoforms), at the level of protein expression they were represented by only two and one isoforms respectively (Table 8). Similarly, the relative enhancement of the transcripts encoding these GSTs was of at least an order of magnitude greater than the changes determined in the abundance of the respective proteins (Table 8). The discrepancy between transcriptome and proteome data for the GSTs demonstrate that the respective genes are subject to major post-transcriptional regulation either at the level of mRNA or translated protein turnover. Similar discrepancies in transcriptome and proteome expression patterns have been reported for GSTs in other plants responding to stress conditions and may reflect the manner in which these genes are evolving through the process of gene duplication (Dixon and Edwards 2010). In this regard, it is interesting that *AmGSTU2* is orthologous to *TaGSTU4*, an enzyme that displays a range of detoxifying activities following minor changes in its coding sequence.
Glutathione Transferases Associated with Non-Target Site Resistance to Herbicides in Blackgrass (*Alopecurus myosuroides*)

Glutathione transferases (GSTs; EC 2.5.1.18) are a superfamily of proteins with multiple functions and a broad association with resistance to pesticides, drugs and herbicides in insects, animals and plants (Sherratt and Hayes 2001). In plants, the most abundant GSTs are soluble proteins that have evolved into six discreet clades classified as the zeta, theta, phi, tau, lambda and dehydroascorbate reductase classes (Dixon and Edwards 2010). Of these, the plant specific phi (GSTF), tau (GSTU) and lambda (GSTL) GSTs have been linked to phytoprotective functions toward xenobiotics and herbicides, with many of their respective members being stress-inducible and highly expressed in crop plants (Labrou et al. 2015). As a protein family, GSTs are well described in terms of their conservation in protein structure, use of glutathione as a substrate and subunit binding to form characteristic dimers (Dixon and Edwards 2010; Labrou et al. 2015). However, our understanding about their functions in planta, particularly their roles in stress tolerance, is surprisingly incomplete. Phytoprotective activities described to date (Cummins et al. 2011), include acting as conjugating enzymes catalysing the S-glutathionylation and detoxification of herbicides and other xenobiotics (GSTF and GSTU), acting as peroxidases that reduce toxic organic hydroperoxides (GSTF and GSTU) and reductases that recycle...
phenolics acting as antioxidants (GSTL) (Dixon and Edwards 2010; Labrou et al., 2015). There is also a growing body of evidence that GSTUs and GSTFs are able to function as ligandins, binding a range of hydrophobic ligands of both natural and synthetic origins (Dixon et al. 2011).

As highly expressed proteins, GSTs have long been associated with herbicide tolerance and selectivity in crops through their ability to rapidly conjugate and detoxify a range of chemistries, including representatives from the triazine, chloroacetanilide, diphenylether, SU, and AOPP classes (Cummins et al. 2011). As key detoxifying enzymes determining herbicide selectivity, changes in the expression of GSTs in weeds is a potential evolutionary route to herbicide resistance. Consistent with this, transcripts encoding GSTFs and GSTUs have been recently identified as being enhanced in populations exhibiting non-target site herbicide resistance (NTSR) in several weed species; notably wild oat (Avena fatua L.; Keith et al. 2017), American sloughgrass (Beckmannia syzigachne (Steud.) Fernald; Pan et al. 2016), shortawn foxtail (Alopecurus aequalis Sobol.; Zhao et al. 2017), annual ryegrass (Duhoux et al. 2017a) and blackgrass (Tétard-Jones et al. 2018). However, the functional significance of this upregulation of GSTs associated with herbicide resistance is not always obvious. Recent studies have demonstrated that the enhanced expression of GSTs able to conjugate and detoxify atrazine, are responsible for the evolved resistance to this herbicide in populations of Palmer Amaranth (Nakka et al. 2017) and waterhemp (Evans et al. 2017). However, in blackgrass and wild oat, the GSTs up-regulated in the NTSR populations have little activity toward herbicides, even though their enhanced expression is intimately linked to resistance (Burns et al. 2017; Cummins et al. 1999). Recent studies in blackgrass demonstrated that the phi_AmGSTF1, which has little conjugating activity toward herbicides, was uniquely associated with all NTSR populations identified to date (Tétard-Jones et al., 2018). Overexpression of the phi_AmGSTF1 protein in...
Arabidopsis (*A. thaliana* ecotype Columbia-0) gave rise to enhanced tolerance to several herbicides, notably to those that did not undergo glutathionylation in the course of their detoxification (Cummins et al. 2013; Tétard-Jones et al. 2018). This suggests that GSTs must possess alternative protective activities in NTSR that do not directly involve herbicide detoxification.

As described above, from the growing body of transcriptome data, it is clear that GSTs from different classes are associated with NTSR in many weed species. Since it is known that the complex NTSR trait in different weed species is multi-genic (Duhoux et al. 2017b; Keith et al. 2017; Tétard-Jones et al., 2018), it is clear that a single GST cannot confer multiple resistance alone. However, GSTs would be prima candidates for having functional roles in NTSR, given their close association in counteracting multiple types of biotic and abiotic stress in plants including broad-ranging xenobiotic tolerance (Dixon and Edwards 2010). In further investigating the roles of GSTs in herbicide tolerance, the detailed characterization of the ‘GSTome’ associated with NTSR in blackgrass is reported using a combination of transcriptomics, proteomics and functional expression of the respective recombinant enzymes.

**Analysis of *AmGSTF1* Variants in Blackgrass.** Recent proteomic studies have revealed the presence of elevated levels of phi-*AmGSTF1* polypeptides in NTSR, as compared with herbicide-sensitive (HS) blackgrass populations (Tétard-Jones et al. 2018). Closer examination of the polypeptides associated with *AmGSTF1* revealed they were derived from two of the four known isoforms, namely the c and d variants, previously identified in the screening of a cDNA expression library prepared from Peldon blackgrass (Cummins et al. 1999). For reference the predicted amino acid sequences of these isoforms are shown in Supplemental Figure S1. Whereas only the c and d variants were expressed as polypeptides, analysis of the transcriptomic
data showed that all four isoforms were present as mRNA transcripts in blackgrass (Tétard-Jones et al. 2018). Differences were observed in the relative abundance of the AmGSTF1 transcripts, with the c form generally more abundant in leaves and the d dominant in the stems. As Southern blot analysis had suggested AmGSTF1 was located at a single location in the black-grass genome (Cummins et al. 2009), the presence of multiple isoforms is suggestive of tandem gene duplication, as found with these GSTs in other plants (Dixon and Edwards 2010). Previous enzymic and transgenesis studies have exclusively concentrated on the characterization of the AmGSTF1c isoform (Cummins et al. 2013). As the proteomic studies now showed that alternative isoforms were also being expressed, it was clearly important to determine whether the variants were all functionally equivalent. AmGSTF1a was selected for comparative enzymatic analysis with AmGSTF1c, as these isoforms showed the least sequence identity (Supplemental Figure S1). The GST sequences were sub-cloned into the pET-STRP3 vector and expressed in E.coli to generate the respective N-terminal Strep-tag II fusion proteins, prior to their purification using streptacin affinity chromatography (Dixon et al. 2009). The affinity-purified enzymes were then assayed for glutathione transferase and glutathione peroxidase (GPOX) activity. Both enzymes showed similar activities and kinetic constants towards the two substrates (Supplemental Table S1). Therefore, it was concluded that the other AmGSTF1 isoforms would possess similar activities. Based on these findings, it is concluded that while AmGSTF1 is present in multiple isoenzymic forms in blackgrass, they are likely to be functionally identical. As AmGSTF1c is the dominant isoform in the resistance proteome, all further references to AmGSTF1 in the paper refer to the c isoform.

**GST Genes Expressed in NTSR Black-grass.** To identify the full range of GST genes associated with NTSR in black-grass, the respective transcriptome contig sequences of NTSR
The HS (Rothamsted) populations were examined, as generated by IonTorrent next-generation sequencing (Tétard-Jones et al. 2018). Using this approach, a total of 53 contigs corresponding to GST genes were identified, which were analyzed by Blast (BlastX) searches against the respective translated proteome database (Tétard-Jones et al. 2018). This approach clustered the contigs to 15 distinct GSTs, which on online protein blast (BlastP) analysis, yielded eight GSTUs, six GSTFs and one GSTL gene (Supplemental Table S2). Of the phi proteins, AmGSTF2 and AmGSTF3 sequences were identified in addition to the AmGSTF1 isoforms. Of the eight GSTU putative genes, one sequence corresponded to the previously identified AmGSTU1 (Cummins et al. 2009). The remaining unigenes were named AmGSTU2 to AmGSTU7, of which AmGSTU2 was the most highly represented in the NTSR populations (Table 8).

AmGSTU2 was selected for further characterization, with multiple sequence variants, termed AmGSTU2a-f, recovered from a previously prepared Peldon cDNA library (Cummins et al. 1999). The predicted protein sequences of all the AmGSTU2 variants were closely related, with AmGSTU2a and AmGSTU2b showing 95% similarity (Supplementary Table 1). Phylogenetic analysis was performed using the predicted protein sequences of the eight putative AmGSTUs, with orthologues identified in wheat, maize and barley (Hordeum vulgare L.). The phylogenetic analysis showed that AmGSTU2a and AmGSTU2b were most likely derived from a lineage specific duplication. Both were members of a tau-class clade, that also contains AmGSTU1, which is only found in monocot species (Brazier-Hicks et al. 2018). In contrast, AmGSTU3 to AmGSTU7 were more evolutionarily diverse, sharing protein sequence identities ranging from 36% to 76% and aligning to several distinct tau class clades (Figure 9). Of the 15 up-regulated GST genes, AmGSTU2a showed the highest level of transcript abundance in the
NTSR transcriptome (Table 8). As the most abundant GSTU protein present in blackgrass, AmGSTU2a was selected for further characterization, after isolating the coding sequence by PCR from the Peldon cDNA library and expressing as the respective Strep-tag II fusion protein in E. coli. Enzyme activity assays of the recombinant protein showed that while AmGSTU2a conjugated CDNB and the structurally diverse herbicides fenoxaprop-ethyl and metolachlor, the recombinant protein showed no GPOX activity (Table 9). Preferential conjugation of herbicides rather than GPOX activity was also determined in the closely related AmGSTU1 (Cummins et al. 2009). Similarly, the closely related (69% identity) TaGSTU4 in wheat has activities toward multiple herbicides (Thom et al. 2002), that can be manipulated by directed engineering of the protein (Govindarajan et al. 2015). Collectively, these results suggest that the multiple variants of AmGSTU2 are likely to have a range of conjugating activities toward different herbicides.

