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Chapter 8

Recent Advances in Deciphering Metabolic Herbicide Resistance Mechanisms

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Introduction

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). The mechanism of resistance to an herbicide in a weed population can greatly determine the effectiveness of resistance management strategies. For example, a target site mutation could endow cross resistance to herbicides with similar mechanism of action, or metabolic resistance can bestow ability to withstand herbicides across more than one mechanism of action. In general, five modes of herbicide resistance have been identified in weeds: (1) altered target site due to a mutation at the site of herbicide action resulting in complete or partial lack of inhibition; (2) metabolic deactivation, whereby the herbicide active ingredient is transformed to nonphytotoxic metabolites; (3) reduced absorption and/or translocation that results in restricted movement of lethal levels of herbicide to point/site of action; (4) sequestration/compartmentation by which a herbicide is immobilized away from the site of action in cell organelles such as vacuoles or cell walls; and (5) gene amplification/over-expression of the target site with consequent dilution of the herbicide in relation to the target site (Nandula 2010). Among these, mutation at target site and gene

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amplification are target site based, and metabolic deactivation, differential absorption/translocation, and sequestration are classified as non-target site-based resistances (NTSR).

The majority of research studies investigating differential absorption, translocation (research procedures of which have been recently summarized by Nandula and Vencill 2015), and metabolism are based on availability and application of ^{14}C-labeled herbicides. Shaner (2009) elegantly described differential translocation of glyphosate as a resistance mechanism. Herbicide sequestration, especially, sequestration of glyphosate, as a resistance mechanism has been investigated and reported on extensively by Ge et al. (2010, 2011, 2012) and reviewed by Sammons and Gaines (2014). This article aims to summarize current understanding of metabolic resistance in weeds by providing a history of related research, reporting recent advances, and identifying future research opportunities.

Herbicide Tolerance

The Weed Science Society of America (WSSA) defines herbicide tolerance as "the inherent ability of a species to survive and reproduce after herbicide treatment" (WSSA 1998). This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant. Differential herbicide metabolism is one of the most common mechanisms by which crop plants tolerate phytotoxic action of herbicides, while wild type/susceptible weeds are controlled. Metabolism of herbicides usually occurs in three phases (Kreuz et al. 1996; Van Eerd et al. 2003): 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 occurs (phase 3) (Delye 2013).

Safeners

In general, safeners (focus of a different chapter in this book) are chemicals applied in combination with herbicides to provide tolerance to grass crops such as wheat, rice, corn, and sorghum against certain thiocarbamate, chloroacetamide, sulfonylurea, and aryoxyphenoxypropionate herbicides that are applied preemergence or postemergence. Safeners enhance herbicide detoxification in 'safened' plants. Safening agents activate/catalyze cofactors such as glutathione and enzyme systems such as cytochrome P450 monoxygenases, glutathione *S*-transferases, and glycosyl transferases (Hatzios and Burgos 2004). The safener-mediated induction of herbicide-detoxifying enzymes appears to be part of a general stress

response (Delye 2013; Hatzios and Burgos 2004). These enzymes deactivate herbicide molecules by modifying side chains, which then are conjugated to biochemical moieties such as sugar and amino acid residues. Some of these conjugates are further deposited in vacuoles and cell walls.

Cytochrome P450 Monoxygenase

Cytochrome P450 monoxygenases (CYP, E.C. 1.14.13.X) are oxidative enzymes that have the most important role in Phase 1 of herbicide metabolism (Barrett 2000). CYP often catalyze monooxygenase reactions, usually resulting in hydroxylation, according to the following reaction: RH + O_2 + NAD(P)H + H^+ -> ROH + H_2O + $NAD(P)^+$ (Van Eerd et al. 2003). CYP can be divided into three classes (Van Eerd et al. 2003). Class I CYP require flavin adenine dinucleotide (FAD), or flavin mononucleotide (FMN), or reduced nicotinamide adenine dinucleotide phosphate (NADPH), and are usually microsomal membrane-bound proteins in plants and filamentous fungi. Class II CYP, while similar to class I, exist only in bacterial and animal mitochondria. Class III CYP occur in plant plastids and do not require auxillary redox partners (Van Eerd et al. 2003).