**GST Protein Expression in Blackgrass.** Having established the inducibility of the GST superfamily in blackgrass at the transcriptome level, it was then of interest to investigate changes in their relative abundance in the proteome of NTSR versus HS plants. As described previously, the NTSR population were either derived from different geographical locations in the UK (Peldon, Essex and Oxford), or by repeated selection with the herbicides pendimethalin or fenoxaprop (Tétard-Jones et al. 2018). Whereas 15 distinct GSTs showed induced transcriptional expression, the up-regulation of only four of these genes resulted in increased protein expression as determined by differential two-dimensional proteomics (Table 8). Relative changes in transcript abundance were not quantitatively predictive of altered protein expression. In the case of AmGSTU1, the 140-fold enhancement in transcripts in Peldon Vs. HS plants resulted in only a 6-fold increase in the respective protein abundance. Changes in GST expression associated with NTSR were primarily associated with the stem tissue, rather than the leaves. Four GST proteins
including AmGSTF2, AmGSTU2, and the AmGSTF1c/d isoforms were present in stem tissue of NTSR plants (Table 8). While all four GST proteins were constitutively upregulated in the Peldon, Oxford and pendimethalin-selected NTSR populations, in the fenoxaprop-selected resistant plants, the expression AmGSTF1c was repressed (Table 8). In terms of relative abundance, the two c and d isoforms of AmGSTF1 were the most highly abundant proteins detected in the leaf and stems by DiGE, being 15- and 11-fold more highly abundant in Peldon than in the HS populations.

As GSTUs and GSTFs are associated with a wide range of stress responses in plants (Dixon and Edwards 2010), it was then of interest to determine if the NTSR-associated GSTs also responded to biotic and abiotic stress in blackgrass. As described previously (Tétard-Jones et al. 2018), HS plants were exposed to a range of stress treatments including wounding, heat and drought treatment, salinity, nitrogen deficiency, rhizobacteria inoculation, and aphid infestation. In addition, the plants were also exposed to the herbicide safener cloquintocet mexyl. The changes in the GST proteome (GSTome) were determined for each treatment. The results demonstrated that the changes in the GSTome associated with NTSR could not be replicated by any of the stress treatments. Of the GSTs in the stem tissue, only AmGSTF1c was responsive to stress, with heat and safener treatments enhancing AmGSTF1c protein abundance by 4.8-fold and 1.6-fold respectively (Table 8). In contrast, the abundance of AmGSTU2 was suppressed by biotic and abiotic stress in as compared with untreated controls (Table 8).

Functional Roles for GSTs in NTSR Blackgrass. The proteomic studies demonstrated that the GSTome of NTSR in blackgrass was distinct from that seen following exposure of the plants to common biotic and abiotic stress treatments experienced under field conditions (Table 8). Taken together with the more extensive proteomic studies recently reported (Tétard-Jones et al. 2018),
this suggests that the complex trait of NTSR is derived from a distinct evolutionary path than

general stress tolerance. On aligning the GSTome of blackgrass with that of crops (Figure 9), the

major genes associated with NTSR have orthologues involved in herbicide tolerance in cereal

crops. Thus, AmGSTF1 is closely related to the major constitutively expressed phi protein

ZmGSTF1, which is involved in the tolerance to chloroacetanilides and triazines in maize (Dixon

et al. 1998). The newly discovered AmGSTF2 was not subject to detailed characterization in this

study but was found to share 83% identity with a phi protein termed TaGSTF3 characterized as

being able to metabolize chloroacetanilide herbicides and act as a GPOX in wheat (Cummins et

al., 2003). AmGSTF2 also shared 70% identity with the safener inducible ZmGSTF4 identified

in benoxacor-treated maize (Irzyk and Fuerst 1993). Of the tau proteins, AmGSTU2 is an

ortholog of TaGSTU4 which is known to play a major role in detoxifying multiple herbicides in

wheat (Thom et al. 2002). Based on transcriptome data, one GST gene from the lambda (GSTL)

subfamily that was up regulated in NTSR compared to HS populations was also identified

(Supplemental Table S2). GSTLs are strongly up-regulated in response to safeners in wheat

(Theodoulou et al. 2003), with with over-expression of the rice OsGSTL2 conferring increased

herbicide tolerance in transgenic plants (Hu 2014). Collectively, these results demonstrate that

changes in the expression of GSTs during the evolution of NTSR in blackgrass show surprising

similarities to the existing GSTome of domesticated crops.

The safener-responsiveness of GSTs in cereal crops was not reflected in their inducibility

in blackgrass. Several reports have suggested that weeds including ryegrass (Duhoux et al.

2017b) and wild oat (Burns et al. 2017), can be partially safened against herbicide injury. Our

previous biochemical studies showed that safening in HS black-grass was associated with a

minor enhancement in the detoxification of the herbicide fenoxaprop-ethyl and that NTSR plants
were less responsive (Cummins et al. 2009). Our current results demonstrate that safening and
NTSR share limited commonality in inducible resistance mechanisms, most likely linked to the
enhancement in the expression of \textit{AmGSTF1}. While \textit{AmGSTF1} was strongly enhanced by
NTSR, this effect was much weaker when HS plants were exposed to safener.

A characteristic feature of the GSTs induced by NTSR were their relative abundance and
multiplicity in isoforms identified in the transcriptome studies, as compared with the proteome
experiments. Thus, the major phi \textit{AmGSTF1} was encoded by at least four sequences and the tau
\textit{AmGSTU2} by six ORFs, yet at the level of protein expression they were represented by two and
one isoforms respectively (Table 8). Similarly, the relative enhancement of the transcripts
encoding these GSTs was of at least an order of magnitude greater than the changes determined
in the abundance of the respective proteins (Table 8). The discrepancy between transcriptome
and proteome data for the GSTs demonstrate that the respective genes are subject to major post-
transcriptional regulation either at the level of mRNA or translated protein turnover. Similar
discrepancies in transcriptome and proteome expression patterns have been reported for GSTs in
other plants responding to stress responses and may reflect the manner in which these genes are
evolving (Dixon and Edwards 2010). Thus, arising from gene duplications the potential to
generate multiple GST variants with slightly different functional properties provides a rich
genetic background for plasticity and rapid evolution to imposed stress. In this regard, it is
interesting that minor changes in the sequence of the \textit{AmGSTU2} orthologue \textit{TaGSTU4} could
give rise to new herbicide detoxifying capabilities (Govindarajan et al. 2015). Based on this
hypothesis and on the respective activities of the enzymes, it is concluded that the \textit{AmGSTF1}
variants have the potential to evolve new signaling related roles in NTSR, while the \textit{AmGSTU2}
isoforms could rapidly develop new herbicide detoxifying activities. Further characterization of
the functional diversity of these GSTs in the future may help explain the multiple herbicide
resistant phenotypes observed in different NTSR blackgrass populations and shed new light on
the importance of these genes in cereal domestication.

Metabolic Herbicide Resistance in *Lolium* spp.

Resistance to small grain-selective acetolactate synthase (ALS) and acetyl CoA
 carboxylase (ACCase) inhibiting herbicides is common in *Lolium* spp. (rigid ryegrass, Italian
ryegrass) in the US, Europe, and especially in Australia (Boutsalis et al. 2012; Broster and
Pratley 2006; Heap 2014; Owen et al. 2014). Both target-site and non-target-site mechanisms
have evolved and these often co-occur within individuals and within populations (Han et al.
2016; Yu and Powles 2014). Metabolism-based resistance in Australian rigid ryegrass has
evolved to wheat-selective ACCase and ALS herbicides with a mosaic of different cross-
resistance and inheritance patterns indicative of multiple resistance genes and pathways
(reviewed in Yu and Powles 2014). Resistance to the ACCase inhibitor diclofop has been
experimentally evolved from initially susceptible individuals (Manalil et al. 2011; Neve and
Powles 2005), and the high level of phenotypic resistance achieved is due to enhanced diclofop
metabolism (Yu et al. 2013). Inheritance of the resistance is polygenic (Busi et al. 2013),
consistent with the hypothesis that multiple genes may contribute to metabolic resistance.
Interestingly, diclofop-susceptible populations were selected for even greater sensitivity (Manalil
et al. 2012), and protection against diclofop due to enhanced metabolism could be induced in
susceptible individuals by 2,4-D application (Han et al. 2013), confirming that both constitutive
 genetic variation and induced gene transcriptional regulation contribute to rigid ryegrass
phenotypic response to diclofop via metabolism. Metabolic resistance to pyroxasulfone was
experimentally evolved from a metabolic resistant population (ACCase, ALS, and mitosis inhibitors) (Busi et al. 2012), but no pyroxsulfone resistance evolved following repeated selection on a susceptible population. The experimentally-evolved pyroxsulfone-resistance appeared to be controlled by a single gene (Busi et al. 2014) and the population was also cross-resistant to prosulfocarb and triallate, with a subsequent selection using prosulfocarb resulting in higher prosulfocarb resistance (Busi and Powles 2013; Busi and Powles 2016). Pyroxsulfone resistance in the experimentally evolved population is due to glutathione conjugation and subsequent metabolic steps to inactive pyroxsulfone, with the first step likely mediated by GSTs (Busi et al. 2018). Some insecticides can reverse metabolic resistance in rigid ryegrass by inhibiting cytochrome P450s, including malathion for chlorsulfuron resistance (Christopher et al. 1994) and amitrole for diclofop resistance (Preston and Powles 1998). Phorate reverses chlorsulfuron, pyroxsulfone, and trifluralin resistance, but antagonizes prosulfocarb and triallate (Busi et al. 2017). Enhanced metabolism for herbicide resistance is still not completely understood, and can complicate rigid ryegrass management due to unpredictable cross-resistance patterns (Preston 2004).

Metabolic resistance in ryegrass has been associated with increased expression of genes with roles in metabolism, such as cytochrome P450s, GSTs, and GTs (Duhoux et al. 2015; Gaines et al. 2014). Although not yet reported, metabolic herbicide resistance could also be due to non-synonymous mutations in metabolism genes, resulting in faster herbicide metabolism due to changes in substrate affinity. An RNA-Seq transcriptome analysis was used to identify genes conferring enhanced metabolism resistance in a rigid ryegrass population experimentally evolved for diclofop resistance (Gaines et al. 2014). Candidate transcripts identified as overexpressed in resistant plants co-segregated with the resistance phenotype in an F2 population, including
CytP450s, GSTs, and GTs. A set of four transcripts (two cytochrome P450s, one nitrogen monooxygenase, and one glucosyl transferase), initially identified from the experimentally-evolved diclofop resistant population, were up-regulated in individuals from nine unrelated rigid ryegrass populations with metabolic resistance (Gaines et al. 2014). A cluster analysis demonstrated the potential of these four transcriptional markers in resistance diagnostics when multiple individuals were sampled from different populations, as herbicide susceptible individuals clustered together and populations containing metabolic resistant individuals could be classified as resistant (Figure 10). Candidate resistance genes from this ryegrass population are being functionally validated, and additional Australian ryegrass populations with different metabolic resistance patterns are being evaluated with transcriptomics for candidate resistance genes. An RNA-Seq analysis of ALS-resistant ryegrass from France identified a set of 19 transcriptional markers associated with metabolic ALS resistance (Duhoux et al. 2015). These 19 transcriptional markers provided diagnostic prediction of herbicide resistance in French ryegrass populations (Duhoux et al. 2017). The functional role of transcriptional marker genes in ryegrass has yet to be evaluated. A major question for metabolic herbicide resistance is that some genes associated with resistance across multiple populations through RNA-Seq studies may not have a functional role in resistance, i.e., they may not be the genes responsible for direct metabolic modifications to herbicides. These genes could be genetically linked to the major resistance gene(s), they could be co-regulated by a common transcription factor, or they could be co-regulated through chromatin structural changes. These genes may have other functions in resistance besides direct herbicide metabolism, or they could have no functional role in resistance at all. Improved basic knowledge...
about the molecular genetic basis of metabolic resistance and linked or co-regulated genes is necessary to develop metabolic resistance diagnostics based on molecular markers.

Mechanisms of Multiple Resistance in *Echinochloa phyllopogon*

*Echinochloa phyllopogon* (Stapf.) Koss. (=*E. oryzicola* Vasing), a predominantly self-pollinating allotetraploid species, is one of the most noxious weeds in rice production (Yamasue 2001). The C₄ photosynthetic pathway of *E. phyllopogon* allows it more efficient photosynthesis and makes it more competitive in summer fields of rice, which is C₃ species (Yamasue et al. 1998). *Echinochloa* genus includes other problematic resistance-prone species such as *E. crus-galli* and *E. colona*, both of which are allohexaploid. Among the three *Echinochloa*, *E. phyllopogon* is the best adapted to flooded condition and do not grow in upland condition. Relatively limited habitat of this species may explain the relatively limited number of resistance reports compared to *E. colona* and *E. crus-galli*.

Herbicide resistance in *E. phyllopogon* has been reported in populations in France, Greece, Brazil, South Korea and the US (Délye et al. 2015; Kaloumenos et al. 2012; Matzenbacher et al. 2014; Song et al. 2017). The populations of France, Greece and Brazil are resistant to ALS inhibitors due mainly to target-site-based mechanisms although additional non-target-site-based mechanism were not excluded. On the other hand, populations of South Korea and the US are known to have non-target-site-based mechanism and exhibit resistance to several herbicides from different modes of action. Here, the study on the resistance mechanism of *E. phyllopogon* found in the US is reviewed.
Multiple-Herbicide Resistant *Echinochloa phyllopogon*. The Sacramento Valley of California is one of the largest rice production areas in the US. Since the introduction of molinate and thiobencarb in the 60's and 80's, respectively, the two thiocarbamate herbicides were preferentially used for *Echinochloa* control (Fischer et al. 2000a). In 1994, another option from group A, fenoxaprop-ethyl, was introduced for postemergent control of *Echinochloa* spp (Williams 2000). Soon after, the failure in herbicide control of *E. phyllopogon* was reported from farmers (Fischer et al. 2000a). Greenhouse experiments conducted on the plants collected from fields in 1997 revealed that the plants exhibited resistance to fenoxaprop-ethyl, molinate, thiobencarb and bispyribac-sodium, a herbicide that was not released yet (Fischer et al. 2000a; Osuna et al. 2002). Later on, the research group revealed that the resistant *E. phyllopogon* also exhibited resistance to cyhalofop-butyl, penoxsulam, bensulfuron-methyl, quinclorac and clomazone, most of which had not been used by 1997 (Table 3).

Earlier studies by Tsuji et al. (2003) revealed that 15 resistant accessions collected in the Sacramento Valley were similar in plant morphology and genetic structure compared to the sensitive accessions. The results suggested that the resistant populations spread from a single multiple-herbicide resistant biotype. Interestingly, the resistant accessions have shorter plant height, narrower and shorter flag leaves, and thinner culms and produce less seeds (Boddy et al. 2012; Tsuji et al. 2003). It should be noted that it is still unknown whether these traits came from costs of adaptation to herbicide stress. Studies using crossed progenies of resistant and sensitive plants as have been conducted in the resistance mechanism study below will help deepen our understanding on the morphological characteristics observed in resistant plants.
Mechanism of Resistance to ALS Inhibitors. Metabolism-based Resistance. Multiple-herbicide resistant *E. phyllopogon* is resistant to three ALS inhibiting herbicides, bispyribac-sodium, penoxsulam and bensulfuron-methyl. The chemical class of the three herbicides are different: bispyribac-sodium, pyrimidinylthiobenzoate class; penoxsulam, triazolopyrimidine class; bensulfuron-methyl, sulfonylurea class. There are several reports of resistance factors which were estimated from different experiments in different cultivation conditions or with different lines (Fischer et al. 2000a, 2000b; Osuna et al. 2002; Yasuor et al. 2009). When compared in the same condition, resistance factor for line 511, which was the line used for molecular analysis, was about 1,100-fold for bensulfuron-methyl, 6.2-fold for penoxsulam (Iwakami et al., 2014a) and 1.8-fold for bispyribac-sodium (unpublished).

No difference was observed in the sensitivity of the target site to these herbicides *in vitro* (Fischer et al. 2000b; Osuna et al. 2002; Yasuor et al. 2009). In accordance with the enzyme assay, the nucleotide sequences of the two copies of ALS genes were identical between resistant and sensitive plants (Iwakami et al. 2012). These studies clearly deny that ALS resistance is not caused by target-site resistance. Studies of metabolism was mainly conducted on penoxsulam using $^{14}$C-penoxsulam. Resistant plants metabolized penoxsulam into polar metabolites 2.8 times faster than sensitive plants (Yasuor et al. 2009). A cytochrome P450 inhibitor, malathion, inhibited the metabolism of penoxsulam. Similarly, faster metabolism in resistant plants was confirmed in bensulfuron-methyl. The resistant plants metabolized bensulfuron-methyl into a de-methylated form (Iwakami et al. 2014a), which is known to lose ALS inhibiting activity (Takeda et al. 1986). Although metabolism was not tested for bispyribac-sodium, Fischer et al. (2000b) revealed application of cytochrome P450 inhibitors partially reversed bispyribac-sodium resistance.
Inheritance of ALS Resistance. Resistance inheritance was investigated in bensulfuron-methyl and penoxsulam using an F2 population derived from a cross of sensitive and resistant plants (Iwakami et al., 2014a). The resistance segregation in F2 generation fitted a model for monogenic inheritance of the resistance gene, when evaluated by the shoot length on herbicide containing medium.

Isolation of CYP81A cytochrome P450 Genes from E. phyllopogon. Cytochrome P450 comprise superfamily with hundreds of genes in plant genomes (Nelson et al. 2004). A recent study by Guo et al. (2017) revealed that a hexaploid close relative of E. phyllopogon, E. crus-galli, possesses as much as 917 P450 genes in its genome, suggesting the existence of approximately 600 cytochrome P450 genes in tetraploid E. phyllopogon. The very large number of genes have made the analysis of genes difficult especially in weed species with no reference genome sequence. No P450 genes involved in weed resistance were identified before the study conducted in E. phyllopogon. On the other hand, a P450 gene involved in rice tolerance to some herbicides were identified (Pan et al. 2006). The P450 gene belongs to CYP81A subfamily (CYP81A6) and is involved in tolerance to various ALS inhibiting herbicides including bensulfuron-methyl.

Attempts to isolate P450 genes from E. phyllopogon were started by nested degenerate PCR using the very few conserved regions of P450 genes, resulting in the isolation of 39 putative P450 genes including seven CYP81A members (Iwakami et al. 2014b). Later, degenerate PCR was used to exclusively amplify CYP81A genes and succeeded in the isolation of five more CYP81A genes from resistant plants (Iwakami et al. 2014a). Among the 12 CYP81A genes, 3 of
them are putative pseudogenes with indels that cause frameshifts. Comparisons of full-length sequences between the resistant and sensitive plants found nonsynonymous substitutions in CYP81A21, CYP81A22 and CYP81A26.

Characterization of CYP81A Genes. Transcript levels of the nine CYP81A genes except the three putative pseudogenes were compared between resistant and sensitive plants (Iwakami et al., 2014a). Two genes, CYP81A12 and CYP81A21, were constitutively highly expressed in the resistant plants. The two genes share extremely high similarity and were estimated as homeologues based on phylogenetic tree analysis, where the two genes appeared next to each other in the phylogenetic tree. The estimation of homeologous relationship was also supported by chromosome linkage analysis of the two genes. The analysis was performed using nucleotide polymorphisms found in both genes between the resistant and sensitive plants. The results indicated there was no linkage between the two genes, which implies that the two genes are on different chromosomes as expected from homeologous relationship. Over expression was also observed in CYP81A22 in addition to the two genes, although, the overexpression was restricted to the roots of resistant plants.

Trangenic Arabidopsis expressing CYP81A12 or CYP81A21 exhibited significant resistance to bensulfuron-methyl (Iwakami et al. 2014a). The resistance factors of the highly expressing lines were as high as more than 1,000-fold, suggesting the P450s metabolize bensulfuron-methyl very effectively. In vivo bensulfuron-methyl metabolism experiment using yeast revealed that the two P450s de-methylate bensulfuron-methyl. The results explain the higher amount of de-methylated form of bensulfuron-methyl in the resistant E. phyllopogon. Association of bensulfuron-methyl resistance and the higher expressions of the two genes were
investigated in F6 generation of the progenies of the resistant and sensitive *E. phyllopogon*. The experiment showed that bensulfuron-methyl resistance was perfectly associated with higher expression in all the F6 lines tested. In contrast to *CYP81A12* and *CYP81A21*, *CYP81A22* did not confer bensulfuron-methyl resistance in Arabidopsis. Higher expression of *CYP81A22* observed in resistant parent did not co-segregate with resistance in the study of F6 generation. Genotyping of F6 lines also revealed that amino acid substitutions found in *CYP81A21*, *CYP81A22* and *CYP81A26* were not associated with resistance. All the results strongly suggest that bensulfuron-methyl resistance in *E. phyllopogon* is caused by overexpression of *CYP81A12* and *CYP81A21*.