A comprehensive list of herbicides subjected to *in vitro* CYP-mediated metabolic reactions was compiled by Siminszky (2006). Described below are selected discoveries on CYP enzymes in selected crops including original reports as well as recently documented cases.

Arabidopsis. Three CYP enzymes, CYP76C1, CYP76C2, and CYP76C4 of CYP76C subfamily specific to Brassicaceae, metabolized herbicides belonging to the class of phenylurea in *Arabidopsis thaliana* (L.) (Hofer et al. 2014). These CYPs also metabolized natural monoterpenols.

Corn. Polge and Barrett (1995) provided evidence of the occurrence of a CYP involved in the metabolism of chlorimuron-ethyl in corn microsomal preparations.

Lupin. Tolerance to metribuzin in mutants of narrow-leafed lupin (*Lupinus angustifolius* L.) was reversed after treatment with CYP inhibitors omethoate, malathion, and phorate (Pan et al. 2012).

Rice. A CYP-mediated *O*-demethylation of bensulfuron-methyl (BSM) played an important role in the metabolism of BSM by rice (*Oryza sativa* L. cv. Lemont) seedlings (Deng and Hatzios 2003). A novel CYP, *CYP81A6*, encoded by *Bel*, a gene found in wild type rice, provided resistance to two herbicide classes, bentazon (PS II inhibitor) and sulfonylureas, in two mutant male sterile hybrid rice parent lines (Pan et al. 2006). A novel rice CYP, *CYP72A31*, was involved in bispyribac-sodium (BS) tolerance (Saika et al. 2014). BS tolerance was correlated with *CYP72A31* mRNA

levels in transgenic plants of rice and *A. thaliana.* Moreover, *Arabidopsis* overexpressing *CYP72A31* showed tolerance to BSM, which belongs to a different class of ALS-inhibiting herbicides. On the other hand, CYP81A6, which has been reported to confer BSM tolerance, was barely involved, if at all, in BS tolerance, suggesting that the *CYP72A31* enzyme has different herbicide specificities compared to *CYP81A6*.

Soybean. The gene product of a CYP cDNA (*CYP71A10*) from soybean, expressed in yeast, specifically catalyzed the metabolism of phenylurea herbicides, converting four herbicides of this class (fluometuron, linuron, chlortoluron, and diuron) into more polar compounds (Siminszky et al. 1999). Analyses of the metabolites suggested that the *CYP71A10* encoded enzyme functions primarily as an *N*-demethylase with regard to fluometuron, linuron, and diuron, and as a ring-methyl hydroxylase when chlortoluron is the substrate. *In vivo* assays using excised leaves demonstrated that all four herbicides were more readily metabolized in *CYP71A10*-transformed tobacco compared with control plants. For linuron and chlortoluron, *CYP71A10*-mediated herbicide metabolism resulted in significantly enhanced tolerance to these compounds in the transgenic plants (Siminszky et al. 1999).

Wheat. Wheat (*Triticum aestivum* L. cv Etoile de Choisy) microsomes catalyzed the CYP-dependent oxidation of the herbicide diclofop to three hydroxy-diclofop isomers (Zimmerlin and Durst 1992).

Glutathione *S*-Transferase

Glutathione *S*-transferases (GST, E.C. 2.5.1.18) are a broadly present, multifunctional family of enzymes that catalyze the conjugation of glutathione to a variety of substrates (Marrs 1996), including herbicides. Glutathione is a tripeptide of glutamate-cysteine-glycine present in the cytosol of several organisms including plants. It acts as an antioxidant by minimizing membrane damage from reactive oxygen species (free radicals, peroxide, etc.) and itself is oxidized to glutathione disulfide in the process. GST isozymes are cytosolic, in general, and exist as homo- and heterodimers with a subunit molecular mass of 25 kDA (Droog 1997). Glutathione-*S*-conjugate uptake into the plant vacuole is mediated by a specific ATPase which is remarkably similar to the glutathione-*S*-conjugate export pumps in the canalicular membrane of mammalian liver (Martinoia et al. 1993).

Described below are selected discoveries on GST enzymes in selected crops including original reports as well as recently documented cases.