The overexpression mechanism of the two genes were investigated from the aspects of the promoter sequence and copy number (Iwakami et al. 2014a). Polymorphisms between the resistant and sensitive lines were observed in the promoter sequences of the two genes, but neither of them co-segregated with resistance in the F6 lines. Also, no difference was detected in the copy number by southern blot analysis. Considering the two genes are on different chromosomes and the resistance is under the control of a single gene (or locus), it is likely that a single trans-element simultaneously regulates the expression of both genes.

**Mechanism of Penoxsulam Resistance.** It is reasonable to infer that the same mechanism as bensulfuron-methyl resistance control the penoxsulam resistance based on the metabolism and inheritance studies above. As expected, bensulfuron-methyl and penoxsulam resistance did not segregate in F6 lines (Iwakami et al. 2014a). Arabidopsis lines transformed with *CYP81A12* or *CYP81A21* exhibited significant resistance to penoxsulam. Interestingly, the *Arabidopsis* that were >1,000-fold resistance to bensulfuron-methyl exhibited ~10-fold resistance to penoxsulam. These results suggest that *CYP81A12* and *CYP81A21* metabolize bensulfuron-methyl 100-fold
more efficiently than penoxsulam. The observation is consistent with the resistance levels to
bensulfuron-methyl (1,100-fold) and penoxsulam (6.2-fold) in *E. phyllopogon*. Taken together,
the two CYP81A genes play a major role in not only bensulfuron-methyl, but also penoxsulam
resistance.

**Bispyribac-sodium Resistance Mechanism is Not Clear.** In contrast to bensulfuron-methyl and
penoxsulam, *Arabidopsis* expressing CYP81A12 or CYP81A21 did not show any significant
resistance to bispyribac-sodium (unpublished). The failure of endowing resistance to *Arabidopsis*
does not necessarily indicate the genes are not involved in bispyribac-sodium resistance.

Knockout rice of CYP81A6, an ALS inhibitor tolerance gene in rice, has slightly higher
sensitivity to bispyribac-sodium although the difference is not very clear (Saika et al. 2014).
Therefore, CYP81A P450s may have functions of bispyribac-sodium metabolism. More detailed
studies will be required to investigate the involvement of the P450 in resistance to bispyribac-
sodium in *E. phyllopogon*.

**Mechanism of Resistance to ACCase Inhibitors.** Resistant plants exhibit resistance to AOPPs,
fenoxaprop-ethyl and cyhalofop-butyl, with a resistance factor 10 and 19, respectively (Bakkali
et al. 2007; Ruiz-Santaella et al. 2006). On the other hand, a cyclohexanedione ACCase
inhibitor, profoxydim, can effectively control resistant plants (Ruiz-Santaella et al. 2003).
Target site sensitivity to fenoxaprop-ethyl did not differ between the resistant and sensitive lines.
Nucleotide sequences of carboxy transferase domain of ACCase, where all the resistance-
conferring amino acid substitutions were found in plants, were compared between the resistant
and sensitive *E. phyllopogon*. In accordance with the enzyme sensitivity, no resistance-conferring mutations were observed in the four copies of ACCase genes in *E. phyllopogon*. These results indicate that the ACCase resistance mechanism is non-target-site based.

A metabolism study was performed for fenoxaprop-ethyl and cyhalofop-butyl. Rapid accumulation of GSH-conjugated metabolites in resistant plants strongly suggest that fenoxaprop-ethyl resistance is caused by enhanced activity of GST (Bakkali et al., 2007). The results suggest that an additional mechanism in addition to overexpressions of *CYP81A12* and *CYP81A21* is behind the multiple-herbicide resistance in *E. phyllopogon*. Resistant plants accumulated more polar metabolites of cyhalofop-butyl than sensitive plants (Ruiz-Santaella et al., 2006).

**Mechanism of Resistance to Clomazone.** Clomazone is one of the modes of action that the resistant plants have never experienced in rice fields (Table 3). The resistance factor is not very high (2x), so clomazone eluded researchers from classification as resistance in the first screening (Fischer et al. 2000a; Yasuor et al. 2008). However, after the introduction of clomazone in the Sacramento Valley, control failures with clomazone have been observed, leading to the findings of the low level resistance (Yasuor et al. 2008). A detailed study of clomazone resistance revealed that the resistant plants more rapidly metabolize clomazone into oxidative forms than sensitive plants (Yasuor et al. 2010).

**Mechanism of Resistance to Quinclorac.** One of the surprising resistance observed in the resistant plants is quinclorac resistance because enhanced metabolism was not proposed as a
resistance mechanism to this herbicide in plants (Yasuor et al. 2011). Therefore, study on the
mechanism of quinclorac resistance was addressed with attention to the mode of action of auxin
herbicides.

The resistance factor to quinclorac is somewhat different between the methods of
quinclorac application: foliar spray application, 6-fold; hydroponic root application, 17-fold
(Yasuor et al. 2011). As in other auxin herbicides, quinclorac is known to induce high level
ethylene production, which is highly related with plant sensitivity to quinclorac (Grossmann and
Kwiatkowski 1993). It is not fully understood, but the correlation is thought to be caused by
HCN accumulation in plants as a byproduct of ethylene production. Yasuor et al. (2011)
compared the ethylene production between the resistant and sensitive E. phyllopogon and found
resistant plants produced significantly lower ethylene. Another interesting finding from their
research was that resistant plants have higher activity of β-cyanoalanine synthase (β-CAS),
enzyme to detoxify HCN. Together, the authors concluded that quinclorac resistance in the
resistant E. phyllopogon is caused by (a) insensitivity along the ethylene production pathway and
(b) enhanced β-CAS activity.

**Future Work.** As reviewed above, extensive work on ALS inhibitor resistance resulted in the
identification of P450 involved in resistance in *E. phyllopogon*. The genes may explain
resistance to other herbicides such as clomazone where enhanced oxidation of clomazone was
reported. On the other hand, the involvement of other herbicide-metabolizing genes namely
*GSTs* are suggested in the case of fenoxaprop-ethyl resistance. Furthermore, even non-
metabolism-based resistance is suggested in quinclorac resistance. A hint to elucidate the
apparently complicated mechanism of multiple-herbicide resistance may come from analyses of
thiocarbamate (molinate and thiobencarb) resistance, which have not yet been investigated. Although findings of resistant populations in the Sacramento Valley occurred right after fenoxaprop-ethyl introduction, the driving force of resistance evolution may be continuous application of thiocarbamates for more than 20 years. Therefore, elucidation of resistance mechanism to thiocarbamates might provide an insight on how the evolution of multiple-herbicide resistance occurred in *E. phyllopogon*. A preliminary work suggested concerted upregulations of several gene families involved in herbicide metabolism (unpublished) as has been reported in other metabolism-based resistant weeds (Duhoix et al. 2015, 2017; Gaines et al. 2014; Gardin et al. 2015). Approaches such as genomics and transcriptomics will shed further light on the mystery of simultaneous evolution of resistance to multiple herbicides in *E. phyllopogon*.

**Multiple Resistance and Metabolic Resistance Mechanisms in Junglerice**

A junglerice ([*Echinochloa colona* (L.) Link] biotype MS1, that was originally misdiagnized as barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.], collected from a rice field in Sunflower County, Mississippi, was resistant to ALS-inhibiting imazethapyr and cross-resistant to other ALS inhibitors such as imazamox (3.3-fold), penoxsulam (9.4-fold), and bispyribac-sodium (7.2-fold) (Riar et. al. 2012). In preliminary experiments, the addition of malathion to penoxsulam and imazethapyr reduced shoot dry weight and/or increased mortality compared to the respective herbicides applied alone, indicating possible involvement of herbicide metabolism driven by cytochrome P450 monooxygenase enzymes (CYP) as a mechanism of resistance. ALS enzyme assays or ALS gene sequencing analysis did not indicate a modified target-site based resistance in the MS1 biotype (Riar et. al. 2013). Lower levels of
translocation of $^{14}$C-bispyribac and $^{14}$C-imazamox in the MS1 biotype compared to a susceptible biotype were recorded, perhaps, indicating a result of metabolism (Riar et. al. 2013).

Additionally, the MS1 biotype was resistant to fenoxaprop-P-ethyl, an ACCase inhibitor, (11-fold; but susceptible to sethoxydim and clethodim at field rates, also ACCase inhibitors) (Wright et. al. 2016). Sequencing of ACCase of MS1 did not reveal the presence of any known resistance-conferring point mutations. An enzyme assay confirmed that the ACCase in the MS1 biotype was herbicide sensitive. Further investigations with two CYP inhibitors, malathion and piperonyl butoxide, and a glutathione-S-transferase inhibitor, 4-chloro-7-nitrobenzofurazan, did not indicate involvement of any metabolic enzymes inhibited by these compounds (Wright et al. 2016).

No reference genome is available for junglerice. RNA-seq analysis of changes in gene expression in the MS1 and a susceptible biotype before and after imazamox treatment was conducted to generate a reference leaf transcriptome (Wright et al. 2017). Differentially expressed transcripts between resistant MS1 and susceptible plants included transcription factors, protein-modifying enzymes, and enzymes involved in metabolism and signaling, which are involved in abiotic stress response in other plants. These results suggest that imazamox exposure induced a stress response. A time course study examining a subset of transcripts showed that expression peaked within 4-12 h and then returned to untreated levels within 48 h of exposure. Two additional biotypes showed a similar change in gene expression 4 h after herbicide exposure compared to the resistant and sensitive biotypes. Thus, within 48 h junglerice mounted a stress response to imazamox exposure (Wright et al. 2017).

The MS1 biotype was resistant to propanil and quinclorac as well (Wright et al. 2018). Differential gene expression analysis of resistant and sensitive plants revealed that 170
transcripts were upregulated in resistant plants relative to sensitive plants and 160 transcripts were upregulated in sensitive plants. In addition, 507 transcripts were only expressed in resistant plants and 562 only in sensitive plants. A subset of these transcripts was investigated further using quantitative PCR (qPCR) to compare gene expression in resistant plants with expression in additional sensitive biotypes. The qPCR analysis identified two transcripts, a kinase and a glutathione S-transferase that were significantly upregulated in resistant plants compared with the sensitive plants. A third transcript, encoding an F-box protein, was downregulated in the resistant plants relative to the sensitive plants. As no cytochrome P450s were differentially expressed between the resistant and sensitive plants, a single-nucleotide polymorphism analysis was performed, revealing several nonsynonymous point mutations of interest. These candidate genes will require further study to elucidate the resistance mechanisms present in the resistant biotype (Wright et al. 2018).

Metabolism-Based Multiple Resistance in Palmer Amaranth

As a result of extensive and intensive selection of PRE- and/or -POST use of most commonly used herbicides in cropping systems, Palmer amaranth has evolved resistance to multiple modes of action, e.g., microtubule-, EPSPS-, ALS-, PS II-, HPPD-, and more recently, to PPO-inhibitors (Heap 2018). Besides herbicide selection, other factors such as biological characteristics of weed species, genetic factors, characteristics of herbicides and agronomic practices also play an important role in the evolution and spread of herbicide resistance in weed species (Powles and Yu 2010). Palmer amaranth characteristics such as high fecundity, germination percentage, wide window of emergence, seed dispersal and short longevity, facilitate the evolution of resistance in response to herbicide selection. Recent advances in
agronomic practices such have increased adoption of no-till or reduced tillage practices in crop production to prevent soil erosion and conserve moisture (Kihara et al. 2012). As a consequence of this practice, the use of herbicides for weed management became indispensable in crop production across many parts globally, creating greater herbicide selection.

**Evolution of Multiple Herbicide Resistance in Palmer Amaranth in Kansas (KS).** Palmer amaranth populations resistant to four modes of action of herbicides, i.e., PS II-, ALS-, EPSPS-, and HPPD-inhibitors have been reported in KS (Heap 2018). Several populations of Palmer amaranth with resistance to at least two of these herbicide modes of action are common in KS. However, a single population of Palmer amaranth (KSR) in central KS (Stafford County), with resistance to PS II- and HPPD-inhibitors was first confirmed in 2012 (Thompson, 2012), in a field where there was no previous history of applications of HPPD-inhibitors but did have a long history of PS II- and ALS-inhibiting herbicides. This population was originally found resistant to Huskie®, a premix of pyrasulfotole (HPPD-inhibitor) and bromoxynil (PS II-inhibitor) in the field. Later, resistance to atrazine (Nakka et al. 2017a; Thompson et al. 2012), chlorsulfuron (Nakka et al. 2017b), and several HPPD-inhibitors (e.g., mesotrione, tembotrione, and topramezone) (Nakka et al. 2017c; Thompson et al. 2012) was confirmed and characterized in this population. Investigation of the mechanism of resistance to atrazine, chlorsulfuron, and mesotrione in this population enabled to address why this population was predisposed to evolve resistance to HPPD-inhibitors even though there was no selection of HPPD-inhibitors.

**Mechanism of Atrazine Resistance in KSR Palmer Amaranth.** The psbA gene in the chloroplast which encodes a D1 protein is the target site of PS II-inhibitors (e.g., triazine,
triazinone, uracil, nitrile, etc.). The D1 protein is an important component of electron transport during photosynthesis as it acts as the plastoquinone binding site. The PS II-inhibitors competitively bind to the D1 protein replacing the plastoquinone during electron transport (Gronwald 1994). Several mutations in *psbA* gene resulting in the evolution of resistance or cross-resistance to different classes of PS II-inhibitors have been reported (Oettmeier 1999). However, in particular, resistance to triazines in a number of weed species is commonly endowed due to a single nucleotide polymorphism in *psbA* gene resulting in the substitution of the amino acid serine264glycine (Gronwald 1994). This point mutation in the *psbA* gene can provide a high-level of resistance to triazine herbicides (Arntz et al. 2000; Gronwald 1994). Furthermore, such resistance to atrazine is associated with fitness costs as well (Conard et al. 1979).

Although the resistance to triazines is commonly associated with mutations in the *psbA* gene, weed species such as velvetleaf (*Abutilon theophrasti* Medik.; Gray et al. 1996), waterhemp (Ma et al. 2013), black grass (Cummins et al. 1999), and rigid ryegrass (Burnet et al. 1993), exhibited enhanced metabolism of atrazine or simazine via glutathione-S-transferases (GSTs) activity. Similarly, crops such as corn or sorghum are also naturally tolerant to triazines due to rapid metabolism of these herbicides mediated by GST activity. Cytochrome P450s and GSTs are multifunctional enzymes that act as substrates in phase I and phase II metabolic detoxification of pesticides (Marrs 1996).

A high level of resistance to atrazine was confirmed in KSR Palmer amaranth exhibiting up to 200-fold resistance relative to a known susceptible population (Nakka et al. 2017a). Upon amplification and sequencing of the conserved fragment (578 bp) of *psbA* gene, no known mutations in this region was found, including the most common substitution serine264glycine in
the *psbA* gene, in KSR Palmer amaranth (Nakka et al. 2017a). Also, when the F₁ progeny (generated by reciprocal crosses between KSR and a known susceptible Palmer amaranth) were screened with 4480 g ai ha⁻¹ of atrazine (twice the field recommended dose), the progeny segregated for atrazine resistance or susceptibility. Such response of F₁ progeny to atrazine treatment clearly indicates that the resistance trait is not maternally inherited, rather transmitted by a nuclear gene. Hence this trait can be spread both via pollen and seed parent (Nakka et al. 2017a). On the other hand, the KSR Palmer amaranth rapidly conjugated [U⁻¹⁴C] atrazine, possibly by GSH mediated by GST activity, within 4 hours after treatment (HAT) (Nakka et al. 2017a). At 4 HAT, about 7.5% and >80% of parent [U⁻¹⁴C] (active form) atrazine remained in KSR and a susceptible Palmer amaranth, respectively. These results confirm predominance of metabolism-based atrazine resistance via GST-mediated conjugation in KSR Palmer amaranth.

**Mechanism of Clorsulfuron Resistance in KSR Palmer Amaranth.** Several classes of ALS-inhibitors, such as SU, imidazolinones (IMI), triazolopyrimidines (TP), pyrimidinylthiobenzoates (PTB) and sulfonylaminocarbonyltriazolinones are extensively used for a broad spectrum of weed control, including the members of Amaranthaceae. The ALS enzyme, which is the target site of ALS-inhibitors, catalyzes an important step in the biosynthesis of branched-chain amino acids, valine, leucine, and isoleucine in plants and microorganisms (Dailey and Cronan 1986; Shaner 1991). Sulfonylurea herbicides such as chlorsulfuron are active on several weed species including Palmer amaranth. However, Palmer amaranth resistance to ALS-inhibitors is widespread across many states in the US, including KS (Heap 2018). The ALS-inhibitor-resistant Palmer amaranth was first documented in KS in 1993 (Horak and Peterson 1995).
A high-level of resistance to ALS-inhibitors due to point mutations resulting in single amino acid substitutions in the *ALS* gene has been reported in many weed species (Shaner 1991). In the last decade, the number of mutations in *ALS* gene conferring resistance to ALS-inhibitors has increased dramatically, and a total of 26 amino acid substitutions were identified across eight amino acid positions (Tranel et al. 2016). Mutations resulting in amino acid substitution at the proline197 position is most commonly reported in many weed species (Guttieri et al., 1995; Foes et al. 1999; Patzoldt and Tranel 2007; Varanasi et al. 2015). Nonetheless, enhanced metabolism of ALS-inhibitors, bestowing resistance has also been found in a number of weed, e.g., rigid ryegrass (Christopher et al. 1991; Cotterman and Saari 1992), wild mustard (*Sinapsis arvensis* L.; Veldhuis et al. 2000), and waterhemp (Guo et al. 2015). Increased activity of cytochrome P450 monooxygenases in the metabolism of this group of herbicides has been reported (Christopher et al. 1994). Also, rapid detoxification of the ALS-inhibitors via a wide array of cytochrome P450 enzymes bestows crop (e.g., wheat, barley, rice or corn) tolerance to these herbicides (Brown 1990).

In a dose-response assay, the KSR Palmer amaranth exhibited >275 times more resistance to chlorsulfuron relative to a known susceptible population (Nakka et al. 2017b). Other *Amaranthus* species, such as redroot pigweed (*A. retroflexus* L.) with >429 and 34 times more resistant to thifensulfuron and nicosulfuron, respectively, compared to a known susceptible population have also been reported (Scarabel 2007). Whereas, Powell amaranth (*Amaranthus powellii* (S.) Wats.) was 1257 more resistant to thifensulfuron compared to a susceptible control (Ferguson et al. 2001). ALS-inhibitor-resistant prostrate pigweed (*A. blitoides* (S.) Wats.) and redroot pigweed had mutations in *ALS* gene resulting in two substitutions, i.e., proline197serine or leucine, respectively (Sibony et al. 2001; Sibony and Rubin 2003). In smooth pigweed (*A.
hybridus L.) and common waterhemp (A. rudis Sauer) amino acid substitutions at alanine122threonine, aspartate376glutamate, tryptophan574leucine, or serine653threonine conferred ALS-inhibitor resistance ranging from 60-to 3200-fold depending on the type of substitution (Patzoldt and Tranel 2007; Whaley et al. 2006, 2007). In KSR Palmer amaranth that survived chlorsulfuron treatment, as well as known susceptible plants, about ~ 2 kb length of the ALS gene covering all known mutations at eight codon positions was amplified and sequenced. Interestingly, only 30% of chlorsulfuron-resistant KSR plants showed the single nucleotide polymorphism, resulting in an amino acid substitution of proline (CCC) to serine (TCC) at the position 197 of ALS gene. The remaining 70% of chlorsulfuron-resistant plants did not show any mutations (Nakka et al. 2017b). Further, whole plant response of KSR Palmer amaranth treated with a combination of chlorsulfuron and malathion (an organophosphate insecticide, known to inhibit the activity of cytochrome P450 enzymes) showed reduction in biomass accumulation when compared to plants that were treated with either malathion or chlorsulfuron alone, or non-treated plants (Nakka et al. 2017b), indicating the synergistic effect of malathion on chlorsulfuron. These data suggest that KSR Palmer amaranth exhibit two different mechanisms of ALS-inhibitor resistance, a) detoxification of chlorsulfuron as a result of P450 activity and b) as a result of a point mutation (proline197serine) in ALS gene that confers resistance. However, metabolism-based resistance appears to exist predominantly in KSR Palmer amaranth (Nakka et al. 2017b). The KSR Palmer amaranth population is a prime example where a weed population exhibits co-existence of both target-site and metabolism-based resistance to ALS-inhibitors.
Mechanism of Mesotrione Resistance in KSR Palmer Amaranth. Herbicides that inhibit HPPD enzyme such as mesotrione are widely used to control a broad spectrum of weeds in agriculture. HPPD-inhibitors are a relatively new class of chemistry discovered about three decades ago and are widely used in agriculture for weed management in crops such as corn.