Arabidopsis. The three-dimensional structure of GST from *A. thaliana* indicated the lack of tyrosine in its active site, as opposed to mammalian GSTs, which share a conserved catalytic tyrosine residue (Reinemer et al.

1996). A transporter responsible for the removal of glutathione *S*-conjugates from the cytosol, a specific Mg21-ATPase, is encoded by the *AtMRP1* gene of *A. thaliana* (Lu et al. 1997). The sequence of *AtMRP1* and the transport capabilities of membranes prepared from yeast cells transformed with plasmid-borne *AtMRP1* demonstrate that this gene encodes an ATP-binding cassette transporter competent in the transport of glutathione *S*-conjugates of xenobiotics and endogenous substances, including herbicides and anthocyanins. At*GSTU19*, a tau class GST, when expressed in *Escherichia coli* was highly active towards chloroacetanilide herbicides (DeRidder et al. 2002).

Corn. The first report of GST activity in plants was on corn (Frear and Swanson 1970). Glutathione conjugation of atrazine, first example of biotransformation of a pesticide in plants, is the primary mechanism of tolerance of corn to the herbicide (Shimabukuro et al. 1970). GST-III was isolated from *Z. mays* var. *mutin* and was cloned, sequenced, and its structure determined by x-ray crystallography (Neuefeind et al. 1997). The enzyme forms a GST-typical dimer with one subunit consisting of 220 residues. Each subunit is formed of two distinct domains, an N-terminal domain consisting of a β-sheet flanked by two helices, and a C-terminal domain, entirely helical. The dimeric molecule is globular with a large cleft between the two subunits.

Rice. A GST from rice showed 44–66% similarity to the sequences of the class phi GSTs from *A. thaliana* and corn (Cho and Kong 2005). The isolated gene product, OsGSTF3-3, displayed high activity toward 1-chloro-2,4-dinitrobenzene, a general GST substrate and also had high activities towards the acetanilide herbicides, alachlor, and metolachlor.

Soybean. GSTs in soybean (*Gm*GSTs) involved in herbicide detoxification in cell suspension cultures were purified (Andrews et al. 2005). With respect to herbicide detoxification, two *Gm*GSTU2-related polypeptides dominated the activity toward the chloroacetanilide acetochlor, while an unclassified subunit was uniquely associated with the detoxification of diphenyl ethers (acifluorfen, fomesafen). The inducibility of the diverent GST subunits was determined in soybean plants exposed to photobleaching diphenyl ethers and the safeners naphthalic anhydride and dichlormid. *Gm*GSTU3, a *Gm*GSTU1-like polypeptide, and thiol (homoglutathione) content were induced by all chemical treatments, while two uncharacterized subunits were only induced in plants showing photobleaching.

Glycosyl Transferase

Glycosyl transferases (GTs, E.C. 2.4) comprise of a large gene family in which proteins conjugate a sugar molecule to a wide range of lipophilic

small molecule acceptors including herbicides (Bowles et al. 2005). The conjugation reactions enable GTs to diversify the secondary metabolites via sugar attachments, to maintain cell homeostasis by quickly and precisely controlling plant hormone concentration, as well as to detoxify herbicides by adding sugars onto molecules (Yuan et al. 2006). Glycosyl transferases exist as a gene superfamily with diverse members. They are found in all kingdoms and can be classified into 78 subfamilies. Two soybean GTs were shown to glycosylate the primary major bentazone metabolite, 6-hydroxybentazone (Leah et al. 1992). Other GTs with activity towards herbicides such as 2,4,5-trichlorophenol have since been cloned and characterized (Brazier-Hicks and Edwards 2005; Loutre et al. 2003).

Metabolic Resistance in Weeds

Metabolic resistance to herbicides in weed species has been studied over the past several decades, but not as extensively or in depth as the research on target site-based resistance. An obvious reason is the difficulty in unraveling the complicated physiological and biochemical processes resulting in the metabolic resistance mechanism. In North (US and Canada) and South American (Brazil and Argentina) crop production fields, the relative ease of cultivation of glyphosate resistant crops drastically minimized adverse economic impact from the evolution of herbicide resistant weeds, until the emergence of glyphosate resistant Palmer amaranth (*Amaranthus palmeri* S. Wats.) (Culpepper et al. 2005). Meanwhile, progress has been made over the past decade, more so in the last two to three years, in understanding metabolic resistance in grass weed species (detailed in following sections) through techniques such as RNA-seq and next generation sequencing.

Blackgrass. Metabolic herbicide resistance, involving CYP, has been identified in several European blackgrass populations (Cocker et al. 1999; De Prado and Franco 2004; Letouzé and Gasquez 2001, 2003; Menendez and De Prado 1997). When a black-grass GST, *AmGSTF1* was expressed in *A. thaliana*, the transgenic plants acquired resistance to multiple herbicides and showed similar changes in their secondary, xenobiotic, and antioxidant metabolism to those determined in multiple herbicide resistant weeds (Cummins et al. 2013). Transcriptome array experiments showed that these changes in biochemistry were not due to changes in gene expression. On the contrary, *AmGSTF1* exerted a direct regulatory control on metabolism that led to an accumulation of protective flavonoids. In addition, a multiple drug resistance inhibiting pharmacophore, 4-chloro-7-nitro-benzoxadiazole, with applications in human cancer tumor research, was active on *Am*GSTF1 and helped restore herbicide susceptibility in multiple herbicide resistant blackgrass. Role of an induced GT in multiple herbicide resistant blackgrass (Brazier et al. 2002) was the first such evidence in weeds.

Rigid ryegrass. Resistance to diclofop in a rigid ryegrass population, selected by recurrent treatment with low doses of the herbicide, was due to rapid metabolism of diclofop acid (likely brought about by CYP) resulting in a 2.6-fold less of the phytotoxic acid compared to a susceptible population (Yu et al. 2013). The major polar metabolites of diclofop acid were similar to that of wheat, a crop naturally tolerant to diclofop. Pre-treatment with 2,4-D, a known CYP inducer, caused up to 10-fold change in LD_{50} and GR_{50} in dose–response to subsequently applied diclofop-methyl in a herbicide susceptible rigid ryegrass population (Han et al. 2013). Metabolism of diclofop acid, following of de-esterification of diclofop-methyl, to non phytotoxic metabolites was 1.8-fold faster in 2,4-D pre-treated plants than on untreated plants. Also, 2,4-D pre-treatment induced cross-protection against the ALS-inhibiting herbicide chlorsulfuron. RNA-Seq transcriptome analysis of diclofop-resistant rigid ryegrass identified four contigs, two CYP, a nitronate monoxygenase (NMO), and a GT, consistently highly expressed in nine field-evolved metabolic resistant populations (Gaines et al. 2014). These four contigs were strongly associated with the resistance phenotype and were major candidates for contributing to metabolic diclofop resistance. More than two-thirds of 33 diclofop resistant populations of rigid ryegrass exhibited both metabolic resistance and target-site ACCase mutations (Han et al. 2015). Duhoux et al. (2015) confirmed four contigs comprising of two CYP enzymes, one GT, and one GST in ryegrass plants resistant to pyroxsulam.

***Echinochloa* spp.** The addition of CYP inhibitors, piperonyl butoxide and malathion, severely increased the sensitivity of a resistant watergrass [*Echinochloa phyllopogon* (Stapf) Koso-Pol.] accession from California to BS, suggesting that metabolic degradation of the herbicide is the primary mechanism (Fischer et al. 2000). Initiation of CYP activity was implicated as the mechanism of multiple resistances in a resistant watergrass biotype from California after greater CYP induction by BS, fenoxaprop-ethyl, and thiobencard in the resistant biotype compared to a susceptible biotype (Yun et al. 2005). Similar CYP-induced multiple resistances endowed cross resistance to clomazone in late watergrass (Yasuor et al. 2010). Clomazone is a proherbicide that must be metabolized to 5-ketoclomazone, which is the active compound. Resistant plants accumulated 6- to 12-fold more of a nonphytotoxic metabolite (containing a monohydroxylated isoxazolidinone ring) than susceptible plants, while susceptible plants accumulated 2.5-fold more of the phytotoxic metabolite of clomazone, 5-ketoclomazone. A late watergrass biotype, resistant to fenoxaprop, metabolized the herbicide to nonphytotoxic polar metabolites and phytotoxic fenoxaprop acid 2-fold more and 5-fold less, respectively, compared to a susceptible biotype (Bakkali et al. 2007). In addition, the resistant biotype exhibited higher rate of glutathione conjugation. Resistant *E. phyllopogan* plants metabolized