Mesotrione inhibits carotenoid biosynthesis resulting in pigment degradation and eventually, plant death. Corn selectivity to these herbicides is endowed because of rapid metabolism, via ring hydroxylation-mediated by cytochrome P450 monooxygenase(s) combined with reduced uptake (Mitchell et al. 2001). To date, only two weed species, i.e., common waterhemp and Palmer amaranth, have evolved resistance to HPPD-inhibitors (Heap 2018). The HPPD-inhibitor resistant common waterhemp was first reported in IL in 2009 (Hausman et al. 2011). This biotype of common waterhemp was also found resistant to atrazine. Detoxification of mesotrione possibly mediated by P450 and atrazine via GST-mediated conjugation has been attributed to mesotrione and atrazine resistance, respectively in this waterhemp population (Ma et al. 2013).

The first case of HPPD-inhibitor-resistant Palmer amaranth (KSR) was documented in KS in 2012 (Thompson et al. 2012). Later, in Nebraska, USA, Palmer amaranth populations were found resistant to these herbicides, in a cornfield, which had a history of continuous use of HPPD-inhibitors (Sandell et al., 2012). As mentioned earlier, the field where KSR Palmer amaranth was found, had no previous history of use of HPPD-inhibitors.

The KSR Palmer amaranth was up to 18 times more resistant to mesotrione compared to a known sensitive population (Nakka et al. 2017c). However, in response to mesotrione treatment, the KSR plants exhibited initial injury (bleached) symptoms on shoot meristem but three weeks after treatment the plants recovered from the injury and continued to grow normally (Nakka et al. 2017c). Investigation of the mechanism of mesotrione resistance in KSR Palmer...
amaranth revealed no difference in uptake or translocation of mesotrione or its metabolites between KSR and a known susceptible Palmer amaranth (Nakka et al. 2017c). However, similar to mesotrione-resistant common waterhemp, KSR Palmer amaranth also rapidly metabolized [14C]mesotrione. Even at 4 HAT ~50% of parent [14C] mesotrione was metabolized, while > 70% was still present in an active form in susceptible samples. At 24 HAT, KSR plants metabolized >90% of mesotrione compared to susceptible plants (Nakka et al. 2017c). Furthermore, the KSR plants were found to detoxify 50 % of mesotrione in a shorter time compared to corn or common waterhemp (Ma et al. 2013; Nakka et al. 2017c).

To assess the possibility of co-existence of target-site and non-target-site resistance to HPPD-inhibitors in KSR Palmer amaranth as observed for ALS-inhibitors, the HPPD gene of KSR and a susceptible Palmer amaranth was sequenced and amplified. However, no mutations nor amplification of HPPD gene that can confer resistance to mesotrione was found (Nakka et al. 2017c). Interestingly, the KSR plants exhibited increased constitutive expression of HPPD transcript and protein (Nakka et al. 2017c). The mesotrione-resistant KSR plants showed at least 8 to 12-fold increase in HPPD mRNA levels (normalized against β-tubulin and CPS) relative to susceptible plants. Also, the HPPD protein expression correlated with the transcript expression (Nakka et al. 2017c). The upregulation of HPPD transcript in KSR plants could have occurred via changes in the cis or trans acting elements or alterations in the promoter region of the HPPD gene. Overall, the mesotrione resistance in KSR Palmer amaranth is conferred predominantly because of rapid detoxification of mesotrione, although increased HPPD gene and protein expression also play a role in the resistance mechanism.
The Predominance of Metabolism-based Multiple Herbicide Resistance in KSR.

Characterization of multiple resistance to PS II-, ALS-, and HPPD-inhibitors in KSR Palmer amaranth clearly demonstrates the predominance of metabolism-based resistance in this population to these groups of herbicides (Figure 11). The exact chronology of the evolution of resistance to PS II- or ALS-inhibitors, in this particular population, is unknown. However, in KS, resistance to ALS-inhibitors in Palmer amaranth was documented prior to PS-II inhibitors (Heap 2018). But the resistance to HPPD-inhibitors evolved more recently in this population (Thompson 2012). It is believed in KSR Palmer amaranth population also, resistance to ALS-inhibitors may have evolved prior to PS-II inhibitors. Regardless, the predominance of metabolism-based resistance to ALS- and PS II-inhibitors mediated by cytochrome P450 or GST activity during phase I and II detoxification process suggest presence of increased activity of these enzymes, which potentially predisposed this population to detoxify other xenobiotics, such as HPPD-inhibitors even though no selection pressure from this herbicide was imposed on this population. The role of specific genes of P450 enzyme family in detoxification of chlorsulfuron or GSTs in atrazine metabolism is yet to be uncovered. Thus, the prevalence of metabolism-based resistance to ALS- and PS II-inhibitors may have predisposed this population to evolve resistance to HPPD-inhibitors. Our results strongly suggest that the metabolism-based herbicide resistance can be a serious threat to weed management, especially in weeds such as Palmer amaranth which is one of the top-ranked economically important weeds across the US.

Summary and Significance. The KSR Palmer amaranth was resistant to most commonly used herbicides (e.g., atrazine, chlorsulfuron and mesotrione) in US Midwestern agriculture. Dose-response experiments indicated up to 230, 275 and 18 fold resistance to atrazine, chlorsulfuron,
and mesotrione, respectively, in KSR Palmer amaranth. Furthermore, no-known mutations in the $psbA$ gene conferring atrazine resistance were found in KSR, ruling out the maternal inheritance of the resistance trait. On the other hand, rapid degradation of atrazine by GST activity was found to provide a high level of resistance to atrazine in this population (Nakka et al. 2017a). Interestingly, co-existence of target-site and metabolism-based resistance to chlorsulfuron and mesotrione was found in KSR, although the primary mechanism appears to be rapid metabolism of ALS-inhibitors (Figure 11). A large frequency of the resistant plants, i.e., 70% exhibited metabolism-based resistance to chlorsulfuron, while 30% of the plants confirmed the presence of nucleotide polymorphisms in the $ALS$ gene (Nakka et al. 2017b). Similarly, KSR plants rapidly metabolized mesotrione even at 4 HAT, nonetheless, the resistant plants also exhibited increased expression of $HPPD$ gene as well as protein (Nakka et al. 2017c). These results suggest co-existence of target-site and metabolism-based resistance to ALS- and HPPD-inhibitors in KSR Palmer amaranth (Figure 11). The prevalence of metabolism-based resistance in Palmer amaranth is a threat to sustainable crop production in this region. Because such a mechanism may confer resistance to other herbicides and even to those that are yet to be discovered. The findings of this research are novel and valuable in order to recommend appropriate weed management strategies in the region and should include diversified tactics to prevent evolution and spread of multiple herbicide resistance in Palmer amaranth.

Biochemical Mechanisms Conferring Multiple Herbicide Resistance in Waterhemp Waterhemp is a problematic, summer annual broadleaf weed species severely affecting maize, soybean, sorghum, and cotton production in the United States (Hager et al. 2002; Heap 2018; Steckel and Sprague 2004). The $C_4$ carbon-fixation pathway and prolonged emergence
period are two factors that allow waterhemp to compete with crops in the field, especially under hot and dry weather conditions (Steckel 2007). No new herbicide modes of action have been commercialized in the past three decades (Duke 2012). Genes conferring resistance are easily spread throughout natural waterhemp populations by pollen flow due to the obligate outcrossing nature of waterhemp, which makes effective herbicide options for management of waterhemp even more limited (Costea et al. 2005; Steckel 2007).

A waterhemp population (named MCR) from central Illinois is the first reported natural weed population to evolve resistance to 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicides (Hausman et al. 2011), which represents the fifth herbicide site-of-action group for resistance in waterhemp (Heap 2018). A different waterhemp population from Nebraska (named NEB) has also demonstrated resistance to HPPD-inhibiting herbicides (Kaundun et al. 2017), and another waterhemp population from Nebraska exhibited resistance to the synthetic auxin herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Figueiredo et al. 2018). In each of these cases, rapid herbicide metabolism contributed to or conferred resistance in the population, as described below.

In addition to HPPD-inhibitor resistance in MCR, this population is resistant to s-triazines, acetolactate synthase (ALS)-inhibiting herbicides, and the foliar applied protoporphyrinogen oxidase (PPO)-inhibiting herbicide, carfentrazone-ethyl (Ma et al. 2015; Obenland et al. 2018). Neither altered target sites (HPPD for mesotrione or \textit{psbA} encoding the D1 protein in photosystem II for s-triazines) nor increased uptake of mesotrione or atrazine was detected in the MCR population. Biochemical studies using excised leaves and whole-plants derived from vegetative clones demonstrated that elevated rates of metabolism, through distinct detoxification pathways, contribute to herbicide resistance in MCR (Ma et al. 2013). These
metabolic pathways include: (1) oxidative metabolism (presumably via P450s) for two HPPD-inhibiting herbicides, mesotrione (Ma et al. 2013) and topramezone (Ma et al. 2018), as well as the ALS-inhibiting herbicide primisulfuron-methyl (Guo et al. 2015; Ma et al. 2015), and (2) GSH conjugation catalyzed by GSTs for atrazine (Evans et al. 2017; Ma et al. 2013; Ma et al. 2016). The use of an excised leaf assay and vegetatively cloned plants ensured that the herbicide-metabolism assay is independent of whole-plant translocation patterns, and identical waterhemp genotypes are analyzed within a biochemical time-course analysis (Ma et al. 2015). The P450 inhibitors, malathion and tetcyclacis, decreased mesotrione metabolism and further reduced the biomass of MCR plants when applied with mesotrione (Ma et al. 2013). Additionally, malathion resulted in a greater injury from ALS-inhibiting herbicides, including primisulfuron, cloransulam, sulfometuron, pyrithiobac, and imazethapyr to MCR (Guo et al. 2015). Treatment with the GST inhibitor 4-chloro-7-nitrobenzofurazan followed by an atrazine PRE or POST application significantly enhanced biomass reduction in another atrazine-resistant waterhemp population from central Illinois (ACR) compared with the atrazine-only treatment (Ma et al. 2016). These increased herbicide activities when applied with metabolic inhibitors to resistant waterhemp further support metabolism-based resistance mechanisms to multiple herbicides in waterhemp. Similarly, rapid mesotrione metabolism via 4-hydroxylation of the dione ring was detected in the NEB population compared to a sensitive population (Kaundun et al. 2017). Rapid metabolism of 2,4-D contributes to resistance in another waterhemp population from Nebraska, which is also potentially mediated by P450 activity (Figueiredo et al. 2018).

Genetic and Molecular Basis of Multiple-Herbicide Resistance in Waterhemp. Metabolism-based herbicide resistance can confer unpredictable and complicated cross- or multiple-resistance
to herbicides with the same or different sites of action, which could be controlled by quantitative
trait(s) (Délye 2013). A genetic study using F2 segregating lines of multiple herbicide-resistant
(MCR) and sensitive waterhemp populations demonstrated that atrazine resistance in the MCR
population is conferred by a single, incompletely dominant nuclear gene, whereas the inheritance
of mesotrione resistance in the MCR population is more complex and appears to be a multigenic
trait (Huffman et al. 2015). Following up on the atrazine resistance trait in the MCR and ACR
populations, traditional protein purification and proteomic methods tested the hypothesis that
enhanced metabolic detoxification of atrazine occurs by a distinct GST isozyme (Evans et al.
2017), which ultimately confers atrazine resistance in the MCR population. Several GST proteins
were identified by liquid chromatography–mass spectrometry (LC-MS) in affinity-purified
fractions from waterhemp using peptide sequence similarity with GSTs from Arabidopsis or
other dicots. Elevated, constitutively expressed transcript levels of one phi-class GST (named
AtuGSTF2) strongly correlated with atrazine resistance in waterhemp (MCR and ACR
populations) as well as an F2 population that segregates for metabolic atrazine resistance
(Huffman et al. 2015). This correlation indicates that AtuGSTF2 is the predominant GST protein
that confers atrazine resistance in waterhemp (Evans et al. 2017), although additional research
involving gene cloning, promoter analysis, and generation of transgenic plants overexpressing
AtGSTF2 is required to prove this hypothesis.

Ongoing Research Investigating Unique Mechanisms Conferring Resistance to
Topramezone and Carfentrazone-Ethyl in Waterhemp. The MCR population also
demonstrated resistance to topramezone (Hausman et al. 2011), another HPPD inhibitor having a
distinct pyrazole structure (Ndikuryayo et al. 2017) compared to the triketone structures of
mesotrione and tembotrione (Figure 12). Biochemical studies with excised leaves and whole plants indicated that an elevated rate of oxidative metabolism confers topramezone resistance in the MCR population relative to two HPPD-sensitive waterhemp populations (Ma et al. 2018). However, the metabolic route of topramezone determined in the MCR population is different than the rapid N-demethylation reaction that occurs in tolerant maize (Grossmann and Ehrhardt 2007), and ongoing research is aimed at determining the precise structure of topramezone metabolites formed in the MCR population (Lygin, Riechers, and Kaundun, unpublished data). Finally, MCR displayed foliar resistance to carfentrazone-ethyl but sensitivity to diphenylethers (DPEs), a different class of PPO inhibitors compared to carfentrazone-ethyl (an aryl triazinone). Carfentrazone-ethyl resistance in the MCR population is not due to a glycine codon deletion or arginine substitution in the PPO2 enzyme; however, it is more likely conferred through a NTSR mechanism such as enhanced oxidative metabolism (Obenland et al. 2018), although additional research is required to directly confirm this hypothesis.

In summary, these biochemical and molecular mechanisms conferring resistance to multiple herbicides with different sites of action indicate waterhemp possesses multiple genes encoding diverse metabolic enzymes, which ultimately result in complex, herbicide-dependent, cross- or multiple-resistance patterns. Cross and multiple herbicide resistance could pose serious challenges for waterhemp management in the future, especially if these resistance mechanisms do not confer a fitness cost to the plant in the absence of herbicide (Délye 2013; Yu and Powles 2014). Additionally, herbicide resistance due to rapid herbicide metabolism via elevated P450 or GST activity, irrespective of herbicide sites of action, has potential to confer resistance to not only existing commercial herbicides but also new or yet-to-be-discovered active ingredients (Yu and Powles 2014). A better understanding of the biochemical, molecular, and genetic
mechanisms conferring metabolic resistance to multiple herbicides in waterhemp provides
insights into evolving weed populations in response to selection pressures, development of
innovative and integrated resistant weed management strategies, and demonstrates an urgent
need for new herbicide site of action discovery (Duke 2012).

Conclusions

Several weed populations around the world have evolved resistance to herbicides by
metabolizing herbicide active ingredients to non phytotoxic metabolites. Characterization of
metabolic resistance mechanisms and underlying biochemical and molecular regulation is a
difficult and arduous process. However, recent strides in OMICS approaches and procedures
have made it possible to delineate roles of enzyme systems such as CYPs, GSTs, and GTs in
metabolic and multiple resistance to herbicides in plants, including both crops and weeds, as
outlined in this monograph.

Acknowledgments

VKN gratefully recognizes funding from the Weed Science Society of America towards
organizing a symposium at the 2018 annual meeting in Arlington, VA. DER acknowledges
expert technical assistance from Dr. Anatoli V. Lygin, Dr. Mayandi Sivaguru, and Dr. Yousoon
Baek, University of Illinois-Urbana. RE acknowledges the joint support of the Biotechnology
and Biological Sciences Research Council (grant BB/L001489/1) and Agriculture and
Horticulture Development Board (RD-2012-3807).

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contributes to resistance in a common waterhemp (*Amaranthus tuberculatus*) population.


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Disruption of OPR7 and OPR8 reveals the versatile functions of jasmonic acid in maize development and defense. Plant Cell 24:1420-1436


Figure Captions

Figure 1. Representative structures of oxidized lipids (oxylipins) formed in plants. Two classes of oxylipins are generated from α-linolenic acid as substrate; either non-enzymatically formed (A, generalized phytofuran, or B, phytoprostane) via interaction with reactive oxygen species or enzymatically synthesized (C, jasmonic acid). For more details on structures and biosynthetic pathways see Cuyamendous et al. (2015), Durand et al. (2011), and Mosblech et al. (2009).

Figure 2. Tissue distribution of GST proteins in a cross section of etiolated grain sorghum seedlings, probed with an antiserum raised against the tau-class TtGSTU1 protein from wheat (Riechers et al. 2003). (A), unsafened (DMSO only) seedling, no primary antiserum (negative control); (B) unsafened (DMSO only) seedling, probed with a 1:500 dilution of primary antiserum raised against TtGSTU1; (C) seedling treated with 10 µM fluxofenim safener for 12 hours, probed with a 1:500 dilution of primary antiserum raised against TtGSTU1. Red arrows in (C) mark the massive accumulations of immunoreactive GST proteins in the outermost coleoptile and epidermal cells. Abbreviations: CL, coleoptile; LP, inner leaf primordia.

Figure 3. Structures of clomazone, 5-OH clomazone and 5-keto clomazone.

Figure 4. Effect of phorate on chlorophyll and carotenoid levels in new leaves of cotton seedlings treated with 100 nM clomazone for 6 days.
Figure 5. Metabolism of $^{14}$C-glyphosate 1 DAT in conventional (A, B) and GR canola (C, D).

The data are expressed as percent distribution of total radioactivity in each leaf on the left side and as actual dpm g$^{-1}$ DW of the leaves on the right. (Black bars) Glyphosate; (gray bars) AMPA, and (white bars) unknown metabolite(s). Error bars are ±1 SE. dpm, disintegrations per minute. With permission for Corrêa et al. 2016.

Figure 6. Various strategies used by organisms that producing protoxins requiring bioactivation prior to interacting with their respective target sites.

Figure 7. A) Example of bioactivation of a protoxin by removing a protective group. Removal of the alanyl-alanyl conjugate from the inactive protoxin bialaphos produced by Streptomyces hygroscopicus to release the active molecule L-phosphinothricin, a potent inhibitor of plant glutamine synthetases. B) Example of hijacking of plant biochemical machinery to bioactivate a protoxin by adding an active functionality. Phosphorylation of 2,5-anhydro-D-glucitol produced by Fusarium solani NRRL 18883 via the action of the plant hexokinase and phosphofructokinase, leading to the formation of a fructose-1,6-diphosphate analog inhibitor of aldolase. G6P = glucose-6-phosphate, F6P = fructose-6-phosphate, F-1,6-DP = fructose-1,6-diphosphate, G3P = glyceraldehyde 3-phosphate, DHAP = dihydroxyacetone phosphate, AhG = 2,5-anhydro-D-glucitol.

Figure 8. Examples of cellular compartmentalizations. A) Leptospernone is a potent HPPD inhibitor produced by a number of plant species including Callistemon spp., Leptospermum spp.
and *Eucalyptus* spp. This natural β-triketone is a potent inhibitor of HPPD. Plant produce it in specialized glands isolated from the rest of the cells to avoid autotoxicity problems. B) Sorgoleone is a potent phytotoxin produced by most members of the *Sorghum* genus. This lipid benzoquinone is produced exclusively in root hairs and exuded into the rhizosphere.

**Figure 9.** Phylogenetic analysis of GSTUs proteins in grass weeds and crop plants. Amino acid sequences of GSTUs from blackgrass (*Alopecurus myosuroides*; red), barley (*Hordeum vulgare*, black), wheat (*Triticum aestivum*, green) and maize (*Zea mays*, blue) were used for maximum likelihood alignment for phylogenetic analysis. The number on the branch represents the bootstrap support values above 50%. The scale bar indicates the inferred number of substitutions per site. Clades comprising exclusively barley sequences were collapsed into triangles.

**Figure 10.** Transcriptional markers for metabolic herbicide resistance can be used to diagnose populations as resistant or susceptible (adapted from Gaines et al. 2014). A cluster analysis of expression levels of four transcriptional markers (increased expression of two cytochrome P450s, one nitrogen monooxygenase, and one glucosyl transferase) differentiates herbicide susceptible individuals (samples ending in S, highlighted in boxes) from populations containing metabolic resistant individuals, which could be classified as resistant based on their clustering. The results highlight the importance of sampling multiple individuals for transcriptional marker diagnostics.

**Figure 11.** Predominance of metabolism-based resistance to PS II-, ALS- and HPPD-inhibitors in a multiple-resistant Palmer amaranth population evolved in Stafford County, Kansas, USA.
Figure 12. Chemical structures of (A) tembotrione, (B) mesotrione, and (C) topramezone.