BSM via *O*-demethylation more rapidly than susceptible plants (Iwakami et al. 2014). Two CYP genes belonging to the *CYP81A* subfamily, *CYP81A12* and *CYP81A21*, were more abundantly transcribed in the resistant plants compared with susceptible plants. Transgenic *A. thaliana*, expressing either of the above two genes, survived BSM or penoxsulam in media, but not wild type plants. Proteins of *CYP81A12* and *CYP81A21*, produced heterologously in yeast (*Saccharomyces cerevisiae*), metabolized bensulfuron-methyl by *O*-demethylation.

A propanil resistant barnyardgrass [*E. crus-galli* L. Beauv.] population metabolized the herbicide to 3,4-dichloroaniline, but not the susceptible population (Carey et al. 1997). Two other polar metabolites found in resistant barnyardgrass were similar to those formed in rice. Further work detected elevated (2- to 4-fold) activity of aryl acylamidase in the propanil resistant barnyardgrass population compared to a susceptible population (Hirase and Hoagland 2006).

***Bromus*.** *Bromus rigidus* (Roth) Lainz populations from Australia that were resistant to sulfosulfuron, an ALS-inhibiting herbicide, were reverted to being susceptible after treatment with the herbicide in combination with malathion, a CYP inhibitor (Owen et al. 2012).

***Avena* spp.** A diclofop-methyl resistant wild oat (*Avena* spp.) population, lacking a resistant acetylCoA carboxylase (ACCase), metabolized the parent methyl ester to the phytotoxic diclofop acid to a lesser extent than a susceptible population (Ahmad-Hamdani et al. 2013). In addition, there was an associated higher level (up to 1.7-fold) of nontoxic polar diclofop metabolites in the resistant plants relative to susceptible plants, indicating a non-target site based mechanism of enhanced rate of diclofop acid metabolism. Three other resistant populations had lower diclofop acid levels in addition to ACCase mutations.

Broadleaf weeds. Resistance to atrazine in velvetleaf populations from Wisconsin and Maryland was found to be due to metabolism of atrazine to glutathione, L-cysteine, and *N*-acetyl-L-cysteine conjugates, metabolites produced in the glutathione conjugation pathway (Anderson and Gronwald 1991; Gray et al. 1996; Gronwald et al. 1989). Resistance to atrazine, applied postemergence, in two waterhemp populations was due to increased levels of GST activity (Ma et al. 2013).

Future Research Direction

A three-step procedure was proposed, based on the use of the 'omics' (genomics, transcriptomics, proteomics or metabolomics), to decipher the genetic bases of NTSR (Delye 2013). Step 1 involves collection of weed genotypes, phenotype determination based on response to herbicide

(resistant/susceptible), and production of genetically homogeneous plant material via controlled crosses. Step 2 is an omics-based approach to identify phenotype-related differences in gene expression to yield NTSR alleles. Step 3 pertains to the validation of NTSR candidate alleles with eventual development of DNA/protein/metabolite-based NTSR makers for NTSR diagnosis or NTSR evolutionary research.

Conclusions

The immediate and urgent challenge for weed scientists is to understand and characterize the reasons of NTSR, which includes metabolic resistance in order to sustain the limited herbicide portfolio and develop integrated weed management strategies (Délye 2013; Yu and Powles 2014). Metabolic resistance research in weeds has mostly been limited to grass species such as rigid ryegrass, blackgrass, and watergrass. However, dicot species such as tall waterhemp has developed resistance to multiple herbicide mechanisms of action by enhanced metabolic degradation (Ma et al. 2013). Thus, both grass and dicot species can develop metabolic herbicide resistance given the high initial frequency of genes responsible for imparting metabolic resistance. Herbicides must be used at full rates (Yu and Powles 2014) to minimize weed escapes, which if left uncontrolled can recharge weed seedbanks or evolve resistance.

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