Mesotrione and tembotrione are examples of triketone HPPD-inhibiting herbicides, while topramezone belongs to the pyrazole subclass of HPPD-inhibiting herbicides.
Figure 1

A

B

C

Figure 1
Figure 3

Clomazone

5-OH Clomazone

5-keto Clomazone
Figure 4

Chlorophyll/Carotenoid (% of the untreated control)

Phorate Concentration (µM)
Figure 5

A

Radioactivity (% distribution)

B

dpm g⁻¹ DW (x1,000)

C

Plant tissue

D

Plant tissue

L1 L2 L3 L4 L5
Figure 6

1- removing a protecting group
2- adding a functionality
3- release from cellular compartment

Inaccessible toxin \(\rightarrow\) Accessible toxin
Figure 7
Figure 9
Figure 11
Table 1. Effect of 50 µM phorate on $^{14}$C-clomazone metabolism in shoots and roots of 7-day-old cotton seedlings. Seedling roots in hydroponic solution were exposed to $^{14}$C-clomazone with and without phorate for 8 h followed by a 16-h chase period before extraction.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Phorate</th>
<th>Clomazone$^a$</th>
<th>Metabolites$^a$</th>
<th>Unextracted$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot</td>
<td>-</td>
<td>54 ± 2</td>
<td>42 ± 2</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>Shoot</td>
<td>+</td>
<td>71 ± 1$^b$</td>
<td>27 ± 10$^b$</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>Root</td>
<td>-</td>
<td>45 ± 4</td>
<td>55 ± 4</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>Root</td>
<td>+</td>
<td>44 ± 8</td>
<td>56 ± 8</td>
<td>14 ± 2</td>
</tr>
</tbody>
</table>

$^a$ Mean ± SD

$^b$ Indicates a significant difference at $P \leq 0.05$ compared to the control (no phorate) within the same tissue and within the same column.
Table 2. Effect of clomazone and phorate on chlorophyll and carotenoid levels in new leaves of 7-day old corn seedlings.

<table>
<thead>
<tr>
<th>Clomazone (nM)</th>
<th>Phorate (µM)</th>
<th>Chlorophyll *a,b (%) of untreated control</th>
<th>Carotenoids *a,b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>51 ± 5 a</td>
<td>56 ± 10 a</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>78 ± 18 b</td>
<td>77 ± 9 b</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>6 ± 5 a</td>
<td>10 ± 2 a</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>15 ± 3 b</td>
<td>17 ± 4 b</td>
</tr>
</tbody>
</table>

* Mean ± SD

b Means within a column followed by different letters and within the same clomazone concentration are significantly different at P ≤ 0.05.
Table 3. Induction of $^{14}$C-clomazone metabolism in corn microsomes by seed treatment with naphthalic anhydride (0.5% w/w), seedling treatment with ethanol (10% v/v) or a combination of the two.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clomazone Metabolite Elution Time</th>
<th>pmole metabolite$^{-1}$ mg microsomal protein$^{-1}$ min$^{-1}$ $^{1+ab}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.6 min</td>
<td>15.4 min</td>
</tr>
<tr>
<td>None</td>
<td>229 ± 13</td>
<td>55 ± 10 a</td>
</tr>
<tr>
<td>Naphthalic anhydride</td>
<td>244 ± 22</td>
<td>117 ± 27 b</td>
</tr>
<tr>
<td>Ethanol</td>
<td>261 ± 31</td>
<td>106 ± 38 b</td>
</tr>
<tr>
<td>Naphthalic anhydride plus Ethanol</td>
<td>207 ± 21</td>
<td>23 ± 23 a</td>
</tr>
</tbody>
</table>

$^{a}$ Mean ± SD  

$^{b}$ Means within a column followed by different letters are significantly different at $P \leq 0.05$.  

Table 4. Effects of NADPH (0.75 mM) and phorate (50 µM) on $^{14}$C-clomazone metabolism in corn microsomes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Clomazone Metabolite Elution Time</th>
<th>pmole metabolite$^{\dagger}$ mg microsomal protein$^{\ddagger}$ min$^{-1}$</th>
<th>12.6 min</th>
<th>15.4 min</th>
<th>23 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NADPH, -Phorate</td>
<td></td>
<td></td>
<td>431 ± 18 a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+NADPH, -Phorate</td>
<td></td>
<td></td>
<td>262 ± 83 b</td>
<td>125 ± 12 a</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>+NADPH, +Phorate</td>
<td></td>
<td></td>
<td>272 ± 37 b</td>
<td>130 ± 7 a</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5. Effect of phorate (50 µM) on 5-OH clomazone (1000 nM) caused reductions in chlorophyll and carotenoid contents of new leaves of cotton seedlings.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chlorophyll</th>
<th>Carotenoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of untreated control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-Phorate</td>
<td>56 ± 13</td>
<td>57 ± 20</td>
</tr>
<tr>
<td>+Phorate</td>
<td>67 ± 7</td>
<td>65 ± 9</td>
</tr>
</tbody>
</table>
Table 6. Distribution of radioactivity in the organic phase from 2-\(^{14}\)C-pyruvate fed chloroplasts treated with 20 µM norflurazone, 5-OH clomazone, 5-keto clomazone, clomazone or fosmidomycin.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HPLC Peak Retention Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.8 min</td>
</tr>
<tr>
<td>Control</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>Norflurazone</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>5-OH Clomazone</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>5-keto Clomazone</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Clomazone</td>
<td>16 ± 7</td>
</tr>
<tr>
<td>Fosmidomycin</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SD.

\(^b\) Means within one column followed by different letters are significantly different at \(P \leq 0.05\).
Table 7. Inhibition of *Catharanthus roseus* DXP synthase activity by 20 µM 5-keto clomazone.

<table>
<thead>
<tr>
<th>DXP Synthase *</th>
<th>5-keto Clomazone</th>
<th>DXP Synthase Activity *b</th>
<th>pmole 1 min 1 mg protein 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>60 ± 18</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>50 ± 13</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>1700 ± 105</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>900 ± 53*</td>
<td></td>
</tr>
</tbody>
</table>

* - extracts from E. coli transformed with vector minus the DXP synthase gene from *C. roseus*. +, extracts from E. coli transformed with vector plus *C. roseus* DXP synthase gene. * Significant difference at P ≤ 0.05 compared to the assay with the same construct and without 5-keto clomazone.
Table 8. Relative abundance of GSTs associated with NTSR-associated proteins in blackgrass determined in stem and leaf tissue in associated with NTSR populations isolated from the field (‘Peldon’, ‘Oxford’) and from forced selection with the herbicides pendimethalin, or fenoxaprop and herbicide group specific resistances. Significant differences in fold-abundance (p<0.05, fold change >1.5) were relative to equivalent herbicide sensitive (HS) plants. The difference in transcript abundances (NTSR vs. HS) were presented in different color codes with red representing enhanced and green representing suppressed transcript abundance. (NA = not analyzed).

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Stem</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>KY172659</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY105 AmGSTF2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KY172655</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY105 AmGSTU2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJ010454</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY106 AmGSTF1d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S125 AmGSTF1c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L131 AmGSTU2</td>
<td>3.7</td>
<td>0.8</td>
</tr>
<tr>
<td>L172 AmGSTF1d</td>
<td>10.8</td>
<td>15.4</td>
</tr>
<tr>
<td>L120 AmGSTF1c</td>
<td>15.4</td>
<td>1.4</td>
</tr>
<tr>
<td>NTSR populations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant</td>
<td>Peldon</td>
<td>Oxford</td>
</tr>
<tr>
<td>Protein fold change in NTSR and HS stressed plants compared to HS control plants</td>
<td>Transcript fold change in NTSR and HS stressed plants compared to HS control plants</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein Identification</th>
<th>Protein spot number</th>
<th>Protein fold change in NTSR and HS stressed plants compared to HS control plants</th>
<th>Transcript fold change in NTSR and HS stressed plants compared to HS control plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein fold change in NTSR and HS stressed plants compared to HS control plants</td>
<td>Transcript fold change in NTSR and HS stressed plants compared to HS control plants</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Protein fold change in NTSR and HS stressed plants compared to HS control plants**

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Stem</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>KY172659</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY105 AmGSTF2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KY172655</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY105 AmGSTU2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJ010454</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SY106 AmGSTF1d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S125 AmGSTF1c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L131 AmGSTU2</td>
<td>3.7</td>
<td>0.8</td>
</tr>
<tr>
<td>L172 AmGSTF1d</td>
<td>10.8</td>
<td>15.4</td>
</tr>
<tr>
<td>L120 AmGSTF1c</td>
<td>15.4</td>
<td>1.4</td>
</tr>
<tr>
<td>NTSR populations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stress conditions applied to HS plants</td>
<td>Transcript fold change in NTSR and HS stressed plants compared to HS control plants</td>
<td></td>
</tr>
<tr>
<td>Pardon</td>
<td>Fenoxaprop</td>
<td>Pendimethalin</td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Protein fold change in NTSR and HS stressed plants compared to HS control plants</td>
<td>Transcript fold change in NTSR and HS stressed plants compared to HS control plants</td>
<td></td>
</tr>
</tbody>
</table>
Table 9. Activities of purified recombinant GST enzymes from black-grassin conjugating (<i>Alopecurus myosuroids</i>) 1-chloro-2,4-dinitrobenzene (CDNB) toward model and herbicides substrates and as a glutathione peroxidase acting on <i>t</i>-butyl hydperoxide activities toward organic hydroperoxides.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (nkat mg&lt;sup&gt;-1&lt;/sup&gt; rGST)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AmGSTF1c</td>
</tr>
<tr>
<td>CDNB</td>
<td>670 ± 36</td>
</tr>
<tr>
<td>Metolachlor</td>
<td>16.9 ± 4.9</td>
</tr>
<tr>
<td>Fenoxaprop-ethyl</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>&lt;i&gt;t&lt;/i&gt;-Butyl hydroperoxide</td>
<td>50.04 ± 13.3</td>
</tr>
</tbody>
</table>
Table 10. Herbicides to which resistant *E. phyllopogon* in the Sacramento Valley exhibits resistance

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Site of action</th>
<th>History of use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenoxaprop-ethyl</td>
<td>ACCCase</td>
<td>Yes</td>
<td>Fischer et al. 2000</td>
</tr>
<tr>
<td>Cyhalofop-butyl</td>
<td>ACCase</td>
<td>No</td>
<td>Ruiz-Santaella et al. 2006</td>
</tr>
<tr>
<td>Molinate</td>
<td>VLCFAE</td>
<td>Yes</td>
<td>Fischer et al. 2000</td>
</tr>
<tr>
<td>Thiobencarb</td>
<td>VLCFAE</td>
<td>Yes</td>
<td>Fischer et al. 2000</td>
</tr>
<tr>
<td>Bipyribac-sodium</td>
<td>ALS</td>
<td>No</td>
<td>Fischer et al. 2000</td>
</tr>
<tr>
<td>Penoxsulam</td>
<td>ALS</td>
<td>No</td>
<td>Yasuor et al. 2009</td>
</tr>
<tr>
<td>Bensulfuron-methyl</td>
<td>ALS</td>
<td>Yes</td>
<td>Osuna et al. 2002</td>
</tr>
<tr>
<td>Clomazone</td>
<td>DOXP</td>
<td>No</td>
<td>Yasuor et al. 2008</td>
</tr>
<tr>
<td>Quinclorac</td>
<td>Auxin</td>
<td>No</td>
<td>Yasuor et al. 2011</td>
</tr>
</tbody>
</table>